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The protective effects of *Nigella sativa*, thymoquinone and bentonite in experimental aflatoxicosis model in broilers

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ABSTRACT

The aim of this study was to determine the preventive efficacy of black seed *(Nigella sativa L.)*, thymoquinone and Na bentonite on biomarkers of tissue degeneration in the liver and other organs induced by aflatoxin (AF) in broilers. One hundred broiler chicks were divided into 10 equal groups and fed for 28 days. The animals received feed with 2 mg/kg total aflatoxin, *Nigella sativa* (NS; 5%), thymoquinone (TMQ; 300 mg/kg) and Na bentonite (BNT; 10 g/kg) both individually and in combination. At the end of the experiment, blood and liver tissue samples were collected. AF treatment significantly increased serum and liver 8-hydroxy-2'-deoxyguanosine(8-OHdG), serum transforming growth factor β (TGF-β), and liver total antioxidant capacity (TAC) levels, whereas it significantly decreased liver TGF-β and serum TAC levels compared to the control. However, there were no changes in the serum and liver hepatocyte growth factor (HGF) levels from AF. Both individual and combined addition of NS, BNT and TMQ to AF-contaminated feed significantly improved liver 8-OHdG, liver TAC and serum TGF-β levels. Serum 8-OHdG levels were ameliorated only in the AF+BNT+TMQ group. In addition, the AF+BNT+NS and AF+BNT+TMQ groups showed significant improvement in liver 8-OHdG, liver TGF-β and liver TAC levels. We conclude that NS and TMQ supplementation to AF-contaminated feed may be beneficial against aflatoxicosis in broilers.

Key words: aflatoxin; broiler; DNA damage; liver biomarker; serum biomarker

Introduction

Aflatoxins (AF) are toxic metabolites synthesized by *Aspergillus* fungi and can be produced in feed, feedstuffs and agricultural foods when the conditions are suitable for the development of fungi. These toxins may be easily formed in carbohydrate and fat-rich agricultural products, such as wheat, corn, rice and peanuts, which are widely used in human and animal nutrition (OĞUZ, 2017). *A. flavus* and *A. parasiticus* are important species that produce aflatoxins. They produce four different types of aflatoxin (B1, B2, G1 and G2) (SARMA et al., 2017).

In the poultry industry, broiler chicks are required to reach the optimal growth weight in approximately 45-60 days since they are raised for meat yield. However, any factor preventing the

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growth of animals causes an important economic problem. AF is considered to be a factor that requires attention because it both slows growth and increases susceptibility to infectious diseases in addition to causing direct mortality in the poultry industry (MONSON et al., 2015). The most important pathological findings in acute and chronic aflatoxicosis are seen in the liver, such as hemorrhage, necrosis, fibrosis and fatty degeneration (ORTATATLI et al., 2002).

AFB1 is metabolized to AFM1 and AFB1-exo-8,9-epoxide, a major hepatocarcinogen, by microsomal enzymes in the liver in mammals and poultry. AFB1-exo-8,9-epoxide binds to DNA and causes guanine (G) mutation. This mutation showed a carcinogenic effect defined as a group 1 carcinogen according to IARC (MARCHESE et al., 2018).

In humans and animals, various disorders occur in many organs, tissues and blood parameters, caused by aflatoxins (MONSON et al., 2015). TGF-β is defined as a cytokine that prevents tumor formation by providing apoptosis of tumor cells in the stages before tumor development, but some extracellular and intracellular signals reverse the transforming growth factor's (TGF)-β task and contribute to tumor development and metastasis (CARMONA-CUENCA et al., 2006). In liver damage, TGF-β and hepatocyte growth factor (HGF) have an important role in liver regeneration. The levels of these growth factors vary depending on the period of liver damage/regeneration (KIM et al., 2002; BREITKOPF et al., 2006; HU and LEE, 2015).

HGF has been identified as a ligand for the c-MET receptor and is reported to activate cell-regulating, cell proliferation, viability and angiogenesis (DIMRI and SATYANARAYANA, 2020). HGF is a growth factor that protects cells against apoptosis. Therefore, it is considered to be a positive factor in tissue regeneration, but is considered a negative factor in the development of AF-induced hepatocarcinoma and tumor cells (PISCAGLIA et al., 2009; DIMRI and SATYANARAYANA, 2020).

Lipid peroxidation causes high levels of reactive oxygen species (ROS) and oxidative stress in the liver (YILMAZ et al., 2017). At the same time, DNA damage develops because of the formation of AF-epoxides and the level of 8-hydroxydeoxyguanosine (8-OHdG) increases (MONSON et al., 2015; MARCHESE et al., 2018). Glutathione (GSH), which is one of the main antioxidants of the organism, is found in all body cells, but the liver has the highest concentration. GSH contributes the immune system of the metabolism and maintains the reduction of free radicals and lipid peroxidation. Thus, it protects cells against apoptosis. GSH levels decrease with aflatoxicosis, and with this decrease AFB1-exo-8,9-epoxide reacts with the N7 atom of guanine to form promutagenic DNA adduct (aflatoxin-N7-guanine) (VERMA, 2004; YILMAZ et al., 2017). AF causes the formation of reactive oxygen species and lipid peroxidation, resulting in degeneration in the liver. Antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, especially GSH, decrease with aflatoxicosis but the use of antioxidant substances may prevent liver damage (YILMAZ et al., 2017).

In recent years, some non-nutritive and inert adsorbents have been used as feed additives to bind AF and reduce the absorption of AF from the gastrointestinal tract. These compounds should not be absorbed from the gastrointestinal tract and must have the ability to bind physically with chemical substances, precluding their absorption (OĞUZ et al., 2018). Bentonite (BNT) as a toxin binder has been effective in preventing aflatoxicosis, and the addition of BNT to broiler feeds reduces liver damage and other toxic effects of the aflatoxicosis (FOWLER et al., 2015). However, some reports have stated that the addition of BNT to an AF-containing diet was insufficient to reduce the toxic effects of aflatoxicosis in broilers, and the protective effect might be related to the amount of BNT (AZIZPOUR and MOGHADAM, 2015).

The fatty acids in Nigella sativa L (NS) are responsible for pharmacological effects in the prevention of aflatoxicosis. The main component of NS is thymoquinone (TMQ). NS seeds and their active components are said to be involved in the modulation of antioxidant, anti-inflammatory, and hepatoprotective activities. In particular,

TMQ has strong antioxidant properties (ATES and ORTATATLI., 2021). It has been determined that lesions were limited in rabbits treated with NS, and it was found to be partially successful in preventing liver fibrosis. Furthermore, the toxic effects of AF on the liver and kidneys were reduced in rats through the cytoprotective and antioxidant properties of NS (AL-GHASHAM et al., 2008).

TMQ, one of the volatile fatty acids obtained by the extraction of NS, has been described as hepatoprotective, anti-inflammatory and antioxidant, and it also reduces the effects of aflatoxicosis. However, in acute TMQ toxicity trials, it was determined that TMQ in higher doses partially decreased GSH levels in the liver, kidney, and heart (BADARY et al., 1998; ATES and ORTATATLI, 2021).

The aim of the present study was to determine the effects of BNT, NS and TMQ on liver and serum TGF-β, HGF, and 8-OHdG, which are important tissue degeneration biomarkers, and the total antioxidant capacity (TAC) in experimental aflatoxicosis in broilers.

Materials and methods

The current research protocol was reviewed and approved by the Ethics Committee of Selcuk University Veterinary Faculty, Experimental Animals Production and Research Center (No:2018/08).

Animals and diets. One hundred 1-d-old male broiler chicks *(Ross)* were obtained from a commercial hatchery. The chicks were divided at random into 10 groups and housed in floor pens with continuous lighting, and were fed *ad libitum*. The basal diet was formulated according to NRC guidelines (1994) and the chicks were fed a commercial starter diet (1-10 days) and a grower diet (11-28 days). The diets were also analyzed by HPLC (Shimadzu, Tokyo, Japan) for possible residual AF before feeding. The limit of detection (LOD) was 0.4 ng/mL for AFB1 and AFG1; 0.2 ng/mL for AFB2 and AFG2; the limit of quantification (LOQ) was 0.8 ng/mL for AFB1 and AFG1; 0.4 ng/mL for AFB2 and AFG2. AF's were not at detectable levels in the diet (86% recovery by the extraction method).

Table 1. Composition of the experimental starter and grower diet

Analytical components (g/kg)			
Starter diet (1 to 10 d)		Grower diet (11 to 28 d)	
Crude protein	220	Crude protein	200
Crude cellulose	37	Crude cellulose	38
Crude fiber	37	Crude fiber	63
Ether extract	63	Ether extract	68
Calcium	12	Calcium	12
Phosphorus	6.5	Phosphorus	6.5
Methionine	5	Methionine	4
Lysine	12	Lysine	13

Aflatoxin and analyze. AF was produced from an *Aspergillus parasiticus* NRRL 2999 culture (USDA, Agricultural Research Service, Peoria, IL, USA) by fermentation of rice (OĞUZ et al., 2000). Successfully fermented rice was then steamed to kill the fungus, dried and ground to a fine powder. AF content in the rice powder was analyzed by HPLC (Shimadzu, Tokyo, Japan). AF detection was performed by the FLD detector (RF 20A) at 360 nm excitation and 430 nm emission wavelengths. The column temperature was set to 30 °C, and the autosampler to room temperature. AF separation was performed with a Supelcosil LC-18 column (150x4.6 mm; internal diameter, 5 µm; Supelco Analytical). The fluorescence feature of AFs was found using electrochemical bromide in a Cobra cell (Coring System Diagnostics GmbH) unit in post-column derivatization. For the test, the mobile phase used water: acetonitrile: methanol, 60:20:20, v/v/v, containing potassium bromide (120 mg) and venitric acid (4 M, 350 µl), and the flow rate was 1 mL/min. Analyses were performed with LC solution software (Shimadzu, Tokyo, Japan).

The aflatoxin within the rice powder consisted of 83.34% AFB1, 2.98% AFB2, 5.37% AFG1 and 2.01% AFG2 (detection limit 1µg AF/kg rice powder, 86% recovery by the extraction method).

Experimental design. The experimental design consisted of 10 dietary treatment groups, and the research continued for 28 days. The rice powder was incorporated into the basal diet to provide the required 2 mg aflatoxin/kg feed. AF was incorporated into the basal diet (Table 1) before NS, TMQ and BNT were added. TMQ was prepared by mixing it into rice flour. Then, NS (Bagdat Baharat®, Istanbul, Turkey), TMQ (CAS 490-91-5, SantaCruz®, USA) and BNT (Karben Ltd®, Ankara, Turkey) were supplemented to the feed. Feed and water were provided *ad libitum*.

The grouping of the research, the administered substances and their levels are presented in Table 2.

Table 2. Experimental design of the research

Group No	Group name	Substances applied	Substances levels
1	Control	-	Basal diet
2	NS	*Nigella sativa*	50 g NS/kg diet
3	TMQ	Thymoquinone	300 mg TMQ/kg diet
4	BNT	Bentonite	10 g BNT/kg diet
5	AF	Aflatoxin	2 mg AF/kg diet
6	AF+NS	Aflatoxin+*Nigella sativa*	2mg AF plus 50 g NS/kg diet
7	AF+BNT	Aflatoxin+Bentonite	2 mg AF plus 10 g BNT/kg diet
8	AF+TMQ	Aflatoxin+Thymoquinone	2 mg AF plus 300 mg TMQ/kg diet
9	AF+BNT+NS	Aflatoxin+Bentonite+*Nigella sativa*	2 mg AF plus 300 mg TMQ/kg diet
10	AF+BNT+TMQ	Aflatoxin+Bentonite+Thymoquinone	2 mg AF plus 10 g BNT plus 300 mg TMQ/kg diet.

Table 3. Effect of *Nigella sativa* (50 g/kg), thymoquinone (300 mg/kg) and Na bentonite (10 g/kg) on serum and liver 8-OHdG, HGF, TGF-β, TAC in broiler chicks fed a diet containing 2 mg total aflatoxin/kg diet at 1 to 28 d of age

Groups/ Parameters	Serum 8OHdG	Liver 8OHdG	Serum HGF	Liver HGF	Serum TGF-β	Liver TGF-β	Serum TAC	Liver TAC
Control	3.29 ± 0.48 ^{d}	2.33 ± 0.82 ^{b}	2436.9 ± 94.9 ^{a}	16.98 ± 6.20 ^{ab}	250.13 ± 53.6 ^{c}	98.91 ± 13.7 ^{ab}	115.00 ± 0.63 ^{a}	99.80 ± 2.22 ^{bc}
NS	6.28 ± 0.86 ^{abc}	1.92 ± 0.56 ^{b}	2728.9 ± 16.8 ^{a}	3.55 ± 3.01 ^{b}	588.13 ± 33.0 ^{b}	105.60 ± 23.6 ^{a}	113.64 ± 0.80 ^{ab}	101.27 ± 2.76 ^{b}
TMQ	3.60 ± 0.74 ^{cd}	2.00 ± 0.61 ^{b}	2500.7 ± 144.5 ^{a}	3.80 ± 3.39 ^{b}	368.38 ± 44.4 ^{c}	66.30 ± 12.9 ^{abc}	112.99 ± 0.50 ^{ab}	90.67 ± 1.27 ^{de}
BNT	4.35 ± 0.88 ^{abcd}	2.52 ± 0.45 ^{b}	2697.6 ± 42.63 ^{a}	6.51 ± 5.37 ^{b}	393.97 ± 60.7 ^{c}	76.35 ± 14.5 ^{abc}	113.75 ± 0.46 ^{ab}	88.14 ± 1.80 ^{e}
AF	6.78 ± 1.24 ^{ab}	4.75 ± 0.60 ^{a}	3002.4 ± 222.1 ^{a}	45.37 ± 25.62 ^{ab}	574.84 ± 87.2 ^{b}	11.25 ± 3.8 ^{d}	112.72 ± 0.28 ^{b}	114.43 ± 3.70 ^{a}
AF + NS	4.50 ± 0.79 ^{abcd}	1.25 ± 0.30 ^{b}	2717.6 ± 22.3 ^{a}	90.75 ± 52.18 ^{ab}	350.90 ± 41.8 ^{c}	54.18 ± 7.7 ^{c}	113.85 ± 0.40 ^{ab}	96.60 ± 2.41 ^{bcd}
AF + BNT	3.99 ± 0.92 ^{bcd}	2.15 ± 0.29 ^{b}	2696 ± 22.58 ^{a}	106.12 ± 47.93 ^{a}	370.74 ± 58.1 ^{c}	5.53 ± 2.2 ^{d}	113.60 ± 0.35 ^{ab}	101.33 ± 2.65 ^{b}
AF + TMQ	4.83 ± 0.85 ^{abcd}	2.81 ± 1.04 ^{b}	2629.5 ± 87.3 ^{a}	49.28 ± 31.33 ^{ab}	291.68 ± 40.5 ^{c}	64.54 ± 5.12 ^{bc}	110.54 ± 1.21 ^{c}	92.96 ± 3.38 ^{cde}
AF + BNT + NS	7.01 ± 1.24 ^{a}	2.98 ± 0.29 ^{b}	3016.6 ± 264.7 ^{a}	37.72 ± 20.85 ^{ab}	734.99 ± 79.7 ^{b}	75.26 ± 17.7 ^{abc}	112.43 ± 0.56 ^{b}	96.44 ± 2.64 ^{bcd}
AF + BNT + TMQ	2.17 ± 0.59 ^{d}	2.6 ± 0.44 ^{b}	3088.7 ± 602.1 ^{a}	75.35 ± 27.30 ^{ab}	1031.87 ± 74.9 ^{a}	54.80 ± 11.2 ^{c}	109.17 ± 0.31 ^{c}	96.78 ± 2.59 ^{bcd}

NS: *Nigella sativa,* TMQ: Thymoquinone, BNT: Na bentonite, AF: Aflatoxin, 8-OHdG: 8-hydroxydeoxyguanosine, HGF: Hepatocyte growth factor, TGF-β: Transforming growth factor, TAC: Total antioxidant capacity, $^{a-e}$: values within columns with no common superscripts are significantly different ($P < 0.05$).Values represent the mean ± SEM of 10 groups of 10 broiler chicks per treatment.

Biochemical analysis and antioxidant capacity. At the end of the 28-day trial period, the blood samples were drawn from the *vena subcutanea ulnaris*, and collected into anticoagulant (K2 EDTA) and serum separating tubes. The tubes were centrifuged at 1600 g, and plasma and serum were separated, respectively. The animals were euthanized by intravenous sodium pentobarbital injection, and liver tissues were collected in the sterile tubes. The serum, plasma, and liver samples were stored at -80°C until ELISA analysis.

The level of transforming growth factor-beta (Chicken TGF-β ELISA Kit, Catalog no: 201-16-0041, Shanghai Sunred® Biological Technology Co., Ltd, China), hepatocyte growth factor (Chicken HGF ELISA Kit, Catalog no: 201-16-2709, Shanghai Sunred® Biological Technology Co., Ltd, China), 8-hydroxydeoxyguanosine (Chicken 8-OHdG ELISA Kit, Catalog no. 201-16-0001, Shanghai Sunred® Biological Technology Co., Ltd, China), total antioxidant (Total Antioxidant Capacity Assay Kit, Catalog no. Ab65329, Abcam Company®, United Kingdom) were determined spectrophotometrically (Bio-Tek Instruments Inc.®, MWGt Lambda Scan 200) using commercially available kit procedures.

Statistical analysis. The data were obtained and evaluated by SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) statistical program with one-way analysis of variance and post hoc Duncan test. $P<0.05$ was considered statistically significant.

Results

The changes in serum and liver 8-OHdG, HGF, TGF-β and TAC parameters caused by single and combined addition of NS, TMQ and BNT in experimental aflatoxicosis in broilers are presented in Table 3.

Feeding animals with a diet of AF alone increased serum-8OHdG, liver-8OHdG, serum-TGF-β and liver-TAC levels compared to the control group ($P<0.05$). At the same time, liver-TGF-β and serum-TAC levels in AF-fed animals were decreased compared to the control group ($P<0.05$).

Addition of NS, TMQ and BNT alone to AF-contaminated feed significantly improved the liver-8 OHdG, serum-TGF-β and liver-TAC levels changed caused by AF ($P<0.05$). However, there was also a partial improvement in serum-TAC level compared to the AF group by the addition of NS and BNT alone ($P>0.05$).

It was determined that BNT + NS addition to AF-contaminated feed provided a significant improvement in liver-8OHdG, liver-TAC and liver-TGF-β levels compared to the AF group ($P<0.05$). BNT + TMQ supplementation to AF-contaminated diet improved the serum and liver 8-OHdG, liver-TGF-β and liver-TAC levels compared to the AF group ($P<0.05$).

Single addition of NS, BNT and TMQ to an AF-free diet did not cause any significant change in the examined parameters compared to the control, with only a minor exception (an increase in serum-8OHdG and serum-TGF-β levels in the NS-alone group).

Discussion

AFs are defined as carcinogenic mycotoxins that also cause growth retardation, malnutrition and suppression of the immune system in humans and animals (OGUZ, 2017; YILMAZ et al., 2017). The aim of the present study was to determine whether BNT, NS and TMQ improved the liver and serum TGF-β, HGF, 8-OHdG and TAC changes induced by AF.

Generally, two approaches are applied to reduce the negative effects of mycotoxins in feed for animal and human health. One of them is to bind mycotoxins in the gastrointestinal tract by adsorbents and to ensure their excretion without passing into the blood circulation (GOWDA et al., 2008; VEKIRU et al., 2015; PAPPAS et al., 2016). The other is the use of feed additives, such as antioxidants, that will interfere in the metabolism of circulating mycotoxins and/or mitigate their effects (UYAR et al. 2016; OĞUZ et al., 2018).

In vivo studies have shown that the epoxide derivatives of AFB1 are formed as a result of biotransformation and DNA alkylation, and consequently a mutation from guanine to

thymine. The increase in reactive oxygen radicals in aflatoxicosis contributes to mutations, and consequently increases the 8-OHdG level in hepatocytes (BEDARD and MASSEY, 2006). In a previous study in rats, the 8-OHdG level was an important finding for DNA damage in AFB1-induced aflatoxicosis. However this level increased in the blood at a later time (14 d after being given AFB1) compared to specific liver enzymes such as ALT and AST (3 d after being given AFB1). It has been reported that liver 8-OHdG levels vary depending on the levels of AF, and that 8-OHdG levels cannot be influenced by antioxidant Vitamin E. However, selenium increases GSH activity and decreases both oxidative damage and 8-OHdG levels (SHEN et al., 1995). The interaction of oxygen radicals with DNA bases also contributes to the formation of mutations. The administration of antioxidant substances reduces liver DNA-adducts and the level of 8-OhdG, and may prevent DNA mutations (YILMAZ et al., 2018). This effect of antioxidants has been reported to be mainly due to inhibition of phase 1 and phase 2 reactions in which liver AF metabolites are formed and reduce the formation of DNA-adducts via glutathione S transferase enzyme (TANG et al., 2007). On the other hand, aflatoxin-induced DNA damage differed according to organs (GUINDON-KEZIS et al., 2014). NSs do not cause any DNA damage and cannot change the level of 8-OHdG (SALAMA, 2011). Oxidative stress (MDA) and liver damage were prevented, and the liver glutathione level increased with prophylactic TMC (9 mg/kg dose, i.p.) therapy in mice, 3 days before experimental aflatoxicosis. However, higher or lower doses than this had no strong effect (NILI-AHMADABADI et al., 2011). On the other hand, TMQ can cause cytotoxic and genotoxic effects through depletion of cellular GSH and induction of DNA damage, depending on its concentration (MASHAYEKHI-SARDOO et al., 2020). NS extracts have been reported to have different effects on various enzymes involved in liver metabolism (IBRAHIM et al., 2008). Bentonite, which was added as a toxin binder, partially reduced AF induced liver lesions, but it caused a significant increase in the level of liver enzyme AST in broilers (SHANNON et al., 2017). In another study, although the liver DNA structure was disrupted in fish with experimental aflatoxicosis, this damage was partially prevented by BNT addition (HASSAN et al., 2010). In the present study, it was determined that AF administration increased liver and serum 8-OHdG levels. The reason for this increase was thought to be due to the fact that epoxide derivatives of AF cause DNA damage in cells. It was observed that the increase in 8-OHdG levels was prevented in the liver, which is the target organ especially in aflatoxicosis, by the addition of NS, BNT and TMQ alone with AF. The addition of NS and its extract, TMQ, may have increased liver glutathione levels and prevented the increase in liver 8-OHdG levels by decreasing the liver DNA-adducts formed during AF biotransformation. BNT may reduce DNA damage to the liver induced by AF, due to partial binding of AF in the gastrointestinal tract. However, in the present study, fluctuations in serum 8-OHdG levels may be a reflection of different effects AF, or NS, BNT and TMQ in different organs. In addition, the AF+BNT+TMQ combination had more positive results in preventing DNA damage. However, the insufficiency of this effect in AF+BNT+NS may be due to non-TMQ substances in the NS structure that reduce the binding activity of BNT.

Administration of AFB1 increases free oxygen radicals and biomolecular oxidative damage. This increase in oxidative stress plays a negative role in cell viability and functionality and cellular signaling pathways. However, it is emphasized that this situation may change depending on the antioxidant capacity of the organs (MARY et al., 2012). AFB1 administration increased MDA levels and decreased SOD levels depending on the dose and duration of AFB1 administration in a peripheral blood mononuclear cell culture (BERNABUCCI et al., 2011). In addition, AFB1 was reported to cause oxidative stress when it transforms into epoxide derivatives with cytochrome enzymes CYP450 in the liver of rats. Furthermore, antioxidants such as GPx, SOD, CAT and GSH decreased, and the level of oxidative stress markers increased, and AFB1 epoxides increased cellular toxicity, especially by conjugating with GSH. The decrease in GSH levels due to this conjugation indicates that it is consumed

in cells (TANG et al., 2007; YILMAZ et al., 2018). MDA levels increase with increased oxidative stress in rats with experimental aflatoxicosis, but this oxidative stress may be suppressed with NS administration where this effect is probably caused by TMQ (SOLIMAN et al., 2012).

In previously conducted research, antioxidant enzyme levels such as liver CAT and GPx were decreased, and lipid peroxide levels were increased in broilers with experimental aflatoxicosis. However, there was no change in the level of SOD, another antioxidant enzyme. In the same study, whilst the addition of BNT to AF-free diet did not have any effect on liver oxidative and antioxidant systems, it was determined that the addition of BNT to AF-containing diet, at the level of 2.5 and 5.0 g/kg diet, did not affect the liver and kidney antioxidant enzymes, but decreased lipid peroxide levels. It has been reported that this effect may be caused by the inability of BNT for optimal binding of AF in the digestive tract (ERASLAN et al., 2004). In another study, different levels of BNT (3.7 and 7.5 g/kg) did not improve plasma and liver total antioxidant levels in aflatoxicosis (0.6 mg/kg) in broilers (BHATTI et al., 2016).

In the present study, aflatoxicosis increased liver total antioxidant levels and decreased serum total antioxidant levels. It was thought that the feed additives (NS, TMK, BNT) caused significant changes in the liver. The main reason for the increase in the level of TAC in the liver, which is a specific organ for AF, may be the release of antioxidant systems against the oxidative stress caused by the animals' metabolism. Addition of NS and TMQ alone to AF-contaminated feed might have achieved significant protective efficacy in terms of TAC levels because it inhibited aflatoxicosis-induced inflammation and cell damage in the liver. Addition of BNT alone to the AF-contaminated feed prevented the increase in liver TAC levels, because BNT may reduce the oxidative stress by binding AFB1 in the gastrointestinal tract. The similar effects of liver TAC levels in combined groups are thought to depend on the stated properties of these substances. In addition, AF mainly causes oxidative stress in the liver, and the antioxidant capacity of the liver is greater than that of other organs. Therefore, the liver TAC levels may have responded more strongly compared to other organs when stimulated with AF. However, it may have decreased the serum TAC level since the antioxidant capacity of other organs is smaller than the liver. In the present study, the responses of preventive substances to aflatoxicosis may not be fully reflected in the serum TAC levels.

HGF has been reported to induce apoptosis and tumor inhibition by jun kinase 1 (JNK1) induction, and by acting on c-met and EGF signaling pathways in AF-induced hepatocyte cell cultures (CONNER et al., 1999). HGF plays a protective role against c-Met expression and apoptosis in AF-induced hepatic cholangiocarcinoma in rats (PISCAGLIA et al., 2009). Serum HGF levels were increased in experimental hepatocarcinogenesis in rats. In the same study, the ethanolic extract of NS prevented the increase of HGF levels by inhibiting angiogenesis, and thereby acted as protection from experimental liver cancer. It was also reported that these effects of NS may be caused by TMQ (FATHY and NIKAIDO, 2018).

In the present study, although there was no statistical difference between serum and liver HGF values, it was observed that there was a significant numerical increase in the experimental aflatoxicosis groups compared to the control. The partial increases in HGF values in aflatoxicosis groups are thought to depend on partial apoptosis because of genetic mutations and degeneration in cells. In addition, the data in the literature are insufficient about HGF in the aflatoxicosis model.

Aflatoxicosis causes immunosuppression, and suppression of liver gene expression of TGF-β cytokine by this immunosuppression (MAHFOUZ, 2015). TGF-β is required for liver regeneration, especially in acute cases (DOOLEY and TEN DIJKE, 2012) and its increase by fibrogenesis is suppressed by stimulation of different pathways (BISSELL et al., 2001). In a study, NS decreased inflammation with close to normal levels of TGF-β expression (NOOR et al., 2015). Although the administration of high doses (0.5 g/kg) of methanolic extract of NS to fish has been seen to regulate TGF-β expression and provide immunomodulation, administration of low doses of NS (0.1 g/kg) decreased TGF-β

expression and increased antioxidant effects (CELIK ALTUNOGLU et al., 2017). In an in vitro study, TMQ has been reported to regulate TGF-β and ensure cell restoration (RAJPUT et al., 2015), and to reduce the over-expression of TGF-β in cancer cells to normal values (KOU et al., 2017). In addition, NS and TMQ has been stated to regulate or increase TGF-β levels in inflammation, fibrosis and cancer cases (HAYAT et al., 2011; KENSARA et al., 2016; ABIDI et al., 2017). The AF binding capacity of BNT may vary depending on the chemical and physical structure, and the amount of BNT. Furthermore, it has been indicated that different results may occur in different biochemical, histopathological and molecular parameters according to the binding rate of BNT, and also assessment of the damage using a single parameter would not be very accurate (AZIZPOUR and MOGHADAM, 2015; FOWLER et al., 2015; SHANNON et al., 2017).

In the present study, liver TGF-β levels may have been decreased by AF depending on DNA damage and immunosuppression. However, aflatoxicosis might have triggered serum TGF-β levels by different pathways. It may also have led to variability in organ and serum TGF-β values, depending on the dose. In addition, single and combined administration of NS and TMQ to AF-contaminated feed may have prevented the degeneration, inflammation, and DNA damage in the liver caused by aflatoxicosis.

Addition of BNT alone to AF-contaminated feed may not have prevented the decrease in liver TGF-β level due to either inadequate binding of AF, or the fact that it may bind amino acids essential for the liver in the intestine. Increased levels of TGF-β can be explained by the dose-dependent immunomodulatory effect of NS in the NS-alone group (BOSKABADY et al., 2011).

In conclusion, aflatoxicosis is defined as an important type of intoxication in humans and animals in the world. NS and TMQ administration may be effective in aflatoxicosis, and they may be important to resolve this intoxication and this should guide many future studies. These data should be evaluated together with other parameters that reflect the effects of AF on target organs and tissues.

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DIK, B., M. B. ATES, H. OGUZ, M. ORTATATLI: Zaštitni učinci crnog kima (*Nigella sativa*), timokinona i bentonita na modelu eksperimentalne aflatoksikoze u tovnih pilića. Vet. arhiv 93, 653-664, 2023.

SAŽETAK

Cilj je rada bio odrediti zaštine učinke crnog kima (*Nigella sativa* L.), timokinona i natrijeva bentonita na biomarkere tkivne degeneracije jetre i drugih organa tovnih pilića u kojih je inducirana aflatoksikoza. Ukupno je 100 tovnih pilića podijeljeno u 10 skupina po 10 jedinki. Životinje su tijekom 28 dana dobivale hranu kojoj je dodano 2 mg/kg ukupnog aflatoksina, crni kim – *Nigella sativa* (NS, 5%), timokinon (TMQ, 300 mg/kg) i natrijev bentonit (BNT, 10 g/kg), u pojedinačnoj dozi i u kombinacijama. Na kraju pokusa prikupljeni su uzorci krvi i tkiva jetre. Aflatoksin je znakovito povećao 8-hidroksi-2'-deoksigvanozin (8-OHdG) u serumu i jetri, serumski transformacijski faktor rasta β (TGF-β) i ukupni antioksidacijski kapacitet jetre (TAC), dok su jetreni TGF-β i razine serumskog TAC-a bili znakovito sniženi u usporedbi s kontrolnom skupinom. Aflatoksin, međutim, nije izazvao promjene u vrijednosti faktora rasta hepatocita (HGF) u serumu i jetri. I pojedinačno i kombinirano dodani NS, BNT i TMQ u hranu kontaminiranu aflatoksinom znakovito su poboljšali vrijednosti 8-OHdG i TAC u jetri te razine TGF-β u serumu. Razine serumskog 8-OHdG povišene su samo u skupini koja je dobivala kombinaciju AF-a, BNT-a i TMQ-a. Također, pokusne skupine koje su dobivale AF + BNT + NS i AF + BNT + TMQ pokazale su znakovito poboljšanje vrijednosti 8-OHdG, TGF-β i TAC u jetri. Rezultati istraživanja pokazuju da dodatak NS-a i TMQ-a hrani za tovne piliće s aflatoksikozom može imati korisne učinke.

Ključne riječi: aflatoksin; brojleri; DNA oštećenje; jetreni biomarker; serumski biomarker