Final report of the

OTKA proposal ‘PD-104823’

‘Investigation of the effects of some fusariotoxins on gene-expression, lipid peroxidation processes and glutathione redox system in chicken and common carp’

Written by

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Introduction

Cereals, the main components of complete feeds for monogastric farm animals, may be contaminated with mycotoxins, secondary metabolites of moulds. *Fusarium* moulds, the most important genus infecting the crops before harvest in temperate climate, produce several mycotoxins, among them the trichothecenes are one of the largest groups.
The occurrence of *Fusarium spp.* is spreading all over the world and the mycotoxin-producing conditions vary yearly according to climatic conditions. Approximately 180 trichothecene derivates have been isolated and characterized (Pestka and Smolinski, 2005).
Chemically, trichothecenes are closely related, low-molecular-weight sesquiterpenoids and usually contain an epoxide ring at C-12 and C-13 and a double bond at position C-9 and C-10 which both are important for their toxicity (Desjardins et al., 1993).
Trichothecenes have been classified into four groups (types A-D) according to their chemical properties and the producing fungi (Ueno, 1977).
Fusarium species produce 'Type A' (e.g. T-2 toxin) and 'Type B' trichothecenes (e.g. deoxynivalenol [DON]), which are distinguished by the absence or presence of a carbonyl group at C-8 position, respectively.
In most countries, a 'Type B' trichothecene, deoxynivalenol (DON), and its main metabolite 15-acetyl DON (15-AcDON) are the most frequent mycotoxin in cereals (Binder et al., 2007), while a 'Type A' trichothecene, T-2 toxin and its main metabolite HT-2 toxin are considered as the most toxic trichothecenes (Bamburg et al., 1968).
High concentration of DON can be found in cereal grains following rainfall at the time of flowering, which might result in so-called 'Fusarium years' (Dänicke, 2003), as years were 2010 and 2015 in Hungary, with higher maximum and mean DON concentrations than usual.
It also has to be noted that most of the *Fusarium* species have the ability to produce more than one fusariotoxin, depending on genetic factors and various environmental conditions (Jouany, 2007).
For DON in complete feeds the European Union has set guidance value of 5 mg/kg feed (2006/576/EC), but for T-2 toxin and HT-2 toxin contamination no recommendations were set for a long time. In year 2013, maximum of 0.25 mg/kg feed was set for T-2 toxin and HT-2 toxin as recommended for farm animals according to the EU proposal (2013/165/EU), while Eriksen and Pettersson (2004) suggest 0.5 mg/kg feed concentration level as tolerable level.

The main aims of the OTKA proposal were the followings:

- To enhance the relative little information about the effect of fusariotoxins on fish species, namely in common carps, focusing on the glutathione redox system and on lipid peroxidation processes.
- To investigate the potential dose-dependent effects caused by the different ('Type A' and 'Type B') feed-borne trichothecene mycotoxins in chickens and carps.
- To investigate the potentially more pronounced adverse effects of the combination of mycotoxins (to explore their potentially synergistic interactions) in chicken and carp.
- Based on the analysis of the expression of glutathione-peroxidase (*gpx4*) gene and the glutathione synthetase gene involved in reduced glutathione (GSH) synthesis in this project to get answers,

(i) whether in the background of the elevation of GSH concentration there is the increased synthesis of this tripeptide or this effect is just a result of the release of the GSH deposits,
(ii) moreover whether the increase of the GPx activity is a result of the increased synthesis of the enzyme, or the elevated co-substrate supply facilitate the more efficient protection of the enzyme against the hydrogen peroxide and organic hydroperoxides.

Materials and methods

In the frame of this OTKA proposal two vertebrate species, the domestic fowl (*Gallus domesticus*) (age: 3 weeks old) and common carp (*Cyprinus carpio carpio morpha domestica*) (age: yearling) were investigated.
In the experiments with chickens the biochemical effects of fusariotoxins were investigated in blood (plasma and red blood cell [RBC] hemolysate), and *post mortem* in those organs which play role in the metabolism (liver and plasma), excretion (kidney) or accumulation (liver and spleen) of the mycotoxins. In the experiments with carps, *post mortem* liver (hepatopancreas) samples were collected at each sampling time.
For the experiments T-2 toxin was produced experimentally on maize by *Fusarium sporotrichioides* strain NRRL 3299, and deoxynivalenol (DON) was produced by *Fusarium graminearum* strain NRRL 5883.
The biochemical analyses of the different tissues collected, namely measurement of malondialdehyde (MDA) (Mihara et al., 1980) and reduced glutathione (GSH) concentration (Rahman et al., 2007); glutathione-peroxidase (Matkovics et al., 1988); glutathione reductase (Smith et al., 1988) and glutathione-S-transferase activity (Mannervik et al., 1985) were done. Analysis of conjugated dienes and trienes were done in liver samples according to the AOAC (1984) method. The GSH concentration and GPx activity was calculated to protein concentration determined with biuret method in case of blood samples and with Folin-Ciocalteu phenol reagent in case of the tissue homogenate 10.000 g supernatant fraction (Lowry et al., 1951).
For measurement of gene expression, method of RNA purification had to be optimized both for carp and chicken. We had to design primers, for the target genes and also for endogenous control genes. In case of chicken, glutathione syntethase (GS) and glutathione reductase (GR) and a glutathione peroxidase (GPX4) were chosen as target, and GAPDH as control genes. In case of carp, B-actin was chosen as control, and *gpx4a* and *gpx4b* as target genes. For qPCR reactions SYBRGreen method was chosen for carps, and TaqMan probe method, for multiple reactions for chicken. Statistical evaluation of the data (one-way ANOVA, Student's t-test) was done by GraphPad Prism 5.04 (GraphPad Inc.) statistical software. Two-way analysis of variance (ANOVA) was performed by MedCalc for Windows 12.3 (MedCalc Software).

Experiments with chickens

Long-term preliminary experiment with T-2 toxin and DON

According to the research plan of the proposal a preliminary experiment was carried out with 2 week-old broilers, in order to decide if DON or T-2 toxin load (higher than the EU guidance level) cause feed refusal in animals. These data were important for deciding whether during the further trials it would be necessary to have a pair-fed control parallel to the group with the highest toxin exposure.
A total of 42 Cobb 500 broiler cockerels was divided into 3 groups: a control, a T-2 toxin treated (3.38 mg/kg feed) and a DON treated one (3.52 mg/kg feed). The experiment lasted for 4 weeks, with sampling at start of the experiment (n=6 served as absolute control), at 14th and 28th day of mycotoxin exposure. At every sampling six birds were exterminated from each group.
Both mycotoxin treatments caused decrease in feed intake, the feed consumption in the T-2 toxin and DON treated groups was 70.8% and 84.3% of the control, respectively.
These data strengthen the previous hypothesis, that during the further trials it would be necessary to have a pair-fed control parallel to the group with the highest toxin exposure.

More information can be found in: Balogh et al. (2015) J. Poultry Sci. 52: 176-182.

Short-term experiment with T-2 toxin and DON

Based on the results of the previously mentioned trial with broiler chicken, a short-term 3-days-trial was carried out with T-2 toxin and DON. To obtain more precise data, during the first day, samplings were done not only in every 12th hour (as it is stated in the proposal), but at start of the experiment, at 2nd, 4th, 8th, 12th, 16th, 20th and 24th hour of mycotoxin exposure, followed by samplings at every 12th hour.
A total of 171 three-week old Cobb 500 broiler cockerels was divided randomly into three groups: control, T-2 toxin treated (4.86 mg/kg feed) and DON treated (5.77 mg/kg feed), consisting of 57 birds and each of these treatments was divided into three groups (n=19). At all samplings 5 randomly selected birds were exterminated from each group (at start of the experiment 6 animal served as absolute control).
Although the T-2 toxin treatment caused 8.6% decrease in feed intake, the DON treated groups consumed 2.2% more feed than the control during this short-term experiment.
GSH concentration of the liver in birds of T-2 treated group was significantly higher than the control at 4th, 8th, 20th and also at 36th hour of mycotoxin exposure. In the same group parallel to the co-substrate (GSH) surplus significantly elevated GPx activity was measured at 4th, 8th and 20th hour of experiment. In the DON treated group significantly higher GPx activity was measured compared to the control at 48th hour, but 12 hours later it sank markedly.
Due to the rapid increase of the amount and activity of the glutathione redox system in liver due to the T-2 toxin exposure, the biological antioxidant system was able to eliminate the harmful free radicals, keeping the lipid peroxidation processes at physiological level, as shown by the MDA concentration, which did not change from the control during the experiment.
The expression of phospholipid hydroperoxide glutathione peroxidase (GPX4) and glutathione-reductase (GR) genes in control group showed circadian changes during the first 24 hour-period, which are similar to the changes in enzyme activities.

The gene-expression of glutathione-S-transferase (GST) in the liver showed higher values (up-regulation) in DON treated groups than the control during the first 8 hours of mycotoxin exposition. Daily fluctuation of gene expression was also measured in the control group, with significant up-regulation in the morning period.

Long-term broiler chicken experiment with different doses of T-2 toxin and DON

A total of 180 Cobb 500 broiler cockerels was divided into 9 groups: a control ('C'), 3 groups which consumed T-2 toxin contaminated feed at different concentrations (0.5; 2.5 and 5.0 mg T-2 toxin/feed in group 'T1', 'T5' and 'T10'), 3 groups which consumed DON toxin contaminated feed at different concentrations (5.0; 10.0 and 15.0 mg DON/feed in group 'D1', 'D5' and 'D5'), and also 2 pair-fed groups ('PT' and 'PD'), which consumed the same amount of feed as the two groups with the highest mycotoxin exposure on the previous day.
The experiment lasted 2 weeks, with samplings at the start of the experiment (n=6 served as absolute control) and at 3rd, 7th and 14th days of mycotoxin exposure. At every sampling six birds were exterminated from each group.
T-2 toxin treatments caused decrease in feed intake in case of 'T5' and 'T10' groups (3.9% and 9.5%, respectively), while none of the DON groups showed the same feed refusal. The reduced feed intake led to lower body weight in both T-2 toxin treated groups compared to control ('T5' *vs*. 'C': $p<0.05$). Comparing to control, significantly ($p<0.05$) higher relative liver weight was measured in 'T5' group after 2 weeks of mycotoxin exposure.
The highest dose of long-term T-2 toxin exposure activated the glutathione redox system, as measured by the significantly ($p<0.01$) higher GPx activity in blood plasma on day 14, comparing to the control. In the same group this activation helped to maintain the level of meta-stable end product of lipidperoxidation processes, as measured by the significantly ($p<0.01$) lower MDA concentration compared to control at day 3 and day 7.
The medium dose of T-2 toxin significantly increased the GSH concentration and GPx activity in liver at day 3, which blocked the initial phase of lipid peroxidation efficiently, as measured by the significantly lower levels of CDs and CTs compared to control. Later, at day 7, depletion was observed in GSH concentration and GPx activity in livers of 'T5' group, while at the same time the CDs and CTs showed the highest values in the 'T10' group compared to control. The elevation of the initial phase lipid peroxidation products in liver at day 7, led to an increase of amount and activity of glutathione redox system at day 14 in 'T10' group, which was able to eliminate the harmful effect of T-2 toxin, as measured by the significantly not different CDs and CTs values and MDA concentration.
The medium dose of DON significantly increased the GSH concentration in liver at day 3, which – as earlier mentioned in case of 'T5' group – led to efficient blocking of the initial phase of lipid peroxidation as shown by the significantly lower CD and CT values compared to control. Later, at day 7, a depletion was observed in GSH concentration and GPx activity in livers of 'D5'group.
In kidney at day 7, significantly elevated MDA concentrations were measured in 'T5' and 'T10' groups, while in case of the highest mycotoxin concentration increased GSH concentration was also observed.

Short-term layer experiment with different doses of T-2 toxin

A total of 24 Bovans Goldline laying hens (49 weeks of age, 90% daily egg production) was distributed to 4 groups: a control and three treated groups fed with 5, 10 or 15 mg T-2 toxin/kg feed. Blood samples were taken from the cubital vein before initiating the treatments (n=6, as absolute control), and 4, 8 and 12 hours thereafter (n=6, from each experimental group). After 12 hours of treatment all birds were exterminated and post mortem liver, spleen and kidney tissue samples were taken.
Blood plasma MDA concentration decreased time-dependently in all T-2 toxin loaded groups, but it was significantly lower only in the group fed with 15 mg T-2 toxin/kg feed as compared to the control. GSH concentration in blood plasma changed time-dependently ($P<0.001$). GPx activity in blood plasma did not show time- and dose-dependent changes.
CD and CT levels were significantly higher at the end of the trial in the 15 mg T-2 toxin/kg feed treatment in liver. However, MDA concentration did not change in the tissue homogenates. GSH concentration and GPx activity were significantly higher at the highest dose T-2 toxin in liver homogenates, but not in spleen or kidney. The results suggest that short-term oral exposure of T-2 toxin initiates lipid oxidation in the liver at the highest dose applied, which activates the antioxidant defence through the Nrf2 transcription factor-mediated process required for activation of the antioxidant gene-cluster (Köhle and Bock, 2007), thus the antioxidant defence system was able to eliminate the free radicals, thus the process did not terminate.

More information can be found in: Bócsai et al. (2015) Europ. Poultry Sci. 79: 1-8.

Long-term broiler experiment with different combined doses of T-2 toxin and DON

A total of 162 Cobb 540 broiler cockerels was divided into 5 groups: a control (‘C’), 3 treated groups which consumed T-2 toxin and DON contaminated feed in combination at different concentrations (‘T1D1’ – the same amount of T-2 toxin and DON as the EU guidance level [0.25 mg T-2 toxin and 5.0 mg DON/kg feed], ‘T5D2.5’ – 5 times higher than the EU guidance level in case of T-2 toxin and 2.5 times higher than the EU guidance level in case of DON [1.25 mg T-2 toxin and 12.5 mg DON/kg feed], and ‘T10D5’ – 10 times higher than the EU guidance level in case of T-2 toxin and 5 times higher than the EU guidance level in case of DON [2.5 mg T-2 toxin and 25 mg DON/kg feed]), and also a pair-fed control group (‘PF’), which consumed the same amount of feed as the group with the highest mycotoxin exposure on the previous day.
The experiment lasted for 2 weeks, with samplings at the start of the experiment (n=6 served as absolute control) and at 1st, 2nd, 3rd, 7th and 14th days of mycotoxin exposure. At every sampling six birds were exterminated from each group.
In case of spleen both combination of the applied fusariotoxins increased significantly the amount (GSH) and activity (GPx) of glutathione redox system at 7th day of exposure. This increase in the amount/activity of the biological antioxidant system was able to protect the tissue against the effect of reactive oxygen species, but not in dose-dependent manner, because in case of the lowest dose (‘T1D1’) significantly elevated MDA concentration was measured at the same time.
In kidney similar effects were observed at 7th day as in case of spleen. The medium and high concentrations of the combined mycotoxins resulted significant increase in GPx activity, which protected the tissue against the lipid peroxidation processes almost until the end of the treatment. But at 14th day of mycotoxin exposure significantly elevated MDA concentrations

were measured in case of the lowest and medium doses of mycotoxins as compared to the control group.
In blood plasma rapid increase of GPx activity was observed as compared to control in case of the highest doses ($p<0.05$ at 1st day), which was followed by the same effect of all the three applied combinations of mycotoxins at 2nd and 3rd day.
The amount of CDs and CTs in the liver were significantly lower in the groups fed with the medium and highest combined doses of trichothecenes as compared to the pair-fed control group at the end of the experiment (day 14). In case of the applied highest combined doses of trichothecenes the level of CTs were significantly lower than in control at 3rd day of exposure.
In the liver the GSH concentration showed dose dependent changes during the experiment. At 3rd day of mycotoxin exposure significantly higher GSH concentration was measured in the group fed with the medium combined doses of mycotoxins, as compared to the control and to the group fed by the lowest applied doses. In the group fed by the highest combined doses of trichothecenes the GSH concentration was significantly higher than in the other groups not only at 3rd, but at the 14th day of mycotoxin treatment.
In GPx activity of the 10.000 g supernatant fraction of liver homogenate similar tendency was found as its co-substrate, but the differences were not significant.
The liver MDA concentration was significantly lower in the group fed by the medium combined doses of mycotoxins as compared to the lowest dose at 3rd day. At 7th day the MDA concentration of the liver was significantly lower in the group fed by the lowest combined doses as compared to the other groups.
GPX4 is described in avian species far more important in the antioxidant defence system than mammals, where GPX1 plays a major role. Elevated GPX4 expression was measured at 1st and 7th day in all treated groups, and showed significant elevation in case of the highest dose (‘T10D5’) on day 1, 2, 3 and 7. Reduced glutathione (GSH) plays a major role in the antioxidant system and also in xenobiotic transformation, protecting cells from oxidative stress. Gene expression of GS, which is the final enzyme in the GSH biosynthesis pathway, showed an inhibition on day 2 in all treated groups which was followed by a continuous elevation. In case of the highest dose (‘T10D5’) on day 3 and 14 this elevation in gene expression was significant as compared to controls. During the observed period expression of GR, which catalyses the reduction of glutathione disulphide (GSSG) to GSH, repairing its antioxidant abilities, showed elevation at 2nd and 3rd days in case of the medium and highest combined doses (‘T5D2.5’ and ‘T10D5’), but decreased later. The 14-day mycotoxin exposure probably emerged the ROS formation, which resulted in the presented responses in the regulation of the antioxidant system. GS showed an inhibition in the beginning of the trial, which was followed by an induction, later. However, GR showed a moderate, but not significant decrease on day 1. The highest applied dose caused the highest induction, which was in parallel with the time of the exposure in case of GS, while it was also in parallel with the time until day 7 in case of GR and GPX4, but in these cases a decrease was observed later, on day 14.

Experiments with common carp

Long-term experiment with T-2 toxin and DON

During this long-term experiment the effect of 'Type A' trichothecene mycotoxin, 4.11 mg T-2 toxin and 0.45 mg HT-2 toxin/kg feed and 'Type B' trichothecene mycotoxin, 5.96 mg DON and 0.33 mg 15-acetyl DON/kg feed treatments was investigated in common carp. The feeding trial lasted for 4 weeks. A total of 144 young, one-year-old (23.26±6.86 g body weight [b.w.]) common carps (Szarvasi P34 hybrid) were obtained from a commercial fish farm (ÖKO 2000, Akasztó) and divided into 6 aquaria (150 L each). All aquaria were filled up with aerated de-chlorinated tap water and were connected to a re-circulating system. Light regimen was maintained at a 12:12 h light:dark schedule. The aquaria were cleaned every day by siphoning out the debris and faeces.
After a week of adaptation period, at the initial of the experiment three groups were formed, a control ('C', 2 aquaria), a T-2 toxin treated ('T', 2 aquaria) and a DON treated ('D', 2 aquaria) group were formed. Each treatment was carried out in two replicates.
Six carps were exterminated from each group weekly.
The applied doses of mycotoxins increased the average mortality in the treated groups compared to control (8.3%) during the experiment. In the T-2 treated group was the highest mortality rate (29.2%), but the mortality rate of DON treatment (16.7%) also was twice as big as of control.
The T-2 toxin treatment increased the GSH concentration in liver at 2nd week of mycotoxin exposition, but later, at 3rd week there was a significant decrease, then increased again during the last week of the trial. Feeding the DON contaminated diet caused significantly elevated GSH concentration at 4th week of mycotoxin exposition, and the glutathione-peroxidase activity was also higher than the control.
Although at the 2nd week T-2 toxin exposition elevated the GPx activity compared to the control, at the 3rd week it resulted significant decrease in glutathione concentration and glutathione-peroxidase activity of liver as compared to the control.
These results suggest continuous activation of the glutathione redox system as effect against oxidative stress caused by T-2 and HT-2 toxin exposure.
The activity of glutathione-S-transferase (GST) in liver of the T-2 toxin treated group was significantly lower than the control at 2nd and 3rd week of mycotoxin exposition.
No significant changes were found in the MDA concentration of liver.
The results showed that the biological antioxidant system was able to eliminate the harmful prooxidant effect of T-2 toxin or DON in common carp.

More information can be found in: Pelyhe et al. (2016) Mycotoxin Res. 32: 77-83.

Short-term common carp experiment with different doses of T-2 toxin and DON

Based on the results of the previous feeding trial a short-term experiment was carried out, with different mycotoxin concentrations and with several samplings to investigate their rapid effect on the lipid peroxidation processes and on the amount/activity of glutathione redox system.
During the experiment the effect of single oral doses of T-2 toxin (0.25 mg and 0.50 mg T-2 toxin/kg b.w.) and DON (0.25 mg and 0.50 mg DON/kg b.w.) treatments was investigated in common carp.
A total of 110 young, one-year-old (35.92±2.82 g b.w.) common carps were divided into 5 aquaria (150 L each).

After a week of adaptation period, at the start of the experiment, 5 groups were formed: a control (‘C’) and 4 treated groups. The treated groups were as follows: lower dose of T-2 toxin (‘TL’), higher dose of T-2 toxin (‘TH’), lower dose of DON (‘DL’) and higher dose of DON (‘DH’).
The complete feed of the treated groups was artificially contaminated with the different mycotoxin doses, and was applied directly into the gut by gavage. The control group was fed by the same way. The applied amount of feed was measured, and fitted the daily feed intake of the animals.
After the single oral mycotoxin exposure, samples were taken at every 8th hour during a 24-hour long experimental period.
The applied doses of mycotoxins, in particular T-2 toxin at high dose level, caused mortality very rapidly, between the 8th and 16th hours after the mycotoxin exposure 4 animals died in group ‘TH’ (18.18% mortality rate).
The amount of CDs and CTs in the liver showed a remarkable, but not significant, increase in group ‘TL’ compared to the control at hour 18 and 24. At 24h the level of CDs and CTs was significantly higher in the ‘TL’ group compared to the ‘TH’ group. The GSH concentration and also the GPx activity in liver was significantly higher in both T-2 treated groups at 16h compared to control. Both DON doses resulted the same increase in GPx activity at 16h.
However, 8 hours later, comparing to control ones, significant depletion of GSH concentration was observed in liver samples of all treated groups (except for group ‘TH’).
At hour 16, comparing to control, the initiation phase parameters of lipidperoxidation processes (CDs, CTs) and also the end-product of it (MDA) showed a significant increase in both DON groups. A remarkable increase in MDA concentration was also observed at 24h in the ‘DH’ group compared to control.
The results show that single oral dose of the investigated mycotoxins caused rapid changes in the lipid peroxidation processes in the liver of carps, the key organ of the metabolism of these mycotoxins, as measured by the increased amount of CDs and CTs in ‘TH’, ‘DL’ and ‘DH’ groups. The emerging free radical burden quickly activated the glutathione redox system in the liver, causing an increase in GSH concentration and GPx activity, which was able to eliminate the harmful peroxidative effect of the mycotoxins investigated, so the end-product of the lipid peroxidation processes, MDA, did not elevate significantly. Except for group ‘DH’, after an increase of GSH concentration, a fast depletion was observed in the co-substrate of GPx, resulting significantly higher MDA concentration in the liver.
Gene expression of *gpx4a* and *gpx4b* showed a dual response during the 24 hour period. After 8 hours of mycotoxin exposure downregulation occurred, which in turn altered into upregulation, in case of the dose of 0.25 mg/kg DON or T-2 toxin at 16h, while in higher doses at 24h. Trichothecene mycotoxins possibly cause oxidative stress which mediates the genes involved in the antioxidant system, through the redox-sensitive Nrf2/ARE pathway, which has central role in the protection against oxidative stress. According to the results this pathway activated only after a particular period, when oxygen free radical formation occurred, which is probably mediated by the activation of Nrf2.
This lag phase of activation was probably blocked by a feedback mechanisms after 8 hours and it may be caused by that trichothecenes has direct inhibitory effect on RNA synthesis. However, this downregulation was followed by an upregulation depending on time and dose, which can be linked to the classic regulatory pathway of Keap1-Nrf2-ARE mechanism induced by oxidative stress. In this case Keap1, which ubiquinilates Nrf2, was inhibited, therefore Nrf2-ARE pathway can be activated.
The results of this experiment suggest that the lower doses of the applied trichothecenes could be absorbed and metabolised rapidly, thus their effect lasted in the period of present experiment, while the higher doses had delayed effects on the gene expression which can

possibly be related to the effect of the bigger absorbed amount of mycotoxin which impairs RNA synthesis and consequently resulted delayed over-reaction of Nrf2-ARE pathway.

Short-term experiment with different combined doses of T-2 toxin and DON

Finally, a short-term experiment was carried out to investigate the effect of single oral administration of different combined doses of DON and T-2 toxin on lipid peroxidation processes and on the amount/activity of glutathione redox system with several samplings.
A total of 112 young, one-year-old (50.42±16.72 g b.w.) common carps (Szarvasi P34 hybrid) was obtained from a commercial fish farm (Balatoni Halászati Ltd., Buzsák-Ciframalom) and divided into 4 aquaria (150 L each).
All aquaria were filled up with aerated de-chlorinated tap water and were connected to a re-circulating system. Light regimen was maintained at a 12:12 h light: dark schedule.
After a week of adaptation period, at the start of the experiment, four groups were formed: a control ('C'), and 3 treated groups. The treated groups were as follows: low doses of T-2 toxin and DON ('T0.5D1' – 0.51 mg T-2 toxin and 1.01 mg DON/kg b.w.), medium doses of T-2 toxin and DON ('T1D2' – 1.01 mg T-2 toxin and 2.01 mg DON/kg b.w.), and high doses of T-2 toxin and DON ('T2D4' – 1.97 mg T-2 toxin and 3.97 mg DON/kg b.w.).
The complete feed of the treated groups was artificially contaminated with the different mycotoxin doses, and was applied directly into the gut by gavage. The control group received the appropriate amount of feed by the same way. The amount of feed was fitted the average daily feed intake of the fishes.
After the single oral mycotoxin exposure, samples were taken at 8th hour intervals during the 24-hour experimental period. Six animals served as absolute control before the mycotoxin treatment.
The highest applied doses of trichothecene mycotoxins increased the mortality rate rapidly; 10% of the animals died between the 8th and 16th hours after the mycotoxin exposure.
In the liver the amount of CTs was significantly higher in 'T2D4' group than in 'T0.5D1' at 8h, showing a rapid prooxidant effect of the applied trichothecenes. Later, at 16h of exposure in case of the 'T0.5D1' group were found significant increase in the initial phase parameters of the lipid peroxidation processes (CD, CT) as compared to control. GSH concentration of the liver significantly ($p<0.05$) increased in case of the medium applied doses ('T1D2') as compared to the group treated with the lowest applied doses ('T0.5D1') at 16h. In the same time also elevated values were found in GPx activity of the liver in the groups treated with the medium and highest combined doses of trichothecenes ('T1D2' and 'T2D4') as compared to the 'T0.5D1' group.
As a result of the activation of the glutathione redox system significantly lower MDA concentrations were measured in group treated with the highest combined doses of mycotoxins ('T2D4') as compared to control at 8h and 24h.
In case of the lowest applied doses ('T0.5D1') the expression of *gpx4a* was significantly lower at 8h and 24h as compared to control, while in case of *gpx4b* gene the significant down-regulation at 8h was followed by and increase at 24h. In case of the medium applied doses of trichothecenes ('T1D2') the expression of *gpx4a* was significantly lower at 8th and 16th hour of experiment, but an up-regulation was observed at 24h. In the same group the expression of *gpx4b* gene showed significant down-regulation at 8h., but it increased later. In case of the highest applied doses ('T2D4') no significant changes were found in the expressions of *gpx4a* and *gpx4b* genes.
In expression of *nrf2* gene significant increase was measured at 8h in case of the group treated with the highest combined doses ('T2D4') as compared to the control, while *keap1*

expression significant down-regulation was observed in all mycotoxin treated groups at 16h. It means that inhibitory effect of Keap1 on Nrf2 was occurred mainly at protein but not at gene expression level, because ARE pathway was down-regulated at early period after mycotoxin exposure but activated later, however, mostly irrespective of the gene expression of both *keap1* or *nrf2*.

In conclusion, the answers to the questions as main purposes of the project are:
(i) The elevation of GSH concentration is caused by both the increased synthesis, as supported by the higher gene expression of glutathione synthetase, and also higher rate of glutathione 'ping-pong' mechanism, as supported by the higher activity of glutathione reductase.
(ii) The increase of GPx activity is only moderately caused by the increased synthesis, because it is not supported consequently the *gpx* gene expression, but probably it is caused by the well-known post-translation modification of the enzyme which may coincide with the actual co-substrate, GSH level.

Gödöllő, 28-07-2016.

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