

Combined effects of aflatoxin B₁ and deoxynivalenol on the expression of glutathione redox system regulatory genes in common carp

Benjamin Kövesi¹ | Csilla Pelyhe² | Erika Zándoki² | Miklós Mézes^{1,2}  | Krisztián Balogh^{1,2}

¹Department of Nutrition, Szent István University, Gödöllő, Hungary

²Mycotoxins in the Food Chain Research Group, Hungarian Academy of Sciences, Kaposvár University, Szent István University, Kaposvár, Hungary

Correspondence

Miklós Mézes, Department of Nutrition, Szent István University, Gödöllő, Hungary.
Email: Mezes.Miklos@mkk.szie.hu

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Abstract

The purpose of the present study was to evaluate the short-term effects of aflatoxin B₁ (AFB₁) and deoxynivalenol (DON) exposure on the expression of the genes encoding the glutathione redox system glutathione peroxidase 4a (*gpx4a*), glutathione peroxidase 4b (*gpx4b*), glutathione synthetase (*gss*) and glutathione reductase (*gsr*) and the oxidative stress response-related transcription factors Kelch-like ECH-associated protein 1 (*keap1*) and nuclear factor-erythroid 2 p45-related factor 2 (*nrf2*) in liver, kidney and spleen of common carp. During the 24-hr long experiment, three different doses (5 µg AFB₁ and 110 µg DON; 7.5 µg AFB₁ and 165 µg DON or 10 µg AFB₁ and 220 µg DON/kg bw) were used. The results indicated that the co-exposure of AFB₁ and DON initiated free radical formation in liver, kidney and spleen, which was suggested by the increase in Nrf2 dependent genes, namely *gpx4a*, *gpx4b*, *gss* and *gsr*. Expression of *keap1* gene showed upregulation after 8 hr of mycotoxin exposure, and also upregulation of *nrf2* gene was found in kidney after 8 hr of exposure, while in the liver, only slight differences were observed. The changes in the expression of the analysed genes suggest that level of reactive oxygen species reached a critical level where other signalling pathway was activated as described by the hierarchical model of oxidative stress.

KEYWORDS

aflatoxin B₁, antioxidant defence system, common carp, deoxynivalenol, gene expression, glutathione peroxidase

1 | INTRODUCTION

Multiple occurrence of mycotoxins is a great concern to feed and food safety worldwide (BIOMIN, 2018). Aflatoxins are secondary

fungal metabolites, which are primarily produced by *Aspergillus flavus* and *A. parasiticus* (Dai, Huang, Zhang, & Zhu, 2017), while deoxynivalenol (DON), a type-B trichothecene, is produced by *Fusarium* moulds, mainly *Fusarium graminearum* (Sobrova et al., 2010). Among

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the more than 20 different aflatoxins, aflatoxin B₁ (AFB₁) is the most toxic one and frequently occurs in nuts, cereals, dried fruits and spices (Taniwaki, Pitt, & Magan, 2018). DON, on the other side, is less toxic than AFB₁ but is the most widely distributed mycotoxin and can be usually found in cereals such as wheat, maize, barley, oat and rice (Wu et al., 2017).

A maximum of 20 µg aflatoxin B₁ kg⁻¹ complete feed is the regulatory limit for farm animals according to the Commission Regulation 574/2011, while for DON, the maximum proposed limit is set at 5 mg/kg complete feed (2006/576/EC).

There are still only limited data about the detrimental effects of AFB₁ or DON on fish health, and the experiments showed marked differences among fish species with different endpoints. However, there is a detailed risk assessment for mycotoxin contamination in fish feeds (Pietsch, 2020).

Both AFB₁ and DON inhibit the protein synthesis at the levels of initiation, transcription and translation, and also DNA synthesis in eukaryotic cells (Holladay, Smith, & Luster, 1995; McLean & Dutton, 1995), and have negative effects on growth rates and immune response, thus both mycotoxins are immune-suppressive or immune-depressive compounds, even in carp (Pietsch, 2015; Sahoo & Mukherjee, 2001). Reduction in feed intake, poor growth rates and feed efficiency in rainbow trout (*Oncorhynchus mykiss*) were reported by Woodward, Young, and Lun (1983) and Hooft, Elmor, and Encarnação (2011) as effect of DON. Döll et al. (2010) also described reduction in feed intake, increase in feed conversion and reduction of growth rate in Atlantic salmon (*Salmo salar*) as effect of 3.7 mg DON/kg feed. Moreover, histopathological changes were observed in the hepatopancreas and intestine in trout (Hooft et al., 2011), and hepatopancreas in carp (Pietsch, Schulz, Pere Rovira, & Kloas, 2014). AFB₁ also significantly reduced growth parameters of carp fingerlings (Akter, Rahman, & Hasan, 2010) and has negative effect on growth of other fish species, such as channel catfish (*Ictalurus punctatus*) and Nile tilapia (*Oreochromis niloticus*) at concentrations ranging from 1.88 to 100 mg AFB₁ kg⁻¹ feed (Ahn Tuan, Grizzle, Lovell, Manning, & Rottinghaus, 2002; Chavez-Sanches, Martinez, & Moreno, 1994; Encarnacao, Srikkhum, Rodrigues, & Hofstetter, 2009; Jantrarotai & Lovell, 1990). Histopathological alterations, like dystrophy of liver, were also described as effect of AFB₁ in carp at high doses, 20 or 200 µg/kg feed (Svobodova & Piskac, 1980). Toxic effects on primary hepatocytes in vitro have also been identified both as effect of AFB₁ and DON (He et al., 2010). Moreover, Zhou et al. (2017) revealed synergistically enhanced toxic effects of AFB₁+DON on fish cell line (BF-2) and zebrafish larvae.

Šišperová et al. (2015) observed that DON causes oxidative stress in rainbow trout and it has also been reported that AFB₁ induces the formation of reactive oxygen species, which may cause oxidative stress (Shen, Shi, Shen, & Ong, 1996). Both AFB₁- and DON-induced oxidative stress affect the enzymatic and non-enzymatic antioxidant defences and alter the transcription factor nuclear factor-erythroid 2 p45-related factor 2 (*Nrf2*), which is the key regulator of the oxidative stress response, and its main negative regulator Kelch-like ECH-associated protein 1 (*KEAP1*) gene expression (Kövesi, Pelyhe,

Zándoki, Mézes, & Balogh, 2018; Pelyhe et al., 2016a, 2016b). Under basal conditions, Keap1 binds Nrf2 and recruits it into the Cul3-containing E3 ubiquitin ligase complex for ubiquitin conjugation and proteasomal degradation (Motohashi & Yamamoto, 2004). However, as effect of oxidative stress, the activation of Nrf2 increases due to conformational changes in Keap1 cysteine side chains; therefore, newly synthesised Nrf2 proteins bypass Keap1 and translocate into the nucleus, bind to the antioxidant response element (ARE) and drive the expression of Nrf2 target, antioxidant system encoding genes (Taguchi, Motohashi, & Yamamoto, 2011). Glutathione peroxidase enzymes have a central role in antioxidant defence, and GPx4 is the most important in fishes, because it gives one third of the total GPx activity (Grim, Hyndman, Kriska, Girotti, & Crockett, 2011). Two *gpx4* genes, *gpx4a* and *gpx4b*, were identified in carp (Hermesz & Ferencz, 2009). GPx4 catalyses the reduction in reactive oxygen species, mainly phospholipid hydroperoxides, using reduced glutathione as co-substrate. Oxidised form of glutathione, glutathione disulphide, can be reduced by the enzyme glutathione reductase, but glutathione homeostasis also depends on its synthesis, catalysed by glutathione synthetase (Halliwell & Gutteridge, 1989).

The purpose of the present study was to evaluate the toxic effects of AFB₁ and DON dual exposure on the oxidative stress response in liver, kidney and spleen of common carp juveniles. Therefore, a short-term (24 hr) in vivo toxicological experiment was carried out where the expression of several genes responsible for the regulation of the glutathione redox system was analysed.

2 | MATERIALS AND METHODS

2.1 | Production of mycotoxins and analyses

For experimental contamination of the feed, AFB₁ was produced in ground corn which was artificially infected with an aflatoxin producing *Aspergillus flavus* strain (SZMC 20750) isolated by Dobolyi et al. (2013) and deposited in the Microbiological Collection of the University of Szeged (SZMC). Aflatoxin content of the complete feeds was analysed with FAML-A-02:2016 AFLAPREP[®] HPLC method after immune affinity clean-up (Food Analytica Ltd.). DON was produced by *Fusarium graminearum* (NRRL 5883) strain on corn substrate according to Fodor et al. (2006). DON and 15-acetyl DON content of the feed were determined by HPLC method after immune affinity purification according to Pussemier et al. (2006).

2.2 | Animals, experimental design, sample preparations

A total of 96 one-year-old common carp (*Cyprinus carpio*) juveniles (body weight: 22.68 ± 6.22 g) was used for the experiment. Animals were randomly divided into four treatment groups (control, MIX1, MIX2 and MIX3) into four aquaria (150 L each) after a seven day long acclimatisation period. The aquaria were used in a semi-static

system with dechlorinated, continuously aerated tap water. A 12 hr light:12 hr dark light regimen was used. The effect of the mycotoxin mixture (AFB₁ and DON) was investigated in three different dosage groups: control; low mix (MIX1: 5 µg AFB₁ and 110 µg DON/kg b.w); medium mix (MIX2: 7.5 µg AFB₁ and 165 µg DON/kg b.w) and high mix (MIX3: 10 µg AFB₁ and 220 µg DON/kg b.w). The dose range was selected based on our previous short-term studies with individual aflatoxin B₁ (Kövesi et al., 2018) or DON (Pelyhe et al., 2016b) exposure in carp. An appropriate amount of mycotoxin-containing fungal culture was mixed with ground growth feed for carp (GARANT Aqua Classic™, Garant-Tiernahrung) and diluted to 5 ml of water immediately before use. The nutrient content of the diet (on dry matter basis) was 30% crude protein, 7% crude fat, 5% crude fibre, 7.5% crude ash and 50.5% nitrogen-free extract.

At the start of the experiment, two animals were taken out from each experimental group and six of them served as absolute control (0 hr). The AFB₁ and DON contaminated diet were applied by gavage directly into the gut once. The transit time of feed particles was also investigated; six fish from the control group received methyl orange dyed (1% w/w) control feed by gavage with an amount of 1% body weight. Water temperature during the experiment was 19 ± 1°C which in case of the common carp means a moderate metabolic rate.

At samplings, namely at 0, 8, 16 and 24 hr after exposure, six carps from each experimental group were investigated. Fish were over-anaesthetised with clove oil and decapitated, and then, liver, kidney and spleen samples were removed and taken into 1.5 ml collection tubes and immediately frozen in liquid nitrogen and stored at -80°C until analysis to prevent RNA degradation.

2.3 | RNA Isolation and quantitative Real-Time PCR

Nucleozol reagent (Macherey-Nagel) was used for total RNA isolation from 5–10 mg liver, kidney and spleen homogenates as described by the manufacturer's instructions, and DNase I treatment was also performed according to the supplier's protocol (Thermo Fisher Scientific) to avoid genomic DNA contamination. The integrity and quality of the RNA samples were verified by agarose gel electrophoresis and NanoPhotometer (Implen GmbH) measurements. Only those RNA samples were accepted for further investigation which had the ratios of absorption 260:280 nm higher than 2.0. Then, pools were formed from equal (1 µg) amounts of DNase treated RNA per 6 individual carp samples for each sampling point per treatment. cDNA production was performed using a standard protocol with RevertAid Reverse Transcriptase and random nanomer primer from 1 µg of total RNA pool.

The real-time PCR measurements were performed in five technical replicates. According to the results of previous experiments, no measurable differences were found if the determination was made from pooled and not individual samples.

The primers for the quantification of the mRNA transcriptional levels of the *gpx4a*, *gpx4b*, *nrf2*, *keap1*, *gss* and *gsr* and endogenous control, *β-actin* gene were chosen based on the literature

(Hermesz & Ferencz, 2009; Jiang et al., 2015; Safari, Hoseinifar, Nejadmoghadam, & Jafar, 2016) and are shown in Table 1. *β-actin* has no known interaction with oxidative stress or mycotoxins and served as an endogenous control gene in other studies screening the effects of mycotoxins in fish species (El-Barbary, 2016).

The applied real-time PCR procedure was described previously by Pelyhe et al. (2016b) and Kövesi et al. (2018). Shortly, Maxima SYBR Green method was used, where no template controls were also performed for each primer pair. SYBR Green signal was detected at the end of the extension period, and the amplified products were verified by melting curve analysis and gel electrophoresis.

The threshold cycle (Ct) of the target genes (*gpx4a*, *gpx4b*, *nrf2*, *keap1*, *gss* and *gsr*) and the endogenous housekeeping control gene (*β-actin*) was determined by StepOne™/StepOnePlus™ Software v2.2 (Thermo Fisher Scientific), and the delta Ct values (ΔCt) and relative quantification (RQ = 2^{-ΔΔCt}) values were calculated by the formula described by Livak and Schmittgen (2001).

2.4 | Statistical methods

All data are presented as mean ± standard deviation (SD). Firstly, the data were tested by Shapiro–Wilk normality test, and to confirm homogeneity of variance, both Bartlett and Browne–Forsythe tests were performed. All data passing both conditions were analysed using one-way ANOVA. Significance of differences between groups was evaluated using post hoc Tukey's test (*p* < .05). In case of those data that did not pass Bartlett and Browne–Forsythe test, a non-parametric Kruskal–Wallis test with pairwise comparisons was used (*p* < .05). Analyses were performed with GraphPad Prism 7.0 (GraphPad Software).

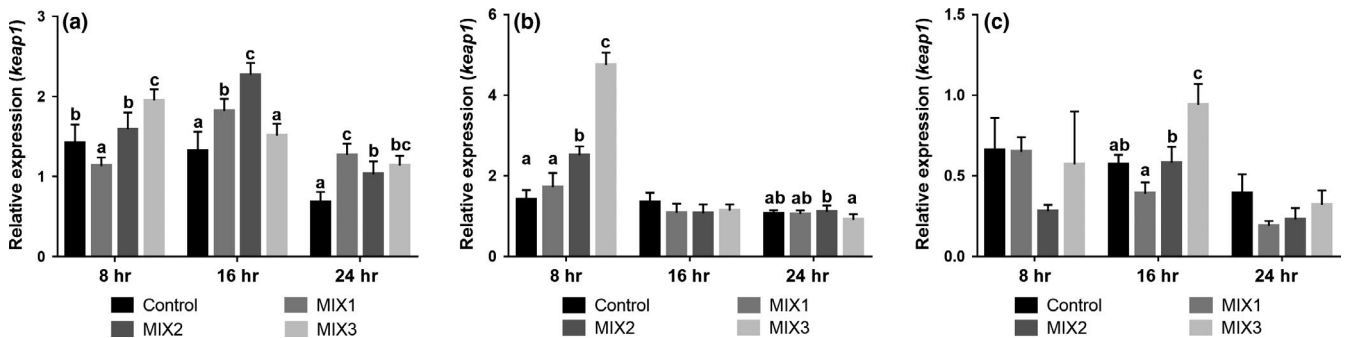
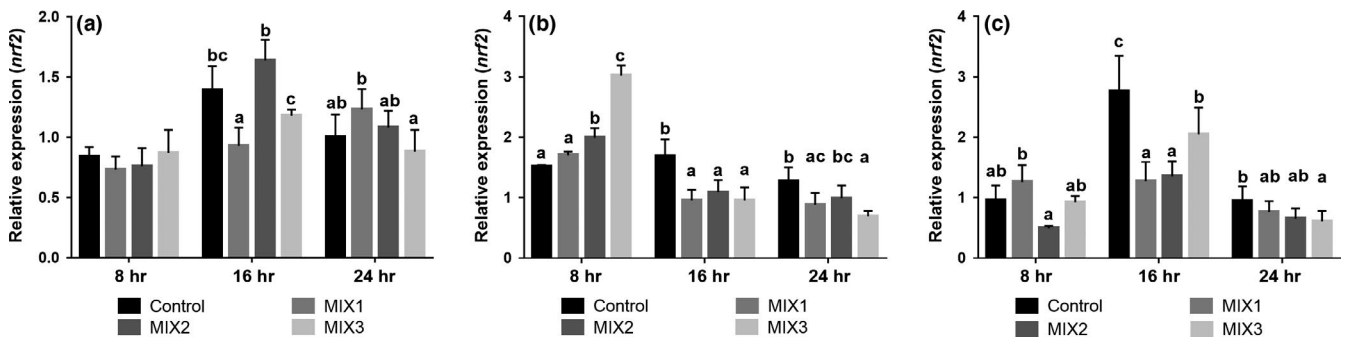
3 | RESULTS

Clinical signs of toxicity and mortality were not observed during the 24 hr long trial in the experimental groups. According to our previous studies (Kövesi et al., 2018), we confirmed that the transit time of feed particles that were coloured with methyl orange dye was 16 hr at the applied water temperature.

The relative expression of *keap1* gene was significantly lower in the liver at 8th hour in the lowest (MIX1) but significantly higher in the highest (MIX3) dose group (Figure 1a), and significantly higher values were observed also in kidney in the moderate and high dose group (MIX2 and MIX3) than the control (Figure 1b), while no significant differences were found in spleen (Figure 1c). After 16 hr of exposure, the *keap1* gene expression was significantly higher in the liver in the lowest (MIX1) and moderate (MIX2), but not in the highest dose groups than the control (Figure 1a), while in kidney, no significant differences were found (Figure 1b). In the spleen, significantly higher values were observed in as effect of the highest dose group (MIX3) at the same sampling (Figure 1c). After 24 hr of exposure, significantly higher *keap1* gene expression values were found in

TABLE 1 Primers of target (*gpx4a*, *gpx4b*, *nrf2*, *keap1*, *gss* and *gsr*) and endogenous control (*β-actin*) genes

Gene	Forward (5'-3')	Reverse (5'-3')	Accession Nr.
<i>β-actin</i>	GCAAGAGAGGTATCCTGACC	CCCTCGTAGATGGGCACAGT	XM_019103102.1
<i>gpx4a</i>	GGAACCAGGAACAAATTCCC	AGATCCTTCTCCACCACGCTTG	FJ656211.1
<i>gpx4b</i>	CTACAAGGCAGAGTTTGACCTC	CTTGGATCGTCCATTGGTCC	FJ656212.1
<i>nrf2</i>	TTCCCGCTGGTTTACCTTAC	CGTTTCTTCTGCTTGTCTTT	JX462955
<i>keap1</i>	GCTCTTCGGAAACCCCT	GCCCCAAGCCCACTACA	JX470752
<i>gss</i>	ACCATGACATACCGCTGACAT	TGTTCCCCATAGATCAGTAGAGGAT	XM_019114684.1
<i>gsr</i>	ACTCGTGCAGGTGTCTATGC	TTTGAGTCTGCTTTGCCCT	HQ174244.1

**FIGURE 1** Effect of AFB₁ and DON treatment on the relative expression of *keap1* gene in the liver (a), kidney (b) and spleen (c) of common carp (mean ± SD; n = 6 in a pool, equal amounts of cDNA per individual). ^{a,b}Different superscripts mean significant difference at *p* < .05 level. MIX1: 5 μg AFB₁ and 110 μg DON/kg b.w; MIX2: 7.5 μg AFB₁ and 165 μg DON/kg b.w; MIX3: 10 μg AFB₁ and 220 μg DON/kg b.w**FIGURE 2** Effect of AFB₁ and DON treatment on the relative expression of *nrf2* gene in the liver (a), kidney (b) and spleen (c) of common carp (mean ± SD; n = 6 in a pool, equal amounts of cDNA per individual). ^{a,b}Different superscripts mean significant difference at *p* < .05 level. MIX1: 5 μg AFB₁ and 110 μg DON/kg b.w; MIX2: 7.5 μg AFB₁ and 165 μg DON/kg b.w; MIX3: 10 μg AFB₁ and 220 μg DON/kg b.w

the liver as effect of all applied dose groups (MIX1, MIX2 and MIX3) than the control (Figure 1a), while in the kidney and spleen, there were no significant differences (Figure 1b,c).

There were no significant changes in the *nrf2* gene expression in liver at 8th hour, but at 16th hour, significantly lower values were observed in the lowest dose group (MIX1) than the control (Figure 2a). In the kidney, upregulation of *nrf2* gene was found as effect of the moderate and highest (MIX 2 and 3) groups after 8 hr of exposure (Figure 2b). After 16 hr of exposure, significantly lower *nrf2* gene expression values were observed as effect of all dose groups in kidney and spleen (Figure 2b,c). After 24 hr of exposure, there was no difference between the treated groups and the control in *nrf2* gene

expression in the liver (Figure 2a), but significantly lower values were found in kidney as effect of MIX1 and MIX3 (Figure 2b), and in spleen as effect of MIX3 (Figure 2c).

In case of *gpx4a* expression, opposite tendencies were found after 8 hr of exposure between the liver and kidney. It was downregulated in the liver (Figure 3a) but upregulated in the kidney (Figure 3b) in all treatment groups, and also in spleen in the lowest (MIX1) dose group, (Figure 3c) as compared to the control. After 16 hr of exposure, significantly higher values were observed in all treatment groups than the control in both liver and kidney (Figure 3b,c), and as effect of moderate dose group (MIX2) in spleen (Figure 3c). Later, at 24th hour, opposite tendencies were found between the liver and

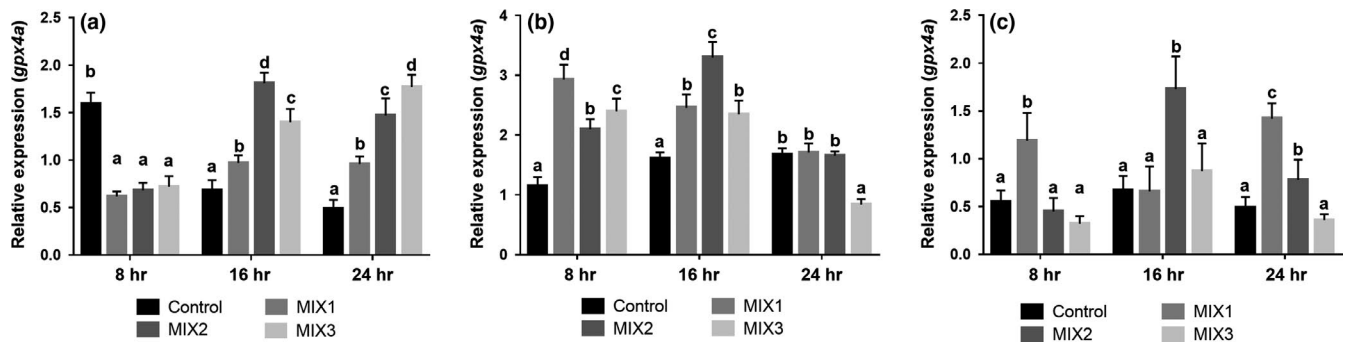


FIGURE 3 Effect of AFB₁ and DON treatment on the relative expression of *gpx4a* gene in the liver (a), kidney (b) and spleen (c) of common carp (mean \pm SD; $n = 6$ in a pool, equal amounts of cDNA per individual). ^{a,b}Different superscripts mean significant difference at $p < .05$ level. MIX1: 5 μ g AFB₁ and 110 μ g DON/kg b.w; MIX2: 7.5 μ g AFB₁ and 165 μ g DON/kg b.w; MIX3: 10 μ g AFB₁ and 220 μ g DON/kg b.w

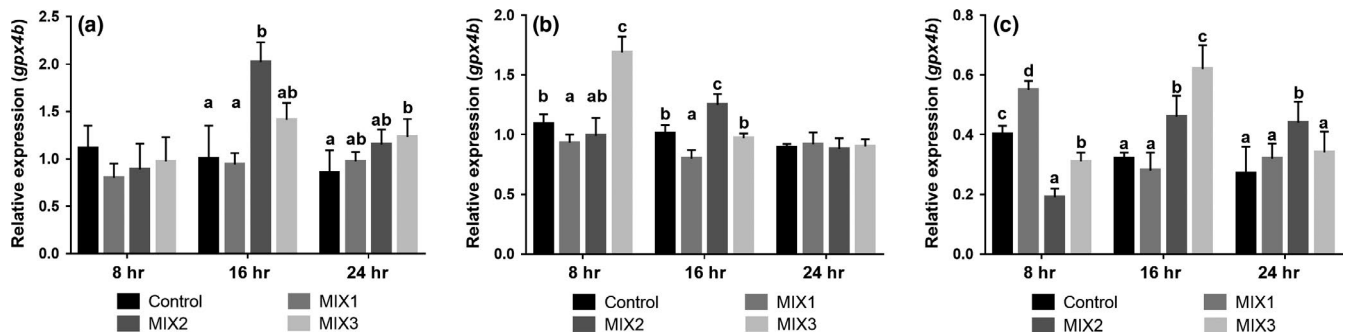


FIGURE 4 Effect of AFB₁ and DON treatment on the relative expression of *gpx4b* gene in the liver (a), kidney (b) and spleen (c) of common carp (mean \pm SD; $n = 6$ in a pool, equal amounts of cDNA per individual). ^{a,b}Different superscripts mean significant difference at $p < .05$ level. MIX1: 5 μ g AFB₁ and 110 μ g DON/kg b.w; MIX2: 7.5 μ g AFB₁ and 165 μ g DON/kg b.w; MIX3: 10 μ g AFB₁ and 220 μ g DON/kg b.w

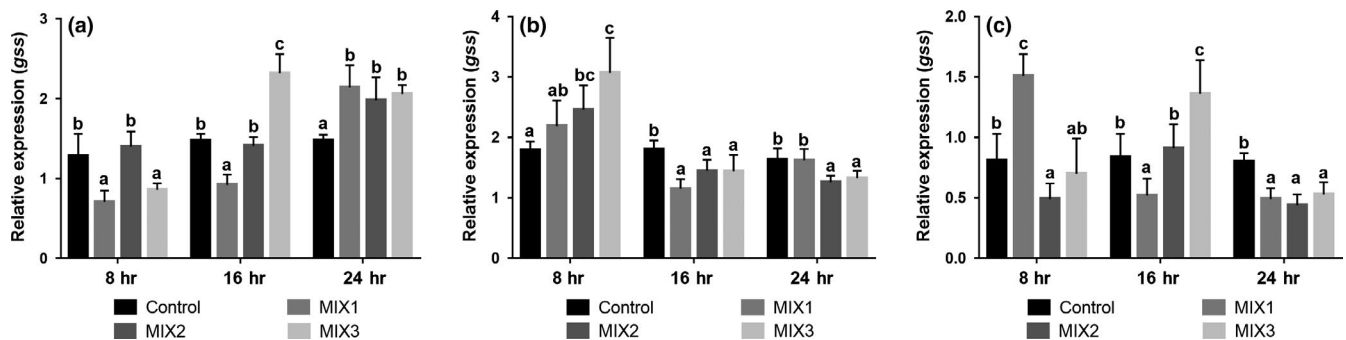


FIGURE 5 Effect of AFB₁ and DON treatment on the relative expression of *gss* gene in the liver (a), kidney (b) and spleen (c) of common carp (mean \pm SD; $n = 6$ in a pool, equal amounts of cDNA per individual). ^{a,b}Different superscripts mean significant difference at $p < .05$ level. MIX1: 5 μ g AFB₁ and 110 μ g DON/kg b.w; MIX2: 7.5 μ g AFB₁ and 165 μ g DON/kg b.w; MIX3: 10 μ g AFB₁ and 220 μ g DON/kg b.w

kidney. The gene expression of *gpx4a* was upregulated in the liver as effect of all treatment groups (Figure 3a), while in kidney, it stayed at control level as effect of MIX1 and MIX2 and downregulated in MIX3 group (Figure 3b). At the same sampling, an upregulation was observed as effect of MIX1 and MIX2 in spleen [Figure 3c].

The expression of *gpx4b* did not change at 8th hour sampling in liver (Figure 4a), but it was downregulated as effect of MIX1, while upregulated as effect of MIX3 in kidney (Figure 4b). At the same sampling in the spleen, downregulation was observed as effect of MIX2

and MIX3 and upregulation in MIX1 group (Figure 4c). After 16 hr of exposure, upregulation was found in all tissues as effect of MIX2, while a downregulation as effect of MIX1 in kidney, and upregulation in the MIX2 and MIX3 groups in spleen (Figure 4a–c). At 24th hour, an upregulation was found in the liver as effect of MIX3 (Figure 4a) and also an upregulation as effect of MIX2 in spleen [Figure 4c].

Gss gene expression was lower in MIX1 and MIX3 groups in the liver after 8 hr of exposure (Figure 5a), while in the kidney, an upregulation was observed as effect of MIX2 and MIX3 (Figure 5b) and in

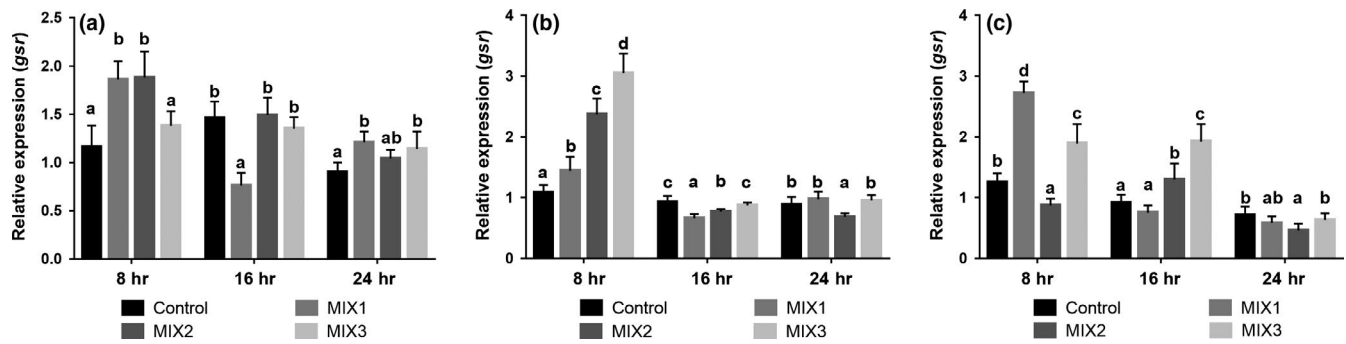


FIGURE 6 Effect of AFB₁ and DON treatment on the relative expression of *gsr* gene in the liver (a), kidney (b) and spleen (c) of common carp (mean \pm SD; $n = 6$ in a pool, equal amounts of cDNA per individual). ^{a,b}Different superscripts mean significant difference at $p < .05$ level. MIX1: 5 μ g AFB₁ and 110 μ g DON/kg b.w; MIX2: 7.5 μ g AFB₁ and 165 μ g DON/kg b.w; MIX3: 10 μ g AFB₁ and 220 μ g DON/kg b.w

spleen upregulation in MIX1 group, but downregulation as effect of MIX2 as compared to the control at the same sampling (Figure 5c). After 16 hr of exposure, similar changes were observed in the liver and the spleen; a downregulation was measured in the lowest dose group (MIX1) while an upregulation as effect of the highest dose group (MIX3) as compared to the control (Figure 5a,c). In case of kidney, a downregulation was found as effect of all applied dose (Figure 5b). After 24 hr of exposure, opposite tendencies were found between the liver and the spleen. The expression of *gss* gene was downregulated in spleen (Figure 5c), while upregulated in the liver (Figure 5a) as effect of all applied dose. In kidney, a downregulation was observed only as effect of MIX2 and MIX3 (Figure 5b).

The expression of *gsr* gene was significantly higher in the liver at 8th hour in the lowest and moderate (MIX1 and MIX2) dose groups (Figure 5a), while in the kidney, significantly higher values were observed as effect of all applied dose (Figure 6b). In spleen, a dual response was observed. Upregulation was measured as effect of MIX1 and MIX3 and downregulation as effect of MIX2 (Figure 6c). After 16 hr of exposure, a downregulation was observed in the liver as effect of MIX1 (Figure 6a) and as effect of MIX1 and MIX2 in the kidney (Figure 6b) as compared to the control. In case of the spleen, significantly higher expression levels were measured as effect of MIX2 and MIX3 at 16th hour (Figure 6c). At 24th hour, significantly higher relative gene expression levels were observed in the liver as effect of MIX1 and MIX3 (Figure 6a), while in kidney and spleen, significantly lower values were measured as effect of MIX2 (Figure 6b,c).

4 | DISCUSSION

The present study revealed that AFB₁-DON co-exposure probably induced oxidative stress in the liver, kidney and spleen of one-year-old common carp juveniles.

In this experiment, it was confirmed that the transit time of feed particles in the gastrointestinal tract was 16 hr in the control group that was fed with methyl orange dyed feed. As fishes are poikilothermic animals, their metabolic rate is defined by the actual water temperature. As there was no further feed intake, this transit time defines the time for the absorption of mycotoxins from the intestine;

therefore, it can be used as an exposure time window. Hence, the changes in the values of the target genes' expression induced by the co-exposure of AFB₁ and DON presumably correlate with that transit time.

The expression of *keap1*, the negative regulator of Nrf2, was up-regulated both in the liver and kidney, but it was slightly affected in spleen; however, in case of kidney, this upregulation was observed only after 8 hr of exposure as the relative gene expression returned to the control levels later. This probably means, that Nrf2, the transcription activator of numerous antioxidant genes, including glutathione peroxidases, glutathione reductase and glutathione synthetase, might have not been dissociated from the binding of Keap1, probably because the level of reactive oxygen species might have reached a high level, where other, for instance NF- κ B signalling pathway (Lingappan, 2018), is activated as described by the hierarchical model of oxidative stress (Gloire, Legrand-Poels, & Piette, 2006). The NF- κ B signalling is different from the Keap1-Nrf2-ARE pathway, and NF- κ B regulates the pro-inflammatory, but not antioxidant genes (Li et al., 2008). This hypothesis is confirmed by the expression of *nrf2* which showed the same changes as *keap1* in kidney; a dose-dependent induction at 8th hour which later turned to downregulation even in case of spleen. Also, expression of *nrf2* in liver showed only slight differences comparing to the control group.

At lower level of ROS formation, cysteine side chains of Keap1 go under conformational changes; therefore, the level of unbound newly synthesised Nrf2 increasing, which then activates the ARE containing genes encoding glutathione metabolism enzymes (*gss* and *gsr*) and glutathione peroxidases, namely *gpx4a* and *gpx4b* (Taguchi et al., 2011). The expression of *gss* (glutathione synthetase), which links glycine to the dipeptide of glutamate and cysteine (Espinosa-Diez et al., 2015), showed similar results in kidney and spleen as *nrf2*, which also suggested that the Keap1-Nrf2-ARE pathway was not activated. In case of the liver, a delayed effect was observed, as *gss* gene expression was upregulated only after 24 hr of mycotoxin exposure. This suggests that higher oxidative stress was induced earlier in kidney and spleen, which activated other pathway. In case of liver, which has a higher steady-state level of antioxidants, showed delayed effect and also lower oxidative stress, which was activated by the Keap1-Nrf2-ARE pathway. The same response was observed

in our previous study with the same dose of AFB₁ alone (Kövesi et al., 2018). The expression of *gsr*, which plays important role in the reduction of glutathione disulphide to reduced glutathione (Espinosa-Diez et al., 2015), showed nearly the same changes in kidney and spleen as *gss*. This means that the reduction in GSSG to GSH was not effective in the last 16 hr of the trial. This was possibly caused by the lack of continuous nutrient supply, because this reaction requires NADPH as hydrogen donor for the reduction, and NADPH synthesis requires optimal carbohydrate supply. In the case of liver, an induction was observed after 8 and 24 hr of mycotoxin exposure. These findings are contradictory with our previous findings with the lower dose of AFB₁ alone, where the induction was found after 16 hr of exposure (Kövesi et al., 2018). These results suggested that there are some synergistic effects between AFB₁ and DON, which modify not only the level of oxidative stress, but also the expression of genes encoding the regulatory elements of oxidative stress response. Phospholipid hydroperoxide glutathione peroxidase (GPx4) plays an important role in the elimination of lipid hydroperoxides in membranes and protection of their integrity from the detrimental effects of lipid peroxidation, in particular in fishes, where it has the highest activity among GPx enzymes (Antunes, Salvador, & Pinto, 1995). Grim et al. (2011) demonstrated that the higher amount of polyunsaturated fatty acids of fish tissue and elevated temperature-dependent oxidative activity are accompanied with higher GPx4 activity. The expression of *gpx4a* and *gpx4b* genes in the liver showed a dual response; downregulation at 8th hour, while upregulation in the last 16 hr of the trial. The same changes were found in our previous short-term studies with AFB₁ (Kövesi et al., 2018) or DON alone (Pelyhe et al., 2016b). These results suggest that the applied doses had delayed effects on gene expression, which may be related to the effect of the higher absorbed amounts of mycotoxins as a function of time. In case of kidney, opposite changes were observed as the relative expression was higher as compared to the control in the first 16 hr, and later dropped to the control level. Spleen showed similar changes than liver as there was an induction at the 16th and 24th hour.

In conclusion, the results of the present study suggested that the co-exposure of AFB₁ and DON induce oxidative stress in liver, kidney and spleen in carp. The results showed that reactive oxygen species formation and consequently gene expression of regulatory and synthesis encoding genes of the antioxidant system were provoked earlier in the kidney than liver and spleen, as the expression of the glutathione redox system-related genes and even the genes of Keap1-Nrf2 pathway was upregulated even after 8 hr of exposure. Lack of Nrf2 activation was probably due to the enhanced formation of reactive oxygen species; therefore, it reached a critical level where another signalling pathway was activated as described the hierarchical model of oxidative stress. The results also suggested a synergistic interaction between AFB₁ and DON, because the response was different than the two mycotoxins used at the similar dose range individually. Moreover, there are no publications indicating the gene expression levels of different antioxidant enzymes in different tissues (liver, kidney and spleen) of common carp intoxicated with

AFB₁ and DON at the same time, and to our best knowledge, this study is the first to address the expression of the above-mentioned genes in three different tissues.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ANIMAL WELFARE STATEMENT

The author confirms that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received (Department of Food Chain Safety, Land Register, Plant and Soil Protection and Forestry of the Pest County Government Office (Hungary) with a permission number PE/EA/1964-7/2017). The authors confirm that they followed EU standards for protection of animals used for scientific purposes.

ORCID

Miklós Mézes  <https://orcid.org/0000-0003-2323-833X>

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