EFFECT OF SHORT-TERM AFLATOXIN EXPOSURE IN COMBINATION WITH MEDICINAL HERB MIXTURE ON LIPID PEROXIDATION AND GLUTATHIONE REDOX SYSTEM IN LAYING HENS

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Abstract: Aflatoxins are well known hepatotoxic mycotoxins, which mainly contaminate the cereal grains. Those induce lipid peroxidation and impair the antioxidant, including glutathione redox system in long-term studies. The purpose of present study was to investigate the short-term (36-hour) effect of feeding aflatoxin B1 (AFB1) contaminated diet alone or in combination with a medicinal herb mixture on lipid peroxidation (conjugated dienes and trienes, and malondialdehyde), and on parameters of the glutathione system (reduced glutathione and glutathione peroxidase) in blood plasma, red blood cell haemolysate, liver and kidney homogenate of 49-week old Bovans Goldline laying hens. The results revealed that AFB1 (125 m /kg feed) did not have effect on feed intake, body and liver weight, but increased malondialdehyde content was observed in blood plasma and red blood cell haemolysate as effect of feeding AFB1 and medicinal herb mixture at 36th hour of the trial. However, the same diet resulted in lower malondialdehyde content in liver, but not in kidney. Reduced glutathione concentrations showed variance among treatments; thus due to inclusion of medicinal herb mixture in the diet lower values were measured in red blood cell haemolysate. Glutathione peroxidase activity was significantly lower in all treated groups as compared to the control at 36th hour of the trial in blood plasma, but not in other tissues. The results are contradictory with previous findings, probably due to the short-term exposure, and/or to medicinal herb mixture supplementation as it could moderately modify the effect of AFB1.

Keywords: Aflatoxin B1, malondialdehyde, glutathione, glutathione peroxidase, medicinal herb

Introduction

Cereal grains and their by-products are important ingredients in poultry diet. Cereals are the main components of complete feeds that may be contaminated with mycotoxins, secondary metabolites of moulds (Binder et al., 2007). Feed spoilage by moulds may result in heating, reduced palatability and loss of nutritive value (Christensen, 1974). Among various mycotoxins, aflatoxins (AF) are often encountered in feed ingredients even at high concentrations in many parts of the world under different environmental conditions (Jelinek et al., 1989). Among avian species, goose is the most sensitive to the prooxidant effect of mycotoxins, followed by duck and chicken, while the most sensitive indicators are liver, blood plasma, and red blood cells (Mézes et al, 1999).

However, mycotoxin contamination levels in animal feedstuffs are usually not high enough to cause an overt disease, but may result in economical loss due to changes in growth, production and immunosuppression (Richard, 2007). In poultry species, the economic losses associated with aflatoxin exposure include poor feed conversion and growth, increased mortality, decreased egg production, leg problems, and carcass condemnations (Smith and Hamilton, 1970; Hamilton, 1971; Huff et al., 1992; Yunus et al. 2011). Aflatoxins are toxic metabolites of certain strains of *Aspergillus flavus* and *Aspergillus parasiticus* moulds (Manafi et al., 2011), and aflatoxin B1 (AFB1) is the most toxic among aflatoxins, which causes a wide variety of adverse effects, such as decrease of feed intake, reduced immune response, impaired production

DOI: 10.18380/SZIE.COLUM.2016.3.1.65

traits, and liver damage depending on the concentration, duration of exposure, animal species, its age, health and nutritional status during the exposure (Diaz 2005). Aflatoxin B1 residues arise food safety problems, because those are present in eggs and flesh of laying hens fed aflatoxin B1 contaminated diet (Herzallah, 2013).

Most of the mycotoxins, e.g. aflatoxin B1, T-2 toxin and ochratoxin A (Balogh et al., 2007; Pál et al., 2009), provoke oxygen free radical formation. The body is protected against reactive oxygen metabolites by the biological antioxidant defence system, which includes antioxidant enzymes and low molecular weight antioxidants (Surai, 2002). It has been suggested that the cellular first line of antioxidant defence is based on the activity of enzymes, such as superoxide dismutases, glutathione peroxidases (GPx) and catalase. Also, the rate of lipid peroxidation is reported to be increased in different tissues as a result of feeding mycotoxin-contaminated diet containing AFB1 or T-2 toxin (Surai et al., 2002) and ochratoxin A (Balogh et al., 2007), which reduces the efficiency of the biological antioxidant defence system.

Phytobiotics, such as essential oils of herbs are considered to be important natural antioxidants both in animal and human nutrition. Compared to synthetic antioxidants, their antioxidant capacity is measurable only at relatively high doses and is mainly proven in the Fe^{2+}/ascorbate system (Bozin et al., 2006). Kim et al. (2009) reported *in vivo* antioxidant effects where dietary supplementation with garlic husk resulted in significantly lower thiobarbituric acid reactive substances (TBARS) values in chicken. In this study a mixture of eight medicinal herb extract was used. Two of them (rosemary [*Rosmarinus officinalis*] and oregano [*Origanum vulgare*]) have antioxidant bioactive component(s), and a third one (Mary thistle [*Silybum marianum*]) is hepatoprotective (Mirzaei-Aghsaghali, 2012).

Our hypothesis, and thus, the purpose of the study was to investigate the short-term effect of AFB1 on lipid peroxidation and some glutathione redox parameters in laying hen and evaluate the efficacy of a herbal mixture (Herbamix™) for counteracting AFB1 in experimentally contaminated layer diet.

Materials and methods

Animals and experimental design

Total of 60 Bovans Goldline laying hens, being at 90% daily egg production at 49 weeks of age were divided randomly to four experimental groups (two replicates each): a control (aflatoxin content < 1.0 μ/kg) and three treated groups fed with aflatoxin (total aflatoxin content was 170.3 μ /kg; AFB2: 39.0 μ/kg; AFG1: 2.0 μ/ kg; AFG2: 4.3 μ/ /kg), herbal mixture, and aflatoxin + herbal mixture, respectively. For experimental contamination of the feed aflatoxin was produced in ground corn which was artificially infected with an aflatoxin producing *Aspergillus flavus* strain (ZT80) isolated by Dobolyi et al. (2011). Appropriate amount of the fungal culture was mixed with the complete feed, according to its total aflatoxin content. Herbal mixture, namely Herbamix Basic Premix™ (Herbamix Trade Ltd., Budapest) was added to the complete feed in powder form at the dose of 600 mg/ kg. Hens were kept in deep litter condition. The feeding trial lasted for 36 hours, after 12 hours of feed deprivation. Because of the short-term exposure the toxin dose was much higher than the regulatory limit of 20 μ /kg for aflatoxin B1,, in complete feed (Commission Regulation 574/2011).

Aflatoxin content of the loaded complete feeds was analysed with AFLAPREP HPLC method after immunoaffinity clean-up (Food Analytica Ltd, Gyula). Five animals from each group were taken randomly at 12, 24 and 36 hours after start the experiment. Body weight and liver weight was measured, and blood and tissue (liver and kidney) samples were taken at extermination.

DOI: 10.18380/SZIE.COLUM.2016.3.1.65

Ethical issues

The experiment was carried out according to the Hungarian Animal Protection Act, in compliance with the EU rules. The experimental protocol was authorised by the Food Chain Safety and Animal Health Directorate of the Pest County Agricultural Office, under permission number XIV-1-001/1880-5/2011.

Biochemical analyses

Level of conjugated dienes (CD) and conjugated trienes (CT) in liver, as markers of initial phase of lipid peroxidation, was measured by the absorbance of samples at 232 nm and 268 nm after extraction in 2,2,4-trimethylpentane (AOAC, 1984). Malondialdehyde (MDA) content, as marker of terminal phase of lipid peroxidation, was measured with the method of Placer et al. (1966) in blood plasma and according to Mihara et al. (1980) in liver and kidney homogenates. Reduced glutathione (GSH) content was measured as non-protein sulfhydryl groups with Ellmann's reagent (Sedlak and Lindsay, 1968), and glutathione peroxidase (GPx) activity according to Lawrence and Burk (1978) in blood plasma, in red blood cell haemolysates, and in the 10,000 g supernatant fraction of tissue homogenates. GSH content and GPx activity were calculated to protein content which was determined with biuret reaction (Weichselbaum, 1946) in blood plasma and red blood cell haemolysate, while Folin-phenol reagent (Lowry et al., 1951) was applied for the 10,000 g supernatant fraction of tissue homogenates.

Statistical analyses

Statistical analysis of data (one-way analysis of variance with Tukey's post-hoc test, calculation of means and standard deviations) was performed with GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA).

Results

There was no morbidity or mortality in the experimental groups during the trial.

Feed intake (Table 1), liver weight and relative liver weight (Table 2) did not change significantly during the experimental period between the treatment groups.

Initial phase of lipid peroxidation, characterised with levels of conjugated dienes and trienes, was not affected by feeding aflatoxin contaminated diets (data not shown), but its terminal phase, described with malondialdehyde concentration, showed increased intensity as a result of combined treatment with medicinal herb mixture and AFB1 containing diet (Tables 3-6). Significantly higher malondialdehyde concentration was found at 36th hour of treatment in blood plasma and red blood cell haemolysate samples of the group treated with the combination of AFB1 and medicinal herb mixture than the Control (Tables 3 and 4). However, variable changes were found in the liver (Table 5). There was significant difference between single Herbamix and combined Herbamix+AFB1 treatments, as the combination showed remarkably lower value. AFB1 alone did not cause significant

Table 1. Individual and combined effect of aflatoxin B1 and medicinal herb mixture (Herbamix™) on calculated individual feed intake (g in each period/hen) of laying hens in different periods of exposure

Groups/Timeframe	0-12 h	12-24 h	24-36 h
Control	146.50	4.06	144.50
Herbamix	150.00	0.00	147.50
Aflatoxin B1	150.00	0.00	146.50
Aflatoxin B1 + Herbamix	146.25	1.56	140.50

Table 2. Individual and combined effect of aflatoxin B1 and medicinal herb mixture (Herbamix™) on body weight, liver weight and relative liver weight (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin B1**	**Aflatoxin B1 + Herbamix**
	Body weight (g)			
12th hour	1986± 116.4	1940± 149.5	1941± 155.2	1859± 100.8
24th hour	1819± 158.1	1782± 276.0	1924± 92.8	1817± 63.5
36th hour	2023± 178.5	2047± 228.5	1945± 183.0	1997± 128.0
	Liver weight (g)			
12th hour	41.91± 6.59	39.85± 2.47	41.54± 3.58	42.88± 3.45
24th hour	34.22± 4.39	34.97± 8.08	41.57± 8.54	37.41± 2.86
36th hour	53.56± 2.98	48.69± 9.45	48.52± 4.49	48.80± 4.97
	Relative liver weight (g/100 g body weight)			
12th hour	2.10± 0.24	2.06± 0.20	2.14± 0.10	2.31± 0.25
24th hour	1.89± 0.25	1.95± 0.20	2.16± 0.44	2.06± 0.17
36th hour	2.66± 0.27	2.37± 0.29	2.50± 0.14	2.44± 0.17

Table 3. Individual and combined effect of aflatoxin B1 and medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of blood plasma (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin B1**	**Aflatoxin B1 + Herbamix**
	MDA (µmol/L)			
12th hour	15.40**a**± 0.79	15.93**ab**± 1.51	18.48**bc**± 1.90	19.13**c**± 1.21
24th hour	19.53± 1.74	21.06± 2.78	21.14± 0.67	18.09± 0.84
36th hour	17.72**a**± 1.32	22.28**ab**± 5.04	25.24**ab**± 2.35	28.71**b**± 3.60
	GSH (µmol/g protein content)			
12th hour	3.43± 0.71	3.51± 0.73	3.71± 0.77	4.14± 0.93
24th hour	4.84± 1.07	3.95± 0.81	5.34± 2.09	4.89± 2.33
36th hour	2.83± 1.05	2.76± 0.74	2.42± 1.15	3.22± 1.81
	GPx (U/g protein content)			
12th hour	4.60± 0.65	4.72± 1.55	5.07± 1.38	4.97± 1.71
24th hour	7.49± 1.79	5.66± 1.02	6.98± 1.88	6.93± 1.94
36th hour	6.93**b**± 1.94	4.17**a**± 0.98	3.82**a**± 0.28	4.04**a**± 0.98

[a,b] Means designated with different letters within the same rows mean significant difference ($p<0.05$)

DOI: 10.18380/SZIE.COLUM.2016.3.1.65

Table 4. Individual and combined effect of aflatoxin B1 and medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of red blood cell haemolysates (mean±SD; n=5)

	Control	Herbamix	Aflatoxin B1	Aflatoxin B1 + Herbamix
	MDA (µmol/L)			
12th hour	29.84b± 1.07	30.47b± 2.66	29.73b± 1.59	26.13a± 1.49
24th hour	38.57± 11.02	35.48± 7.03	37.32± 8.46	50.35± 14.13
36th hour	44.88a± 0.84	52.92ab± 5.58	45.32ab± 5.55	54.00b± 6.91
	GSH (µmol/g protein content)			
12th hour	8.92± 0.82	7.23± 0.67	7.88± 1.34	7.90± 0.87
24th hour	6.91± 3.46	6.18± 2.05	5.16± 1.59	4.36± 2.41
36th hour	5.79b± 0.42	4.11a± 1.04	4.65ab± 0.40	4.87ab± 0.99
	GPx (U/g protein content)			
12th hour	6.09± 1.01	6.25± 1.89	6.08± 0.79	6.05± 0.15
24th hour	5.55± 1.97	4.80± 1.19	4.04± 0.99	3.50± 1.03
36th hour	4.62± 0.56	4.14± 0.87	4.30± 0.46	4.24± 0.98

[a,b] Means designated with different letters within the same rows mean significant difference ($p<0.05$)

difference as compared to other experimental groups. In case of kidney, there were no significant changes in malondialdehyde concentration (Table 6).

Reduced glutathione concentration showed significant variance across the groups only in red blood cell haemolysate (Table 4) where medicinal herb mixture treatment resulted significantly lower level as compared to untreated Control, but in blood plasma (Table 3), liver (Table 5) and kidney (Table 6) no significant changes were found during the 36-hours period of experiment. Glutathione peroxidase activity was significantly lower in all treated groups as compared to the Control at 36th hour of experiment in blood plasma (Table 3), but there were no measurable changes in the other tissues during the experimental period (Tables 4-6).

Discussion

The result of present study showed that short-term aflatoxin B1 exposure did not have effect on feed intake and liver weight, which is probably due to short period of investigation, because long-term experiments showed marked feed refusal effect of AFB1 and liver weight was also increased (Aly Salwa and Anwer, 2009). However, AFB1, even after short-term exposure, induced lipid peroxidation, which was confirmed by the significant increase of the termination phase parameter (MDA) in blood plasma and red blood cell haemolysate, but not in liver or kidney. MDA concentration in blood plasma and red blood cell haemolysates revealed significant differences between the treatment groups at hors 12 and 36 but not at hour 24. This results probably due to the lack of feed intake in the period between hours 112 and 24, because of dark period. Additionally MDA concentration in liver and kidney was numerically lower in aflatoxin B1 contaminated feed fed group as compared to the control, which is probably duet to the impairment of lipid metabolism as effect of AFB1 (Siloto et al., 2013), while

Table 5. Individual and combined effect of aflatoxin B1 and medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation of liver homogenates (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin B1**	**Aflatoxin B1 + Herbamix**
	MDA (µmol/g wet weight)			
12th hour	8.28± 1.12	8.37± 2.41	10.59± 1.34	8.33± 1.68
24th hour	8.01± 2.66	7.21± 4.24	7.32± 2.96	6.76± 1.54
36th hour	13.61**ab**± 1.74	14.28**b**± 2.39	11.68**ab**± 1.59	10.70**a**± 1.65
	GSH (µmol/g protein content)			
12th hour	4.66± 1.20	4.64± 0.76	5.25± 1.87	6.06± 2.22
24th hour	3.78± 0.68	3.83± 0.93	3.17± 0.53	4.00± 0.71
36th hour	6.84± 0.68	7.79± 0.83	7.28± 0.41	6.61± 1.72
	GPx (U/g protein content)			
12th hour	4.22± 0.91	4.57± 0.83	5.13± 1.80	5.74± 2.20
24th hour	3.41± 0.88	3.75± 0.86	3.28± 0.49	4.01± 0.56
36th hour	7.01± 0.70	7.30± 0.50	7.04± 0.55	6.61± 1.77

a,b Means designated with different letters within the same rows mean significant difference ($p<0.05$)

Table 6. Individual and combined effect of aflatoxin B1 and medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation of kidney homogenates (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin B1**	**Aflatoxin B1 + Herbamix**
	MDA (µmol/g wet weight)			
12th hour	7.50± 1.58	9.84± 2.76	10.10± 1.07	10.29± 2.73
24th hour	12.44± 2.82	15.98± 3.46	19.36± 7.25	12.97± 2.56
36th hour	15.40± 2.86	11.57± 2.39	13.26± 2.50	15.28± 4.01
	GSH (µmol/g protein content)			
12th hour	5.29± 0.42	5.19± 0.66	5.79± 0.62	5.24± 0.31
24th hour	6.08± 1.15	4.58± 1.53	5.81± 0.89	6.11± 0.40
36th hour	6.70± 0.31	7.08± 0.86	6.83± 0.50	7.55± 1.84
	GPx (U/g protein content)			
12th hour	5.17± 0.44	5.62± 0.60	6.39± 1.13	6.06± 0.43
24th hour	4.79± 0.78	3.96± 0.94	4.81± 0.83	4.94± 0.34
36th hour	4.67± 0.68	5.14± 0.41	5.49± 0.59	5.55± 0.53

DOI: 10.18380/SZIE.COLUM.2016.3.1.65

MDA concentration partly depend on the actual lipid content of the sample (Dworschák et al., 1988) The results in case of liver are contradictory to previous findings which are showed significant MDA formation in liver, but after long-term aflatoxin B1 exposure or application of higher doses (Surai, 2002). Aflatoxin B1 in combination with medicinal herb mixture revealed lower value of MDA, thus lower rate of lipid peroxidation in liver and kidney, which alludes to reduced oxygen free radical formation when aflatoxin was combined with the herbs.

Among the parameters of the glutathione redox system, GSH content changed only in red blood cell haemolysate, as medicinal herb mixture resulted in significantly lower value than in the other groups. The exact cause of this difference is not known yet, but it probably caused by the changes of the intensity of glutathione synthesis in the red blood cells. It is an important finding, because erythrocyte glutathione plays an important role in mitigating the damaging effects of reactive oxygen species (ROS) present in the circulation (Mak et al., 1994) which causes continuous oxidation of haemoglobin within the cytosol of the erythrocyte (Hsieh and Jaffe, 1975). GSH reacts with ROS and degrades hydrogen peroxide and lipid peroxides by glutathione peroxidase or modifies toxic xenobiotics by glutathione *S*-transferase (Pace et al., 2003). The activity of glutathione peroxidase did not vary significantly in red blood cell haemolysate, liver and kidney, but it was reduced in all treated groups in blood plasma. This result is different than previous finding where AFB1 exposure resulted in significant drop in GPx activity in liver, however that was a long-term trial (Shi et al., 2012).

Acknowledgement

The present study was supported by the Stipendium Hungaricum Scholarship to MN and Research Centre of Excellence 11476-3/2016/FEKUT grant. The authors thank to Dr. Csaba Senánszky (Herbamix Trade Ltd.) for providing the Herbamix Basic premix.

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