SZENT ISTVÁN UNIVERSITY

EFFECT OF TRICHOTHECENE MYCOTOXINS ON THE REGULATION OF THE GLUTATHIONE REDOX SYSTEM

PhD thesis

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1 INTRODUCTION AND OBJECTIVES

1.1 Introduction

The primary aim of animal nutrition is to demand nutrient requirement with balanced mixture of ingredients to foster the maintenance, growth, reproduction, meat quality and health of the animals. Cereal grains play an important role in the nutrition of monogastric and ruminant animals as well, but those may contain toxic compounds, which can affect the animals.

Trichothecene mycotoxins are secondary metabolites of *Fusarium* molds. Deoxynivalenol (DON) and T-2 toxin are the most relevant members of this mycotoxin family, based on their economic significance and occurrence.

The toxic effects of these mycotoxins are well known, which are based on changes in parameters of biological roles and their regulation mechanisms. Although most of these results are concluded of *in vitro*, or *in vivo* sublethal, long-term mycotoxin exposure studies.

Nevertheless, there are still many questions about the effects and mechanism of T-2 toxin and DON on the production of reactive oxygen species (ROS) and the biological antioxidant system. After a long-term mycotoxin exposure the biochemical changes indicate only a final state of a process, which can be in relation to increasing levels of damage or even adaptation.

The time of the manifestation of the toxic effects of DON and T-2 toxin after feed intake is one of the primary questions, including the level of the oxidative stress induced, and the order and level of induction of the biochemical and molecular markers of the antioxidant system. These questions are still open and clear answers cannot yet be given.

In addition the short term effects of mycotoxins, such as T-2 toxin or DON in avian and fish species are less known yet, thus broiler chicken and common carp juveniles were chosen as models in present studies.

The aim of the series of experiments presented in my PhD thesis was to evaluate the *per os* effects of graded doses of T-2 toxin or DON on the level and steps of induction of lipid peroxidation, the members of the antioxidant and xenobiotic transformation system. These biological processes were studied at enzyme/protein and gene expression levels in common carp and broiler chicken, in short term mycotoxin exposure studies.

1.2 Objectives

1. The main objective of my studies was to investigate the effect of trichothecene mycotoxins (T-2 toxin or DON) on the lipid peroxidation processes, regulation and changes of the members of the glutation redox system in the first 24 hour exposure in two animal species with different physiological characteristics, common carp and broiler chicken.

2. The evaluation of age-dependent changes of the parameters was also a goal in broiler chickens, in experiments with similar dose ranges of the mycotoxins.

3. Transit time study was also aimed, thus the time of passage of feed particles in the gastrointestinal tract was observed in common carp and broiler chickens, which may influence the available time for absorption of mycotoxins from the intestine. Based on the transit time, the evaluation of correlations between the observed changes in the parameters and the period of absorption of mycotoxins was also an objective of present studies.

4. The investigation of the applied mycotoxin doses on increased ROS production, elevated lipid peroxidation and the order and time-interval of changes in the glutathione redox system in mRNA and protein/tripeptide levels was also objective my research.

The following experiments were designed and parameters were measured to achieve the objectives:

I. Evaluation of short term effects (24 hours) of single, sublethal doses of T-2 toxin or DON in the liver of one-year-old common carp:
   a) on the lipid peroxidation processes, including the markers of initiation phase (conjugated dienes (CD) and conjugated trienes (CT)) and metastable end-product of terminal phase (malondiladehyde, (MDA));
   b) on the changes of parameters of the biological antioxidant system (glutathione peroxidase (GPx) activity, reduced glutathione (GSH) content);
   c) and the gene expression of phspholipid hydroperoxide glutathione peroxidase genes (glutathione peroxidase 4 a and b (*Gpx4a és Gpx4b*)) and genes of transcription factors of Keap1/Nrf2-ARE (*kelch-like ECH-associated protein 1/ nuclear factor E2-related factor 2*/Antioxidant Response Element) pathway

II. Evaluation of short term age-dependent effects (24 hours) of sublethal doses of T-2 toxin or DON in the liver of broiler chickens (1 and 3 week-old):
   a) on the lipid peroxidation processes, including the markers of initiation phase (CD and CT) and metastable end-product of terminal phase (MDA);
   b) on the changes of parameters of the biological antioxidant system (GPx activity, and GSH content);
   c) and the gene expression of members of glutathione redox system (glutathione peroxidase 4 (*Gpx4),* glutathione synthetase *(Gss),* glutathione reductase *(Gsr*)).

2 MATERIALS AND METHODS

2.1 Mycotoxin production and artificial contamination of feed

2.1.1 Artificial mycotoxin contamination of the feed

DON was produced by *Fusarium graminearum* (NRRL 5883) and T-2 by *Fusarium sporotrichioides* (NRRL 3299) strains on corn substrate in collaboration with Mycotoxins in the Food Chain Research Group, Hungarian Academy of Sciences-Kaposvár University.

In broiler chicken experiments the corn substrate, with known trichothecene mycotoxin doses was mixed to broiler chicken feed in order to reach the final concentration of intended doses.

Commercial carp feed (Aqua Garant Classic™) was grinded and mixed with corn substrate, with known trichothecene mycotoxin doses in order to create the final concentration of intended doses. Appropriate amount of feed was mixed with water in 1:4 ratio immediately before use for appropriate application by gavage.

Concentration of DON and T-2 toxin was determined in both control and contaminated feeds by HPLC method, after immunoaffinty clean-up.

2.2 Experimental protocols and sampling method

2.2.1 Studies with common carp juveniles

Control and experimentally contaminated feed was given by gavage directly into the gut once, at the start of the experiment. Samples were taken from 6 carps before start of the trial and from randomly chosen 6 of each group at every 8th hour during a 24-hour period. *Post mortem* liver samples were taken for biochemical and molecular biological measurements.

Another group was formed to observe the transit time, where methyl orange dyed (1% w/w) control feed was given by gavage directly into the stomach once, at the start of the experiment.

2.2.2 Studies with broiler chickens

In the age-dependent experiments with broiler chickens *ad libitum* feeding trial was designed with 1 and 3-week-old chickens. Samples were taken at the beginning of the trial from 5 chickens and from randomly chosen 5 of each group at every 4th hour during a 24-hour period. *Post mortem* liver samples were taken for biochemical and molecular biological measurements.

2.3 Biochemical analysis

Malondialdehyde concentration was measured in the native 1:9 liver homogenate with colorimetric method according to Placer et al. (1966) as modified by Matkovics et al. (1988). Reduced glutathione concentration of the 10,000 supernatant fraction of liver homogenate was determined on the basis of complex formation of free non protein SH-groups with 5,5'dithiobis-2-nitrobenzoic acid (Sedlak and Lindsay, 1968). Glutathione peroxidase activity was measured in 10,000 supernatant fraction liver homogenate using the endpoint direct assay of Matkovics et al. (1988). Enzyme activity and GSH content were calculated to protein concentration which was determined by Folin phenol reagent in the 10,000 supernatant fraction tissue homogenates (Lowry et al., 1951), where bovine serum albumin was used as standard.

2.4 Gene expression

2.4.1 RNA purification and reverse transcription

Total RNA was purified with Trizol reagent from 5 mg liver homogenates, according to the instructions of the manufacturer. RNA was DN-ase treated according to the protocol of the supplier to avoid any genomic DNA contamination. The quality and integrity of total RNA was verified. All samples were accepted with the ratios of absorption 260:280 nm higher than 2.0. A standard protocol was used for cDNA production with RevertAID Reverse transcriptase and random nanomer primer from 1 μg of total RNA. Equal amount of individula cDNA was pooled per sample group in, which was used as template in qPCR measurements.

2.4.2 Real-time PCR measurements in common carp

The gene expression of target genes (*Nrf2, Keap1, Gpx4a* and *Gpx4b,*) and endogenous housekeeping control gene (*β-actin*) was measured by qPCR with SYBRGreen method. Measurements were carried out with Step One Plus™ Real Time PCR system using Maxima SYBR Green qPCR Master Mix, in 5 technical replication. The PCR profile for *Gpx4a* and *Gpx4b* target genes consisted of 95 °C for 10 minutes for pre-amplification denaturation (PAD), and 95°C 15 sec, 55°C 30 sec and 70°C 30 sec for 45 cycles, for *Nrf2* and *Keap1* target genes 95 °C for 10 minutes PAD, and 95°C 15 sec, 60°C 30 sec and 70°C 30 sec for 45 cycles, where SYBR Green signal was detected at the end of the extension period.

2.4.3 Real-time PCR measurements in broiler chicken

The gene expression of target genes (*Gpx4*, a *Gss* and *Gsr*) and endogenous housekeeping control gene, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was measured by duplex qPCR method. Via specific primers and dual labelled (MGB-NFQ) TaqMan probes with different fluorescent dyes for the target and house keeper genes duplex qPCR was used for measurements of each target-house keeper gene pairs. Measurements were carried out with Step One Plus™ Real Time PCR system using Maxima Probe qPCR Master Mix, in 5 technical replication. The PCR profile for *Gpx4a* and *Gpx4b* target genes consisted of 95 °C for 10 minutes for pre-amplification denaturation (PAD), and 95°C 15 sec, 58°C 30 sec and 72°C 30 sec, for 45 cycles, where signals were detected at the end of the extension period.

2.4.4 Real-time PCR evaluation and calculation

The amplified products were verified by melting curve analysis, and gel electrophoresis in SYBRGreen measurements, and by gel electrophoresis in TaqMan duplex measurements. The threshold cycle (Ct) of target genes and endogenous housekeeping control genes, was determined by StepOne™/ StepOnePlus™ Software v2.2, the delta Ct values (ΔCt), delta-delta Ct values (ΔΔCt) and relative quantification (RQ = 2ˆ-ΔΔCt) values were calculated by the formula described by Livak and Schmittgen.

2.5 Statistical analysis

Statistical analysis of data (calculation of means and standard deviations and one-way analysis of variance (ANOVA)) was performed by GraphPad Prism 5.04 software (GraphPad Software Inc., San Diego, USA); two-way analysis of variance (Two-Way ANOVA, Student-Newman-Keuls post-hoc test) was performed by MedCalc for Windows, version 12.3 (MedCalc Software, Ostend, Belgium). In ANOVA the groups of each sampling point were compared to each other, while in Two-Way ANOVA the time and treatment effects were examined. In this analysis the effect of time (H), treatment (T) and their common effect (TxH) can be evaluated in the changes of the parameters.

3 RESULTS AND DISCUSSION

3.1 Experiments with common carp

The transit time was observed via methyl orange dyed feed, which was applied by gavage to an untreated group of carps and the first appearance of colored feces indicates the passage time.

The transit time was 16 hours in common carp juveniles at 19±1 °C water temperature in an untreated control group.

In experiments with a single oral dose of contaminated feed, given by gavage, the measured transit time of feed particles in the gut determines the biological availability of mycotoxins, thus this is the available period of time for the absorption of mycotoxins, because there was no further feed uptake. Based on this, it is presumable, that the transit time correlates with the changes in biochemical parameters and gene expression induced by the mycotoxin exposure.

3.1.1 Summary of short term effect of T-2 toxin exposure

1. table The applied T-2 toxin doses in each treatment group (mg/kg body weight)

Treatment group	Control	T1 (low)	T2 (medium)	T3 (high)
T-2 toxin	< 0.02	0.15	0.33	1.82

There is no available LD_{50} value for T-2 toxin for common carp. Thus, doses were chosen based on literature data, which reach the toxic level in other animal species. The applied doses are presented in table 1.

In the 24 hour period mortality (19%) was observed only in the T3 group, which was treated with the highest T-2 toxin dose, between 8^{th} and 16^{th} hours. Other studies suggest that T-2 toxin is the most toxic trichothecene mycotoxin in animals, including fishes, which support this result together with my previous experiences.

Parameters of the initial and terminal phase of lipid peroxidation, CD and CT and MDA levels, were less affected, only a slight elevating trend was observed at 8^{th} and16^{th} hour. However, in the group treated with the highest dose of T-2 toxin (T3) CD level was significantly higher at 8^{th} hour, as compared to the control. Based on the present changes in lipid peroxidation, T-2 toxin induced mild ROS formation in the applied dose range, which was caused only minor oxidative damage of the lipids in the 24 hour period.

Parameters of antioxidant defense showed a rapid and marked response to ROS formation in all of the mycotoxin challenged groups. GSH content was significantly higher in all experimental groups as compared to the control in all of the T-2 toxin treated groups at 16^{th} hour. GPx activity also elevated at 16^{th} hour, and the difference was significant as compared to the control in T1 and T3 groups, and earlier, at 8^{th} hour in the highest dose, T3, group. However, it is important to note, the results suggest that not only the dose, but also the time of exposure and the common effect of these two factors have effect on the developing changes. After more or less complete absorption of mycotoxins (16 hour transit time), these parameters turned back to the control level by the 24^{th} hour.

These results suggest that ROS formation occurred in the liver of common carp as an early effect of the single oral dose of T-2 toxin, but the emerging oxidative stress was effectively eliminated by the antioxidant system. This statement supported by the results, namely lipid peroxidation processes were less affected because of the effect of the antioxidant defense system.

These results can be compared indirectly to previous studies with common carp, as most of them based on long term sublethal studies.

In the 24 hour experiment, the 16th hour appeared as a key time of exposure, which can be related to the transit time of feed particles in the gastrointestinal tract, which determines the available time for mycotoxin absorption. This is supported by the observations in the mild elevating trends of lipid peroxidation processes and the marked induction of the glutathione redox system in the 16th hour, which parameters turned back to the control level at 24th hour.

Expression of genes encoding the proteins play a role in the Keap1-Nrf2 pathway were also measured. In these parameters a delayed and fluctuating changes was found, namely *Keap1* gene expression first decreased, and increased later in a dose dependent manner, but significant elevation as compared to the control occurred only at 24th hour. Time had also significant effect on the fluctuation. *Nrf2* gene expression also changed in a delayed manner. At 8th hour no significant changes were observed, but dual response was found later, in 16 and 24th hour, thus dose dependency also did not appear. In the T3 group *Nrf2* expression was lower at 16th hour, which was followed by an induction in the T1 group at 24th hour. *Keap1* expression was also induced at 24th hour in the T2 and T3 groups. These changes caused by sublethal doses, and mild oxidative stress can be hypothesize according to the hierarchical model of oxidative stress.

In fish species phospholipid-hydroperoxide glutathione peroxidase plays an important role in the antioxidant defense system, which is encoded by the *Gpx4a* and *Gpx4b* genes. Changes in the expression of these genes were different, depending on the period of exposure. *Gpx4a* elevated, while *Gpx4b* decreased at 8th hour by T-2 toxin exposure, which was followed by an induction, which was occurred only at low dose and at 16th hour, while in case of the higher doses the induction appeared in a delayed manner, at 24th hour. This difference can be related to the induction or inhibition of Keap1-Nrf2 pathway.

There is limited data in the literature for the effects of T-2 toxin in fish species, even in subchronic long term studies. Nevertheless, the presented results can be supported by the results of a previous study of our research group, where subchronic exposure of T-2 toxin resulted in mortality, but other results of changing parameters of oxidative stress can be compared only indirectly, because of the difference in the period of time of the exposure.

The single oral dose of T-2 toxin resulted in significant differences between the sampling times in each treatment groups in the parameters of lipid peroxidation, glutathione redox system and the expression of the regulating genes. These changes are not only triggered by the mycotoxin treatment, but appeared in the control group, as well. This can be in connection with the biological circadian rhythm, the single feed intake, and other unknown factors.

Also, it is important to note, that the aim of this study was not related to the circadian rhythm, the applied lightning regimen was 12:12 hour, which was different than the natural day-night period. However this difference affected all of the experimental groups, thus the effect of T-2 toxin as compared to the control can be studied accurately. The natural circadian rhythm could have been disturbed by the light regimen, which can affect the members of the antioxidant system in protein and mRNA levels, as well.

The common effect of treatment and time was also proved statistically in several parameters, which indicates that not only the dose, but the time of exposure is also key question in the developing changes. Other studies also described dose- and time- dependent effects of T-2 toxin, but mainly in *in vitro* studies.

3.1.2 Summary of short term effect of DON exposure

There is no available LD_{50} value for DON for common carp. Thus, doses were chosen based on literature which reach the toxic level in other animals. The applied doses are presented in table 2.

2. table The applied DON doses in each treatment group (mg/kg body weight)

Treatment group	Control	D1 (low)	D2 (medium)	D3 (high)
DON	< 0.02	0.13	0.31	1.75

In the 24 hour period mortality did not occurred in any group, which can be related to the lower toxicity of DON as compared to T-2 toxin.

Parameters of the initial phase of lipid peroxidation was induced by DON exposure, CD and CT levels elevated at 16th hour in all doses, and D3 also induced the terminal phase at 16 and 24th hour.

Based on the present changes in lipid peroxidation, DON induced mild ROS formation as effect of the dose range applied, and those have medium level of oxidative damage of the lipids in the 24 hour period. In conclusion lipid peroxidation was induced by T-2 toxin in a milder way in the 24 hour experiment as compared to DON.

Although, parameters of antioxidant defense showed a rapid and marked response to ROS formation in all of the mycotoxin challenged groups. GSH content showed elevating trend in all experimental groups as compared to control at 16th hour, where D3 caused significantly higher level as compared to the control. GPx activity also showed elevated levels at 16th hour, and the difference was significant as compared to the control in the D2, and D3 groups. However, it is important to note, that the results suggest effect of not only dose, but also time of exposure, and also the common effect of these factors. After nearly complete absorption of mycotoxins from the intestine (16 hour transit time), these parameters turned back to the control level by the 24th hour sampling.

In the 24 hour experiment, the 16th hour appeared as a key time of exposure, which can be related to the transit time of feed particles in the gastrointestinal tract, which determines the available time for the absorption of mycotoxins. This is supported by the induction of both lipid peroxidation processes and glutathione redox system at the 16th hour, which parameters turned back to the control level at 24th hour.

Expression of genes encoding the proteins play role in the Keap1-Nrf2 pathway were also measured. *Keap1* was induced in D2 dose at all sampling time, while D1 and D3 doses induced dual response. *Keap1* gene expression decreased at 8th hour in D1 group, which was followed by an elevation at 16th hour in D1 and D2 groups, and at 24th hour only in the D2 group. However, the highest, D3, dose revealed only minor changes in the 24 hour period. *Nrf2* also showed a dual response for DON exposure. At 8th hour D1 resulted in inhibition, while D2 and D3 resulted in induction, which was followed by a decreasing, dose dependent, trend at 16th hour. However, this was occurred only at the highest dose, D3, at 24th hour, but dose dependent trend was also observed. In summary, *Keap1* and *Nrf2* genes showed dual response, similarly to the T-2 toxin exposure, but in a different trend, but neither of them showed unequivocal correlation to the doses or time.

Gpx4a and *Gpx4b* expression was inhibited at 8th hour by DON exposure, which was followed by induction. This was occurred at the low dose, D1, at 16th hour, while in case of the higher doses, D2 and D3, the induction appeared in a delayed manner, at 24th hour. The observed changes were similar to T-2 toxin, but in a less marked manner, because the elevation was not significant as compared to the control, only in some doses and sampling time.

There is limited data in the literature about the effects of DON in fishes, even in subchronic long term studies. Nevertheless, the presented results can be supported by the results of a previous study of our research group, where subchronic exposure of DON resulted in mortality, but other results, such as changing of the parameters of oxidative stress, can be compared only indirectly, because of the difference of the time of the exposure.

The single oral dose of DON resulted in significant differences between the sampling times in each treatment groups in the parameters of lipid peroxidation, glutathione redox system and the expression of regulating genes. These changes are not triggered by the mycotoxin treatment only, but appeared in the control group, as well. This can be in connection with the biological circadian rhythm, the single feed intake, and other unknown factors as well, as was mentioned previously.

The common effect of treatment and time was also proved statistically in several parameters, which indicates that not only the dose, but the time of exposure is also key factor in the developing changes. Other studies also described dose- and time- dependent effects of DON, but mainly in *in vitro* studies.

3.2 Age-dependent experiments with broiler chickens

The transit time was observed via methyl orange feed consumption, which was available *ad libitum* for both age-group and the first appearance of the colored excreta indicates the period of passage through the gastrointestinal tract. The transit time was 5 hours 40 minutes in 1–week-old, and 5 hours 45 minutes in 3–week-old chickens.

During the 24 hour experiment the chickens had free access to the control or artificially contaminated feed and water. The daily feed intake and the transit time both can affect the first manifested changes in the observed parameters as the effects of T-2 toxin or DON. The time of the beginning of the feed intake and the transit time determinates the available time for mycotoxin absorption, which can be correlated with the changes in the biochemical parameters and gene expression induced by the mycotoxin exposure. Nevertheless, this can be relevant only in the first part of the trial and the first changes can be inked to it, as the continuous feed intake changes the effects in the parameters were measured.

The applied doses in the age-dependent trial with broiler chickens is showed in table 3.

3. table The applied doses in the age-dependent experiment with broiler chickens

Dose in feed (mg/kg feed)		
Control	T-2 toxin	DON
T-2 < 0.02; DON < 0.02	5.77	4.86
1-week-old (mg/body weight kg)		
Control	T-2 toxin	DON
T-2 < 0.02; DON < 0.02 (mg/kg feed)	1.35	1.75
3-week-old (mg/body weight kg)		
Control	T-2 toxin	DON
T-2 < 0.02; DON < 0.02 (mg/kg feed)	0.77	1.29

3.2.1 Summary of short term effect of T-2 toxin exposure in one-week-old broiler chickens

In the experiment, did not occur mortality in one-week-old chickens, as effect of T-2 toxin exposure. This founding is supported by an earlier study of our research group with similar dose range. Poultry species in general, especially chicken is less sensitive for trichothecene mycotoxins, nevertheless T-2 toxin is considered as the most toxic member of this mycotoxin family.

Well known effect ot trichothecene mycotoxins is feed refusal, which was also observed in this trial, as the applied dose of T-2 toxin (1.35 mg/b.w.) resulted in 11.25% feed refusal in the 24 hour period.

Parameters of the initial and terminal phase of lipid peroxidation, CD and CT and MDA levels, were only slightly affected by the T-2 toxin. Although, the antioxidant defense showed a rapid response to mild level ROS formation in the T-2 toxin treated group. GSH content was significantly higher in all experimental group as compared to the control after 4^{th} and 8^{th} hour. GPx activity also showed elevated levels at 8^{th} hour. Thus, the glutathione redox system was activated within the first 8 hours of the experiment, but later turned back to the control level.

Several changes were also observed in the regulation of the glutathione redox system as effect of T-2 toxin exposure. *Gpx4* gene expression elevated at 8^{th} hour, then turned back to the control level. *Gss* expression showed similar changes, elevated in the first 8 hours, but decreased later, and it was significantly lower as compared to the control only at 24^{th} hour. This dual response was also observed in *Gsr* expression, which was elevated at 4^{th} hour, then decreased as compared to the control at 16^{th} hour.

In summary, short term effects of T-2 toxin in 1-week-old chickens revealed no significant changes in lipid peroxidation, but the glutathione redox system was activated quickly, but returned to the control level thereafter. Gene expression also showed changes, and the tendency was similar in all target genes. Induction was found in the first 8 hours, followed by a decrease. Continuous long term exposure of T-2 toxin possibly can cause decreased activity/amount of the members of glutathione redox system, via inhibition of gene expression, also this can induce "feedback" mechanisms, which could regulate the expressions of the above mentioned genes.

Effects of trichothecene mycotoxins on broiler chickens are widely described, but most of these results depend on subchronic, long term studies. As effect of T-2 toxin short- and long term *in vivo* and also in *in vitro* in primer hepatocyte culture in 48 hours, where the ROS production was elevated, which activated the enzymatic antioxidant system in gene expression and protein level, which supports my results of the 24 hour exposure.

In most of the measured parameters T-2 toxin exposure resulted in significant differences between the sampling times in each treatment groups. Previously it was reported, that GPx and GSH are regulated by the circadian rhythm in chicken brain, which possibly occurred in other tissues, such as in liver, as well.

Also, it is important to note, that the aim of this study was not related to the circadian rhythm, therefore continuous lightning regimen was applied, but this affected all of the groups, thus the comparison of the effect of T-2 toxin to the control can be accurately evaluated. The natural circadian rhythm could have been disturbed by the light regimen, which can affect the members of the antioxidant system in protein and mRNA levels, as well.

The common effect of treatment and time was also proved statistically in several parameters, which indicates that not only the dose, but the period of exposure is also key factors in the developing changes. Other studies with poultry species also proved dose- and time- dependent effects of the T-2 toxin in the antioxidant parameters.

3.2.2 Summary of short term effect of T-2 toxin exposure in three-week-old broiler chickens

In the experiment, did not occur mortality in three-week-old chickens, but 12.77% feed refusal was observed as effect of 0.77 mg T-2 toxin/kg feed exposure, similarly to the one-week-old chickens. This founding is supported by an earlier study of our research group with similar dose range. Poultry species in general but, especially chicken is less sensitive for trichothecene mycotoxins, nevertheless T-2 toxin is considered as the most toxic member of this mycotoxin family.

Parameters of the initial phase of lipid peroxidation, CD and CT levels, were not affected in the trial, but the terminal phase, MDA level was elevated at 8^{th} hour. This suggests that lipid peroxidation was induced quickly, but in a minor level. The observed changes were similar in trend to the one-week-old chickens.

The antioxidant defense showed a rapid response to mild ROS formation in the T-2 toxin treated group. GSH content was significantly higher in all experimental group as compared to control at the first 8^{th} hour. GPx activity also showed elevated levels at 8^{th} hour, which then turned back to the control level, but a reactivation was observed at 20^{th} hour, which did not occur in the one-week-old chicken.

In the regulation of the glutathione redox system changes were also observed as effect of T-2 toxin exposure. *Gpx4* gene expression did not change relevantly as compared to the control level. However, expression of *Gss* showed continuous elevation, which was significant as compared to the control at 12^{th}, 20^{th}, and 24^{th} hours. This tendency was contradictory to the observed trend in the one-week-old chickens in case of *Gss* expression. Expression of *Gsr* decreased at 16^{th} and 24^{th} hours, which was also different in the two age groups, because dual response was found in the one-week-old chickens.

Previously it was reported in different animal species that the age of the model animals influences the ROS production and also the synthesis of antioxidant proteins.

Effects of trichothecene mycotoxins on broiler chickens are widely described, but most of these results depend on subchronic, long term studies. Effect of T-2 toxin on ROS production and glutathione redox system was described in 3.2.1..

In most of the measured parameters T-2 toxin exposure resulted in significant differences between the sampling times in each treatment groups which is linked to the circadian rhythm, as it was described in 3.2.1. above.

The common effect of treatment and time was also proved statistically in several parameters, which indicates that not only the dose, but the time of exposure is also key factors in the developing changes. Other studies with poultry species also described dose- and time- dependent effects of T-2 toxin in the antioxidant parameters, which was mentioned in 3.2.1.

3.2.3 Summary of short term effect of DON exposure in one-week-old broiler chickens

In the experiment, mortality or feed refusal did not occur in one-week-old chickens, as effect of 1.75 mg/kg feed DON exposure. This founding is supported by an earlier study of our research group with similar dose range. Chicken is less sensitive for trichothecene mycotoxins, in particular to DON, and much less toxic than T-2 toxin.

Parameters of the initial and terminal phase of lipid peroxidation, CD, CT and MDA levels, did not change significantly as compared to the control in the 24 hour trial, which was similar to the effect of T-2 toxin. Indeed, the antioxidant defense also did not show response to DON exposure, which is in contrary to the effect of T-2 toxin in both age groups.

Even so, the regulation of the glutathione redox system responded to DON exposure quickly. *Gpx4* gene expression elevated at 8^{th} hour, then turned back to the control level, similarly to the effect of T-2 toxin in one-week-old chickens. Conversely, *Gss* expression showed a continuous decrease in trend, which was significant as compared to the control at 8^{th} , 16^{th} , 20^{th} , and 24^{th} hours, but elevated at 12^{th} hour, in contrary to all other sampling time, thus it showed a dual response, but the inhibition was more dominant. Dual response was also observed in case of *Gsr* expression, which elevated at 4^{th} and 12^{th} hour, then decreased as compared to the control at 16^{th} hour.

Effects of trichothecene mycotoxins in broiler chickens are widely described, but most of the changes related to lipid peroxidation are effects of T-2 toxin in the literature, and most of them based on subchronic, long term studies. Nevertheless, some studies describe elevated oxidative stress in broiler chickens as effect of feeding DON contaminated feed, which was related to the changes in the expression of hemoxygenase and xanthine-oxidoreductase genes. However, there are confusing data in the literature about the relation between DON exposure and ROS formation, and most of these studies described only DNA damage.

In summary, it can be concluded, that one-week-old chickens are less sensitive to DON, according the results of the changes in the markers of lipid peroxidation and glutathione redox system, however gene expression profiles showed more sensitive responses in the observed 24 hour period. It is important to emphasize that the decreasing tendency of gene expression of *Gss* was similar to the tendency as was observed in one-week-old chicken fed with T-2 toxin contaminated diet.

In most of the measured parameters DON exposure resulted in significant differences between the sampling times in each treatment groups. Previously it was reported, that GPx and GSH are regulated by the circadian rhythm in chicken brain, as it was mentioned previously, but such changes possibly occurs in the liver, as well.

As it was mentioned before, continuous lightning regimen was applied in the feeding experiments, but this affected all of the groups, thus the effect of DON exposure can be compare accurately to the control group, because the environmental parameters were the same in all treated groups. The natural circadian rhythm could have been disturbed by the light regimen, which may affect the members of the antioxidant system in protein and mRNA levels, as well in physiological processes.

The common effect of treatment and time was also proved statistically in several parameters, mostly in gene expression data, as lipid peroxidation and glutathione redox system did not change relevantly in DON groups compared to the control.

3.2.4 Summary of short term effect of DON exposure in one-week-old broiler chickens

In the experiment, did not occur mortality in three-week-old chickens, as effect of 1.29 mg/kg feed DON exposure. It is well known that chicken is less sensitive for trichothecene mycotoxins, in particular to DON, and less toxic as compared to T-2 toxin.

Parameters of the initial and terminal phase of lipid peroxidation, CD and CT and MDA levels, did not elevate, but slightly decrease as compared to the control in the 24 hour trial. In these parameters there was no major changes as effect of DON in one-week-old chickens. This difference between the age-groups can be in association with the different dose of DON per kg body weight, the rhythm of the feed intake during the day, and possibly the different activity of the antioxidant system. Thus, the different toxin exposure, and intensity of ROS production can be different in different age groups.

The antioxidant defense also did not show response to DON exposure in 3-week-old chickens, similarly to one-week-olds, in contrary to the T-2 toxin in both age groups, which can be linked to the lower toxicity of DON as compared to T-2 toxin.

Although the regulation of the glutathione redox system responded to DON exposure significantly, like in the younger age group. *Gpx4* gene expression elevated at 20th hour, similarly to the one-week-old group, but it was occurred later. *Gss* expression showed a continuous elevation in trend, which was significant as compared to the control group at 16^{th} and 20^{th} hours. The trends to the changes as effect of mycotoxin exposure were similar in both age groups, but the age-dependent changes were in contrary, in tendency. Dual response was observed in *Gsr* expression, which was the same in both age-groups as effect of both mycotoxin exposure, but the tendency and level of changes were different, thus neither of them showed unequivocal correlation to the dose or time.

Age-dependent changes can be affected by several factors, such as different levels in ROS production and protein synthesis, as was mentioned previously.

In summary, it can be concluded, that three-week-old chickens are less sensitive to DON, which statement supported by the changes in the markers of lipid peroxidation and glutathione redox sytem, and also by different changes as was observed in the younger agegroup. Indeed, lipid peroxidation levels were lower as compared to the control in the older age group, which is in association with the slightly induced antioxidant system by DON exposure.

However, gene expression showed sensitive responses in the observed 24 hour period. It is important to highlight that the tendency of gene expression of *Gss* was elevating, while *Gsr* revealed dual response.

The common effect of treatment and time was also proved statistically in several parameters, but glutathione redox system, also it did not change relevantly in DON groups compared to the control.

4 NEW SCIENTIFIC RESULTS

Evaluating the effects of the applied doses of trichothecene mycotoxins on common carp, in the observed 24 hour period, I concluded that:

1. The glutathione redox system activated as effect of both DON and T-2 toxin exposure, while lipid peroxidation induction occurred only as effect of DON.
2. Gene expression of *Keap1* and *Nrf2* changed in both mycotoxin treated group, but neither of them showed unequivocal correlation to the doses or time.
3. Gene expression of *Gpx4a* and *Gpx4b* showed dual response at the beginning, which was followed by induction, and delayed tendency was found in the higher doses of both DON and T-2 toxin.

Evaluating the age-dependent effects of trichothecene mycotoxins in broiler chickens, in the observed 24 hour period, I concluded that:

4. As effect of T-2 toxin, the glutathione redox system was activated in the first 8 hours of exposure in both age groups, but not as effect of DON. On the other side lipid peroxidation was not induced as effect of DON or T-2 toxin in either of the two age groups.
5. *Gss* gene expression showed opposite direction in tendency in the two age-groups, it decreased in one-week-old, and elevated in three-week-old chickens as effect of both trichothecene mycotoxins.
6. Gene expression of *Gsr* also changed as effect of both mycotoxins and in both age group, but it did not show unequivocal correlation to the doses or time.

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5 CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

The aim of the presented studies was to evaluate the *per os* effects of T-2 toxin or DON on the initial and terminal phase of lipid peroxidation processes, the amount/activity of members of the antioxidant system and the changes in gene expression of the regulation of glutathione redox system in common carp juveniles and broiler chicken of different age groups, in short term mycotoxin exposure studies. In the 24 hour studies the effect of the period of exposure was also observed in the measured parameters.

Evaluation of transit time of feed particles in the gastrointestinal tract was also aimed, in common carp and broiler chickens, which may influence the available time for the absorption of mycotoxins from the intestine. Based on the period of transit time, the study of correlations between the first observed emerging changes in the parameters was also the objective of present studies.

Single oral treatment was chosen by gavage, in case of experiments with common carp, while *ad libitum* feeding design was carried out with different age groups of broiler chickens.

Parameters of the lipid peroxidation were measured in the liver of both animal species, including the markers of initiation phase (conjugated dienes and conjugated trienes) and metastable end-product of the terminal phase (malondiadehyde). In addition the changes of parameters of the biological antioxidant system (glutathione peroxidase activity and reduced glutathione content) was also measured.

For evaluating the changes in the regulation of glutathione redox system, gene expression studies were carried out. Based on the literature and gene bank databases, the available gene sequences were N*rf2*, *Keap1*, *Gpx4a* and *Gpx4b*, for common carp, while in case of broiler chicken *Gpx4*, a *Gss* and *Gsr* genes were chosen.

Transit time was 16 hours in common carps at 19±1 ^{o}C, while it was approximately 6 hours in both age groups (one and three-week-old) of broiler chicken. Genetic selection through hundreds of generations aimed to reach the highest growth rate in chicken next to the best feed conversation ratio, which indicates intensified metabolism. Thus nowadays the metabolism of broiler chickens is extremely intense. In contrary, common carp populations has been less intensified and selected. Otherwise fish are poikilothermic animals, thus their metabolic activity is determined by the water temperature.

The applied doses were 0.15; 0.33 and 1.82 mg T-2 toxin/kg body weight and 0.13; 0.31 and 1.75 mg DON/kg body weight in the experiments with common carp. In case of broiler chicken the applied doses were 1.35 mg T-2 toxin/kg body weight, and 1.75 mg DON/kg body weight in one-week-old, while 0.77 mg T-2 toxin/kg body weight, and 1.29 mg DON/kg body weight in three-week-old birds.

Based on the above mentioned doses it can be concluded that, the applied doses of the trichothecene mycotoxins were in a similar dose-range in both animal species. The aim of using sufficiently high doses was to induce acute toxicity. However, it is important to note that, the applied 5.77 mg DON/kg feed dose in broiler chicken experiments can be appear in the nature in some years, while the applied 4.86 mg T-2 toxin/kg feed dose represents an extremely contaminated feed. In case of common carp, the applied T-2 toxin doses (10.79; 23.67; and 130.82 mg kg feed) do not occur in the practice, while the low and medium DON doses represents extremely contaminated feeds (9.0; 22.15; and 125.92 mg/kg feed).

It is well known that, broiler chickens are less sensitive to trichothecene mycotoxins, and also the least sensitive among domesticated poultry species. Sensitivity of fish species is less known in relation to trichothecene mycotoxins, however common carp described as a moderately sensitive species in general, but the available data specific about mycotoxins is limited.

Mortality occurred only in the highest applied dose of T-2 toxin in common carp, which was 19%. Though, mortality did not appeared in broiler chickens, but feed refusal was observed in both age groups as the effect of T-2 toxin, in similar level, which was approximately 10%.

DON induced lipid peroxidation as effect of all applied doses in common carp, while T-2 toxin had only mild effect on these parameters. This can be in association with the higher induction of ROS production by DON or its metabolites in the liver as compared to T-2 toxin, despite the similar chemical structure of the two mycotoxin. Although, the intensity of ROS formation was different, and the antioxidant glutathione redox system was markedly induced by both mycotoxin. The sampling at 16^{th} hour appeared a key time of exposure in changes of the biochemical parameters in the experiments with common carp, which can be correlated to the measured transit time, thus this period available for the absorption of mycotoxins and transported to the liver, thus develop measurable effects after the feed intake.

The regulation of glutathione redox system was also affected by the mycotoxin exposure. Expression of *Gpx4a* and *Gpx4b* genes showed elevation, after an early inhibition, which induction occurred in a delayed manner at the higher doses. This can be related to the difference in the period of absorption and metabolism of the different mycotoxin doses.

It is important to emphasize that DON and T-2 toxin showed similar effects and tendencies in the measured parameters of glutathione redox system and gene expression, which indicates that changes in the lipid peroxidation itself is not an adequate indicator of the effects of mycotoxins, since the activated antioxidant system can obscure the initiation or progression of lipid peroxidation, as it was observed in case of T-2 toxin in common carp.

In the age-dependent experiments with broiler chickens lipid peroxidation did not change relevantly in the mycotoxin treated groups in both age groups. Differences between the changes in the two model species can be in association with the difference in intensity and pathways of metabolism of the mycotoxins in the liver.

However, T-2 toxin activated the glutathione redox system quickly in both age groups in the first 8 hours, which was followed by a re-activation in three-week-old animals at 20^{th} hours. This effect was observed in common carp as well, as the glutathione redox system is activated by the mycotoxin exposure, thus lipid peroxidation do not elevate, even if ROS forming is induced by the mycotoxin exposure. Nevertheless, DON did not activate the glutathione redox system in broiler chickens, which can be linked to the high tolerance of trichothecene mycotoxins. DON may induce only minor level of ROS formation as compared to T-2 toxin, but this level was adequate for triggering to induce the regulation of the glutathione redox system at gene expression level.

Gpx4 showed only minor changes, and did not show unequivocal correlation to the mycotoxin doses or time showed unequivocal correlation to the time or age. Gene expression of *Gpx4* elevated in both mycotoxin group at 8^{th} hour in one-week-old animals, but decreased in T-2 toxin at 20^{th} hour, while it only showed elevation at 20^{th} hour in the DON treated three-week-old chickens.

Dual response was observed in *Gsr* gene expression, which was the same in both age-groups as effect of both mycotoxin exposure, but the tendency and level of changes were different, thus neither of them showed unequivocal correlation to the doses or time.

I would like to highlight that the trends in *Gss* gene expression in the mycotoxin exposure were similar in both age groups, but the age-dependent changes were in contrary in tendency in this parameter. It decreased in one-week-old, and elevated in three-week-old animals as effect of both mycotoxin exposure. It can be concluded that, among the measured molecular markers, *Gss* appeared the most efficient indicator to evaluate the effects of DON or T-2 toxin in age-dependent aspect.

The observed changes in gene expression support our hypothesis, that T-2 toxin induces ROS formation quickly, but it is eliminated rapidly by the antioxidant system, while DON induces ROS formation mildly, but continuously, which reaches a trigger point to induce the glutathione redox system in mRNA level, but not in protein level.

5.2 Suggestions

The sequences of the studied genes in the thesis are completely available for both applied model animal species recently, however, some of them were not available at the beginning of my PhD studies. Thus, based on present results, I suggest to continue the research with extended set of target genes in mycotoxin studies, with other type of mycotoxins, as well.

I also suggest to evaluate the effects of multi-mycotoxin exposure, as mycotoxins occur in a mixture in animal feed, and the results can imply additive, synergistic or antagonistic effects of common mycotoxin effects.

Lastly, I would like to suggest to design experiments which aim to eliminate the negative effects of mycotoxins and prevent the emerging oxidative stress via natural antioxidants or antioxidant mixtures. Thus, the results can provide more information about natural active substances to support the farm animals in the elimination of mycotoxins.

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