INVESTIGATION OF ZEARALENONE TOLERANCE LIMIT IN THE FEEDSTUFFS FOR WEANED PIGS

Ionelia Taranu[1*], M.A. Gras[1], Gina Cecilia Pistol[1], Monica Motiu[1], Daniela Marin[1], Mariana Stancu[1]

[1]Laboratory of Animal Biology, National Research and Development Institute for Biology and Animal Nutrition, Romania

Abstract

In the present study an in vivo feeding experiment was carried out to evaluate the immunotoxic and genotoxic effect of a diet contaminated with 100 ppb zearalenone (the maximal tolerance level recommended by CE/576/2006) in young pigs. Six weaned pigs received the diet contaminated with 100 ppb of zearalenone (ZEA), while other six pigs receiving a non-contaminated diet were used as control for 33 days. The effect of ZEA on pro-inflammatory cytokines and several molecules involved in oxidative stress (CAT, SOD, GPx, iNOS, eNOS) and related pathways (NF-kB1, p38-MAPK, Nrf2) was investigated in internal organs (liver, spleen, kidney and intestine) collected at the end of the experiment. Real-time PCR and ELISA analyses were used to perform gene expression and protein concentration of the above mentioned markers.

Our qPCR results showed that the level of 100 ppb of ZEA in the pig diet decreased the gene expression of markers involved in inflammatory process in intestine (duodenum) and liver, but not in spleen, kidney and colon in which an increase in the expression of these genes was found. The modulation of gene expression was not statistically different for the majority of pro-inflammatory cytokines compared to control. Also, throughout ELISA analyses no differences were detected at the protein level for these markers. However, a significant difference was found in gene expression for IL-8 in spleen (+320%) and duodenum (-89%), IL-6 in spleen (+127%) and kidney (+120%) and for COX2 in liver (-88%) and colon (+279%) respectively, when compared to control. Gene encoding for enzymes involved in the oxidative stress were affected (up-regulation) especially in liver and kidney and less in the other organs.

The corroboration of these results with other markers of metabolic and physiological processes might contribute to the establishment of the dose accepted as maximal limits in the diet designated to young pigs.

Key words: zearalenone, pig, limit of tolerance

INTRODUCTION

Mycotoxins are the most frequent natural contaminants of cereals (Placinta et al., 1999). For several mycotoxins such as *Fusarium* toxins, the legal tolerance limits in farm animal feed compounds are regulated only through the recommendation CE/576/2006 of the European Commission [1], meaning that new experimental evidence are required in order to confirm the proposed/recommended limits. Recently, EFSA [2], suggested more in-depth studies to document that the doses accepted as maximal limits have no genotoxic and immunotoxic effects. The pig, which is a great consumer of cereal grains, is considered by EFSA the most sensitive species to mycotoxins action like zearalenone, deoxynivalenol, etc. The most known pathological effects produced by ZEA are reduced fertility, increasing abortion, embryo and foetal death [3]. *In vitro* and *in vivo* studies showed also that ZEA presents immunotoxic properties acting either as stimulator or suppressors of immune response [4, 5, 6]. This mycotoxin is among the contaminants no regulated by CE. However, the recommendation of tolerance limit in feed for ZEA issued by CE/575/2006 guideline for young pigs is 100 ppb. In the present study an *in vivo* experiment was conducted in pig in order to provide new data which could contribute to the establishment of the maximal tolerance levels (legal regulation) for zearalenone. The effect of 100 ppb of ZEA on

[*]Corresponding author: ionelia.taranu@ibna.ro
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several inflammatory and oxidative stress markers (pro-inflammatory cytokines, antioxidants enzymes and related pathway signaling molecules) was investigated.

MATERIAL AND METHODS

Experimental design. Two groups of 12 weaned piglets (6 pigs/group, TOPIGS-40 crossbred pigs, 5-week-old) with an initial average body weight of 9.5 ± 0.6 Kg were fed *ad libitum* for 33 days with a control or a ZEA (100 ppb) contaminated diet. At the end of the experiment animals were slaughtered and tissue samples were taken for assessment of gene expression of several key markers of inflammatory response, oxidative stress and signaling molecules.

Determination of gene expression by quantitative real-time. The gene expression of cytokines, antioxidants enzymes and signaling molecules was analyzed by fluorescent real-time PCR (qPCR) as described by Marin et al., [5].

Extraction of total RNA and cDNA synthesis. Organ tissue samples snap-frozen were homogenized in liquid nitrogen and stored at -80ºC until RNA extraction. Total RNA was extracted and measured as described by Pistol et al., [3] using Qiagen RNeasy midi kit (QIAGEN GmbH, Germany), according to the manufacturer's recommendations. The total RNA isolated from each sample was further used to generate cDNA using M-MuLV Reverse Trascriptase kit (Fermentas, Thermo Fischer Scientific, USA) according to the manufacturer's protocol.

qPCR. Briefly, the PCR reaction was set up in Rotor-Gene-Q (QIAGEN GmbH, Germany) apparatus using 5µl of cDNA, 12.5 µl Maxima SYBR Green/ Fluorescein qPCR Master Mix 2X (Fermentas, Thermo Fischer Scientific, USA) and 0.3µM each of gene-specific primer [5]. The PCR cycling conditions consisted in: UDG pre-treatment at 50°C for 2 min, initial denaturation step at 95°C for 15s, followed by 40 cycles of 95°C for 15s, 60°C for 15s and 72°C for 15s with a single fluorescence measurement; a final elongation step was carried out at 72°C for 10 min. Two reference genes were used for data normalization. Results were expressed as relative fold change (Fc) compared with control.

Determination of cytokine concentration by ELISA. Organ lysate was prepared as described by Taranu et al., [7] and cytokine concentration was determined in supernatant by ELISA. Briefly, purified fraction of anti-swine cytokines (IL-1β, IL-6, IL-8, TNF-α, IFN-γ) were used as capture antibodies in conjunction with biotnylated anti-swine antibodies (R&D system). Streptavidin-HRP (Sigma) and TMB (Sigma) were used for detection. Absorbance was read at 450 nm using an ELISA plate reader (Tecan, Sunrise, Austria). Recombinant swine cytokine proteins were used as standard and results were expressed as picograms of cytokine/mL.

Statistic analyses. Result data are expressed as mean ± standard error of the mean (SEM). One way ANOVA analysis was used to determine the statistical differences between groups for all parameters analyzed. Further differences between means were determined by the least square difference Fisher procedure. Values of $P< 0.05$ were considered significant.

RESULTS AND DISCUTIONS

Our qPCR results showed that the presence of 100 ppb of ZEA in the pig diet decreased the gene expression of some mediators of inflammatory process (IL-1β, IL-6, IL-8, TNF-α, COX2) slightly in liver and more pronounced in duodenum, but not in spleen, kidney and colon in which an increase in the expression of these genes was found (Table 1). The modulation of gene expression was statistically different ($P<0.05$) for IL-8 in spleen (+320%) and duodenum (-89%), IL-6 in spleen (+127%) and kidney (+120%) and for COX2 in liver (-88%) and colon respectively (+279%) when compared to control. The highest down-regulation was observed for COX2 expression (88%, $p<0.05$) (Table 1). The down-regulation of inflammatory genes expression in liver and duodenum and their up-regulation in spleen, colon and kidney suggests that the modulatory effect of ZEA is organ specific with a dual effect on inflammatory response: stimulation of inflammation in spleen, colon and kidney and suppression of inflammatory response in liver and duodenum which might have important repercussions on immune homeostasis. Similar results was also observed by Wang et al., [8], Pistol et al., [9], Choi et

al., [10] etc. The modulation of inflammatory genes expression was confirmed also at protein level. The concentration of IL-1β, IL-6, IL-8, TNF-α were higher in spleen and kidney and lower in liver and duodenum in comparison with those in control organs, but not statistically different (data not shown).

In the most of mycotoxin contaminated cases the induced inflammation is strong correlated with oxidative stress [5] which in fact is responsible for the induction of inflammatory mediators [11]. Recent studies revealed that ZEA is able to produce oxidative damages [13]. Indeed, the results of the present study showed that ZEA modulated the gene expression of some enzymes involved in oxidative stress. Thus, an up-regulation of GPx, iNOS and eNOS gene expression was observed in liver, spleen and kidney and of SOD1 and CAT in kidney, duodenum and colon. Noteworthy, the expression of all investigate antioxidant enzymes was up-regulated in kidney. Among them the most significantly ($p<0.05$) over-expressed were iNOS and eNOS with an increase of 200 and 268% respectively. Gresakova et al., [13] reported also that ZEA determined an increase of GPx activity in the duodenal mucosa and kidney tissue in chicken. By contrast, ZEA down-regulated the SOD1 and CAT expression in liver, GPx in colon and eNOS expression in duodenum and colon. This down regulation was significant ($p<0.05$) for SOD1 which decreased with 79% in liver.

To see if 100 ppb of ZEA exert its modulatory effect by acting on some signaling molecules involved in the regulation of the immune response especially inflammation, the expression of p38-MAPK, NFkB and Nrf2 was investigated in organ tissues. A decrease in the expression of these genes in duodenum and an increase in colon and kidney (over-expression of p38-MAPK gene) respectively were registered which might correlate positively with the activation or non-activation of pro-inflammatory genes in these organs (Table 3). No effect in spleen and by contrast an up-regulation in liver of piglets fed contaminated diet was observed which suggest that the modulation of pro-inflammatory and oxidative stress response were mediated by other transduction pathway molecules. The gene expression pattern of signaling molecules observed in liver is in contrast with results of Pistol et al., [9] who found a significant decrease of p-38MAPK, NF-kB and related pro-inflammatory molecules under the effect of a diet contaminated with a higher concentration of ZEA (316ppb).

Table 1 Effect of *in vivo* exposure of piglets to ZEA on gene expression of pro-inflammatory molecules in organ tissue

Cytokine expression (Fc) [b]	**Group**	**Organ**[a]									
		Liver		**Spleen**		**Kidney**		**Duoden**		**Colon**	
		Fc	SEM	**Fc**	SEM	**Fc**	SEM	**Fc**	SEM	**Fc**	SEM
IL-1β	Control	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0
	ZEA	**0.72**	0.3	**2.31**	0.2	**2.16**	0.1	**0.62**	0.2	**1.25**	0.2
IL-6	Control	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0
	ZEA	**0.87**	0.2	**2.27***	0.2	**2.75***	0.2	**0.51**	0.2	**0.89**	0.1
IL-8	Control	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0
	ZEA	**0.80**	0.2	**4.20***	0.0	**1.15**	0.1	**0.11***	0.0	**0.35**	0.1
TNF-α	Control	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0
	ZEA	**0.84**	0.1	**2.07**	0.2	**1.59**	0.2	**0.92**	0.2	**1.65**	0.1
IFN-γ	Control	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0
	ZEA	**1.15**	0.3	**1.03**	0.2	**1.28**	0.1	**0.55**	0.2	**1.94**	0.1
COX2	Control	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0	**1.00**	0.0
	ZEA	**0.12***	0.0	**0.73**	0.2	**1.02**	0.1	**0.83**	0.2	**3.79***	0.0

[a] Pigs were fed for 33 days with a control or a ZEA (100 ppb) contaminated diet. At the end of the experiment, organ samples from all animals (n=6) were collected and analyzed for cytokine mRNA expression by quantitative Real-Time PCR.

[b] Results are expressed as fold change-Fc (mean ± SEM).

*Significant different compare with control ($P<0.05$).

Table 2 Effect of *in vivo* exposure of piglets to ZEA on gene expression of antioxidant enzymes in organ tissue

Enzymes expression (Fc)[b]	Group	Organ[a]									
		Liver		Spleen		Kidney		duoden		colon	
		Fc	*SEM*	**Fc**	*SEM*	**Fc**	*SEM*	**Fc**	*SEM*	**Fc**	*SEM*
GPx	Control	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00
	ZEA	**1.73**	0.09	**1.30**	0.20	**2.10**	0.14	**1.24**	0.21	**0.66**	0.25
SOD1	Control	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00
	ZEA	**0.21***	0.03	**1.12**	0.21	**1.99***	0.21	**1.39**	0.26	**1.52**	0.13
CAT	Control	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00
	ZEA	**0.69**	0.08	**0.78**	0.23	**1.99**	0.16	**1.48**	0.22	**1.62**	0.12
iNOS	Control	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00
	ZEA	**4.76**	0.12	**1.43**	0.21	**3.00***	0.22	**0.89**	0.10	**0.73**	0.09
eNOS	Control	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00
	ZEA	**2.18**	0.09	**1.47**	0.20	**3.68***	0.15	**0.37**	0.14	**0.62**	0.10

[a] Pigs were fed for 33 days with a control or a ZEA (100 ppb) contaminated diet. At the end of the experiment, organ samples from all animals (n = 6) were collected and analyzed for enzymes mRNA expression by quantitative Real-Time PCR.
[b] Results are expressed as fold change-Fc (mean ± SEM).
*Significant different compare with control (P<0.05).

Table 3 Effect of *in vivo* exposure of piglets to ZEA on gene expression of signaling molecules in organs tissue

Signaling molecules expression (Fc)[b]	Group	Organ[a]									
		Liver		Spleen		Kidney		duoden		colon	
		Fc	*SEM*	**Fc**	*SEM*	**Fc**	*SEM*	**Fc**	*SEM*	**Fc**	*SEM*
p38-MAPK	Control	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00
	ZEA	**1.52**	0.21	**1.13**	0.32	**1.51**	0.15	**0.45**	0.17	**1.43**	0.24
NFkB	Control	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00
	ZEA	**1.89**	0.20	**0.84**	0.31	**0.82**	0.13	**0.57**	0.15	**1.53**	0.20
Nrf2	Control	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00
	ZEA	**1.57**	0.02	**1.36**	0.05	**0.93**	0.14	**0.56**	0.16	**1.65**	0.10

[a] Pigs were fed for 33 days with a control or a ZEA (100 ppb) contaminated diet. At the end of the experiment, organ samples from all animals (n = 6) were collected and analyzed for signaling mRNA expression by quantitative Real-Time PCR.
[b] Results are expressed as fold change-Fc (mean ± SEM).
*Significant different compare with control (P<0.05).

CONCLUSIONS

In summary the results of this study showed that ZEA at 100ppb concentration (EU maximal limit recommended for piglets feedstuffs) modulated several genes involved in inflammation and oxidative stress and the modulation is organ specific. Indeed, the diet contaminated with ZEA induced a significant down-regulated of COX2 gene expression in liver and an up-regulation of this gene in colon; also ZEA increased in spleen the expression of IL-8 and decreased its expression in duodenum. The effect was similar for gene encoding for antioxidant enzymes which were affected (up-regulation) especially in liver and kidney and less in the other organs. However, through ELISA assay no differences were detected at the protein level for cytokines.

The corroboration of these results with those issued from studies on a higher number of animals might contribute to the establishment of the dose accepted as maximal limit of ZEA in the diet designated to young pigs.

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