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The effects of aflatoxin B_1 on growth hormone regulated gene-1 and interaction between DNA and aflatoxin B_1 in broiler chickens during hatching

E Gülbahçe Mutlu[1], E Arslan[2], Y Öznurlu[3], H Özparlak[2]

[1]*Department of Physiology, Faculty of Medicine, KTO Karatay University, Karatay,* [2]*Department of Biology, Faculty of Science, Selçuk University, Selçuklu, Konya, Turkey, and* [3]*Department of Histology-Embryology, Faculty of Veterinary, Selçuk University, Selçuklu, Konya, Turkey*

Abstract

Many types of aflatoxin cause problems for both public and animal health. Aflatoxin B_1 (AFB_1) is the most toxic and commonly encountered fungal toxin that appears in poultry feed and in feeds stored under unsuitable conditions. AFB_1 decreases feed quality, egg production and fertility of hatching eggs. Also, AFB_1 alters the development of embryos by infecting eggs. We investigated using sequence analysis the changes caused by different concentrations of AFB_1 on the promoter sequences of the growth hormone regulated gene-1 (GHRG-1) in chick embryo at 13, 17, 19 and 21 days incubation. DNA isolated from the liver of chick embryos treated with different concentrations of AFB_1 was separated using agarose gel electrophoresis to detect apoptosis, and DNA interaction with AFB_1 was investigated using plasmids to detect changes in electrophoretic mobility and their effects on DNA. Base changes of the promoter sequences of GHRG-1 in 5 ng/egg, 15 ng/egg and 40 ng/egg doses of AFB_1 were increased on day 19 compared to base changes of the same AFB_1 doses on day 13. We also found that AFB at different concentrations changed the mobility of DNA by binding to it, and that high doses of AFB1 destroyed DNA. The DNA interaction study using plasmid demonstrated that AFB_1 at high doses was bound to plasmid DNA, slowed its mobility and inhibited restriction cuts.

Key words: aflatoxin B_1, broiler chickens, DNA interaction, growth hormone regulated gene-1, sequence analysis

Aflatoxins (AFs) are metabolites produced by *Aspergillus parasiticus* and *Aspergillus flavus*. AFs may occur as natural contaminants of poultry foods (Edds and Bortell 1983, Giambrone et al. 1985a,b, Leeson et al. 1995, Oğuz et al. 2002). In many areas of the world, AFs have been identified in foods for poultry and domestic animals (Arafa et al. 1981, Jindal et al. 1994). There are 18 AF types, but AFB_1, AFB_2, AFG_1 and AFG_2 are commonly encountered as fungal toxins in food (Madhusudhanan et al. 2006); aflatoxin B_1 (AFB_1) is the most toxic and most common (Busby and Wogan 1981, Neldon-Ortiz and Qureshi 1992, Oğuz et al. 2002). The toxic effects of AFB_1 are a major concern for poultry production because of serious economic losses resulting from poor feed utilization, anorexia, decreased egg production, slowed body weight gain, increased susceptibility to diseases and increased mortality (Qureshi et al. 1998, Tessari et al. 2006, Oğuz et al. 2002, Oguz 2011, Oznurlu et al. 2012).

AFs exhibit mutagenic, teratogenic, carcinogenic and growth inhibitory effects. AFB_1 is the most hepatotoxic and hepatocarcinogenic AF. Animals fed contaminated food can pass AFB_1 products into milk products, eggs and meat. These products accumulate in the fat depots and soft tissues of the chicken (Leeson et al. 1995, Bintvihok et al. 2002). AF residues also are found

Correspondence: E . Arslan, Selçuk University, Faculty of Science, Department of Biology, Selçuklu, Konya, Turkey. Phone: 90 332 2231884, e-mail: earslan@selcuk.edu.tr

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in the egg (Jacobson and Wiseman 1974, Sudhakar 1992, Qureshi et al. 1998). Jacobson and Wiseman (1974) found 9 ng AFB_1/egg on day 10 of feeding in chickens that consumed diets that contained 100 mg/kg AFB_1. Trucksess et al. (1983) fed laying hens a diet containing 8,000 mg/kg AFB_1 for 7 days and found that livers and ova contained the greatest amounts of AFB_1 and its metabolite, AF Ro. Trucksess et al. (1983) and Qureshi et al. (1998) reported that the carryover ratio of AFB_1 from layer hen food into eggs was between 1:2,000 and 1:2,500. The legal upper levels in Turkey for food for laying hens are 10 µg/kg for AFB_1 and 20 µg/kg for AF, but these limits often are exceeded (Nizamlıoğlu 1996, Oguz et al. 2011).

Although the mechanism of the effects of AFB_1 in adults is well known, there is less information about the effects of AFB_1 in embryonic cells. The effect of AFB_1 is caused by interaction of the toxin with reactive sites on DNA and two types of interaction are known. One of interaction is weak, reversible, non-covalent binding, while the other is irreversible covalent binding that forms AF-DNA adducts.

AFB_1 is metabolized by the liver. AFB_1 biotransformation produces metabolic products, especially hydroxylated derivatives. Epoxidation of AFB_1 to exo 8, 9-epoxide is considered responsible for the carcinogenic effect. The epoxide is extremely unstable and reacts with nuclear DNA; it binds with high affinity to guanine bases to form aflatoxin-N (7) guanine (Hatch 1988). The formation of AF-DNA adducts causes malignant transformations and deletions (Laurent-Puig et al. 2001) and sister chromatid exchanges (Qureshi et al. 1998).

Oznurlu et al. (2012) and Gündüz and Oznurlu (2014) reported developmental retardation in the skeletal and muscular systems as well as general developmental retardation in high-dose AFB_1 treated chicks. Therefore, we investigated the potential base changes in the regulatory promoter region of growth hormone regulated gene-1 (GHRG-1) caused by different concentrations of AFB_1. We also detected apoptosis by electrophoretic mobility of total DNA isolated from the liver of the groups that were treated with different concentrations of AFB_1; the interaction between AFB1 and plasmid DNA also was examined. The known sequence of pBR322 plasmid DNA and restriction map were used to determine whether or not AFB_1 was bound to DNA and if so, to identify the base to which it was linked. Although much is known about the toxic effects of AFB_1, there are few reports concerning its effects on chicken DNA.

Material and methods

Preparation of AFB_1 solutions

AFB_1 (Makor Chemical Co., Jerusalem, Israel) solutions were prepared in 30% ethanol according to Oznurlu et al. (2012). Pure AFB_1 was diluted in benzene to prepare a 20 µg/ml stock solution. The solution was transferred to vials to contain the desired concentrations of AFB_1 for each dose group and the benzene was allowed to evaporate overnight. The AFB_1 residue was dissolved in absolute ethanol (99.9%), which then was diluted to 30% with sterile double-distilled water. The AFB_1 concentration of these solutions was measured in duplicate using a thin layer chromatography (TLC) densitometer equipped with a fluorescence detector (MPF 43A; Perkin Elmer, Santa Clara, CA) at 365 nm excitation and 425 nm emission wavelengths, and by a UV-visible recording spectrophotometer (UV 2100; Shimadzu, Nakagyo/KU, Kyoto, Japan).

Treatment groups and injections

We used 420 fertile eggs of Ross parent stock. The eggs were weighed, then divided into five groups: control group, 45 eggs; 30% ethanol injected group (solvent group), 50 eggs; 5 ng AFB_1/egg group, 74 eggs; 15 ng AFB_1/egg group, 99 eggs; and 40 ng AFB_1/egg group, 152 eggs. The injections were performed just before placing the eggs in the incubator. The egg shell was drilled at the blunt end and 20 µl of the respective solution was injected into the air space (Parlat et al. 1999) using micropipettes. After injection, the hole was sealed with melted paraffin. The eggs were placed in an incubator maintained at 37.8° C and 65% relative humidity and turned 90° every 2 h.

Determining stage of embryonic development

From each group and on days 13, 17, 19 and 21 of incubation, five randomly selected eggs each containing a developing embryo were weighed with a digital balance and opened. The developmental stage of each embryo was determined according to the Hamburger-Hamilton scale (1951). Liver samples were obtained from each embryo.

DNA isolation and polymerase chain reaction (PCR) amplification of GHRG-1 gene region

Genomic DNA was isolated from embryonic liver using the technique described by Chatigny (2000).

Tissues were ground to a fine powder in liquid nitrogen using a small mortar and pestle. Approximately 50 mg of ground tissue was placed in microcentrifuge tube together with 1 ml STE buffer (0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.001 M EDTA), 25 µl of 20 mg/ml proteinase K and 50 µl of 20% SDS. The mixture was mixed gently, then incubated on a water bath for 24 h at 55° C. The mixture then was placed in a 15 ml centrifuge tube and extracted using 25:24:1 phenol:chloroform:isoamyl alcohol. The aqueous layer was placed in a clean tube and 1/10 volume of 2 M sodium chloride was added. The mixture was precipitated with 1 volume cold 95% ethanol and left at −20° C overnight. Each tube then was centrifuged for 30 min at 7,000 x g and the supernatant removed using a micropipette. The remaining liquid was evaporated in a convection oven for 2 h at 55° C. The DNA was re-suspended in 50 µl TE buffer (0.001 M Tris HCl, pH 7.5, 0.0001 M EDTA).

Primers were designed based on GHRG-1 (promoter) of the nuclear DNA sequence of *Gallus gallus* obtained from the GenBank™ database at the National Center for Biotechnology Information (NCBI; accession no. S75126) (Agarwal et al. 1995). The sequences of forward and reverse primers were: 5′-GGTCAGTGCTTTGTCCTGATGG-3′ and 5′-CTGAGCGTTTTCACATGTTCAGG-3′, respectively.

The PCR reaction volume was 15 µl and contained 50 pmol of each forward and reverse primer, 10 x PCR buffer (200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl, pH, 8.8, 0.1% Tween 20), 25 mM $MgCl_2$, 20 mM dNTP, 5 units Taq polymerase and 50 ng genomic DNA. PCR reactions were performed in a thermocycler as follows: an initial denaturation at 95° C for 5 min was followed by 30 cycles of denaturation at 94° C for 1 min, annealing at 57° C for 1 min, elongation at 72° C for 1 min and final extension at 72° C for 5 min.

Agarose gel electrophoresis

DNA isolated from chicken embryo samples for measuring apoptosis and amplification products (503 bp) for sequence analysis were run in 0.7 and 1% agarose gel with ethidium bromide, respectively. The gels were photographed on a UV transilluminator. The gel images were transferred to a computer using a DNA ima ging system (Vilber Lourmat, Eberhardzell, Germany).

AFB_1-DNA interaction

Interactions between AFB_1 and pBR322 plasmid DNA were examined using agarose gel electrophoresis (Nyxtechnik, San Diego, CA). Doses of 40, 20, 10, 5 or 2.5 ng/20 µl AFB_1 were prepared using 30% ethanol. The supercoiled pBR322 plasmid DNA was treated with these doses of AFB_1 in 30% ethanol for 24 h at 37° C. So that the DNA interactions of AFB_1 alone were determined, we also examined whether the 30% ethanol, in which aflatoxin was dissolved, had an effect on DNA (solvent group). One microliter plasmid DNA (0.5 µg/µl) was added to each concentration of AFB_1 and solvent. The mixtures were left in an incubator (Nuve, Ankara, Turkey) at 37° C overnight in the dark. Fifteen microliter aliquots of AFB_1-DNA were loaded onto 1.5% agarose gel with loading buffer (0.1% bromophenol blue and 0.1% xylene cyanol). Electrophoresis was performed in TAE buffer (0.05 M Tris base, 0.05 M glacial acetic acid, 1 mM EDTA, pH 8.0) for 1 h at 70 V. After electrophoresis, the gel was visualized using UV light and a transilluminator (DNA image system; Vilber Lourmat).

Bamhi and HindIII restriction enzyme digestion

After both the AFB_1-DNA mixtures and the 30% ethanol-DNA mixtures were incubated overnight, they were restricted by digestion by enzymes, *BamH*I or *Hind*III (1 Unit) for 1 h at 37° C. The restricted DNA was run in 1% agarose gel electrophoresis for 2 h at 60 V in TAE buffer (Scheideler 1993). The gels were viewed using a transilluminator in the image system.

DNA sequencing and data analysis

The GHRG-1 gene products (503 bp) of all specimens were sequenced by Macrogen Inc. (Seoul, Korea). The sequences of GHRG-1 gene obtained were aligned (481 bp) (Codon Code Aligner computer program) and sequence differences were detected.

Results

Total DNA isolated from chick embryo liver treated with different doses of AFB_1 (5, 15 and 40 ng/egg) was determined using 0.7% gel electrophoresis (Fig. 1). Total DNA of the tissue treated with ethanol used as solvent had the same mobility as the DNA of the control group. The 5 ng/egg AFB_1 dose

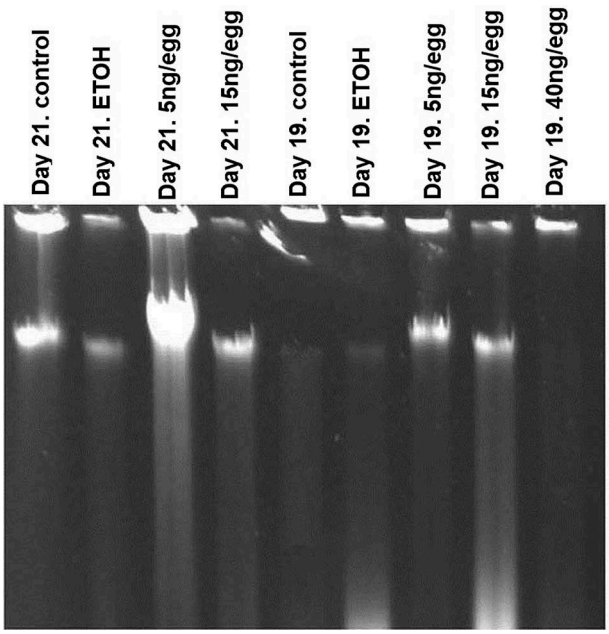

Fig. 1. DNAs of AFB_1 treated chick livers.

produced a DNA band with slower mobility in the gel (Fig. 1) than the other doses and controls, which suggested that AFB_1 had bound to DNA and slowed its mobility. Therefore, we induced DNA interactions to verify whether AFB_1 was bound to DNA and to which bases. Although the DNA band of the 5 ng/egg dose group exhibited slower mobility than the control DNA band, the DNA band of 15 ng/egg dose exhibited faster mobility than the control DNA band and the DNA band of 5 ng/egg dose (Fig. 1), i.e., DNA mobility in the 5 ng/egg AFB_1 dose was decreased. We believe that either the AFB_1 treated embryos did not reach the high dose level (15 ng/egg), because AFB_1 doses were not injected directly into the embryo, or low AFB_1 (5 ng/egg) bound to DNA more effectively than the high dose (15 ng/egg). DNA isolated from embryo liver after the highest dose (40 ng/egg at day 19) was not observed (Fig. 1), but amplification of the GHRG-1 gene region could be performed. The reason why the DNA was not observed in electrophoretic image from the 40 ng/egg dosed samples (Fig. 1) may be that the high dose destroyed the DNA. Electrophoretic images reveal that cells did not undergo apoptosis, because characteristic DNA fragmentation of apoptotic cells was not observed. Instead, embryos treated with 40 ng/egg dose day at 21 failed to survive.

A part of the sequence analysis of the GHRG-1 gene region (promoter) with many base changes is shown in Fig. 2. All sequence analyses exhibited increased DNA base changes in embryos treated with AFB_1 on day 19 compared to day 13; this increase was more pronounced at higher doses. Furthermore, base changes were largely transversion mutations: G→A and G→T. The fact that all embryos in the 40 ng/egg group died on day 21 may be associated with changes caused by the AFB_1 effects on DNA. Our findings concerning the interaction of different doses of AFB_1 with plasmid DNA (pBR322) are shown in Fig. 3.

When circular plasmid DNA was subjected to electrophoresis, the fastest migrating supercoiled form I and the slower moving open circular form II were observed. In both forms I and II, electrophoretic mobility of plasmid treated with 40 ng/20 µl AFB_1 was slower than the control plasmid. For the 20, 10, 5 and 2.5 ng/20 µl concentrations, DNA mobility was close to the control plasmid

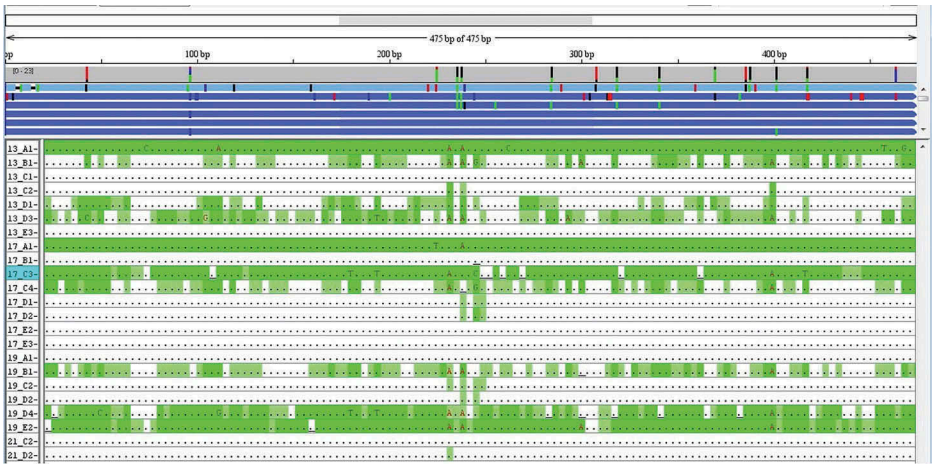

Fig. 2. Sequence analysis of GHRG-1 gene region of treated chick embryos (dense parts of the base changes are shown).

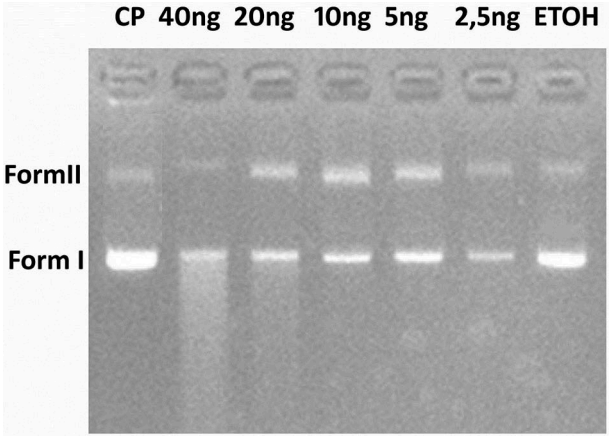

Fig. 3. Plasmid DNA treated by different doses of AFB_1. CP, control plasmid.

mobility. The plasmid mobility of ethanol, the solvent of AFB_1 was the same as for the control plasmid. Therefore, ethanol had no effect on DNA or AFB_1 binding to DNA.

BamHI and *Hind III* enzymes recognize DNA at the 5 '…G ↓ GATCC … 3' and 5 '… A ↓ AGCTT … 3' restriction sites and hydrolyze phosphodiester bonds between nucleotides. The digestion of plasmid DNA by these enzymes converts supercoiled plasmid form I DNA into linear form III DNA. Figure 4 shows that *BamHI* restriction enzyme did not digest DNA in the 40, 20 and 10 ng/20 µl AFB_1 groups, because AFB_1 bound to nucleotides at the restriction site and enzyme digestion was prevented. For the 5 and 2.5 ng/20 µl concentrations, the *BamHI* enzyme digested partially, which allowed form III to be seen as well as forms I and II by electrophoresis (Fig. 4).

Our tests using *Hind* III restriction enzyme revealed that all concentrations of AFB_1 inhibited enzyme digestion. The fact that *Hind* III restriction enzyme did not digest in electrophoresis indicates that AFB_1 exhibits greater affinity for adenine bases than for guanine bases. AFB_1 can bind guanine bases only at high concentration (40, 20 and 10 ng/20 µl). We suggest that AFB_1 may have different affinities for binding the plasmid DNA that is used for open circular vs. supercoiled structures. Neither restriction enzyme, *BamHI* or *Hind III*, digested the plasmid DNA treated with ethanol that was used as the solvent in our study (Fig. 4). We found that 30% ethanol used as a solvent had no negative effect and did not suppress the effects of AFB_1; it showed the same result as the control plasmid.

Discussion

Contamination of poultry food may cause serious problems in fertilized eggs (Dietert et al. 1985). The transmission of AFB_1 from feed to the fertilized egg causes economic loss, because the residual AFB_1 can cause organ malformations that affect embryo viability (Sambrook et al. 1989, Sur et al. 2011, Cilievici et al. 1980). Although the legal upper limits in Turkey are 10 µg AFB_1/kg and 20 µg AF/kg in food for laying hens, some investigators have detected levels ranging from 5 to 100 µg/kg in poultry food (Oğuz and Kurtoğlu 2000, Ozturk et al. 2012).

Aflatoxicosis caused by AF in poultry is characterized by poor food utilization, increased susceptibility to disease, increased mortality and poor body weight gain (Huff et al. 1986, 1988, Kubena et al. 1993, 1998, Potchinsky and Bloom 1993, Bailey et al. 1998, Smela et al. 2001, Oğuz et al. 2002). The mechanisms of AF effects include inhibition of RNA and DNA synthesis, and RNA polymerase activity (Hatch 1988). Inhibition of RNA and DNA synthesis reduces protein synthesis, which ultimately reduces growth. Oznurlu et al. (2012) suggested that AFB_1 administered *in ovo* affected embryonic development adversely. Gündüz and Oznurlu (2014) reported that large amounts of AFB_1 administered *in ovo* affected muscle development adversely.

AFB_1 is oxidized by the mixed function oxidase (MFO) enzyme system, which depends on liver cytochrome P-450 (P-448 in goats); it is converted to more powerful cytotoxic, mutagenic and carcinogenic epoxy derivatives (AFB1-8,9-epoxide) (Kubena et al.

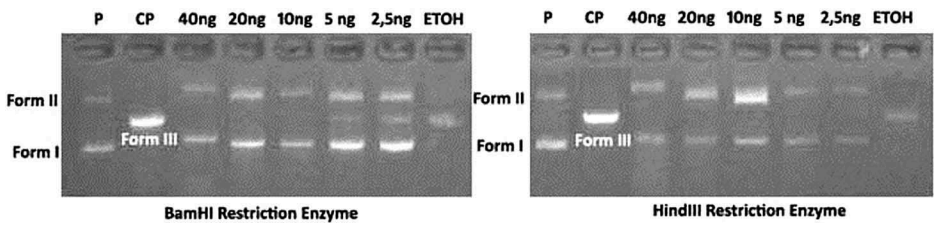

Fig. 4. Restriction enzyme cutting of plasmid DNA using different doses of AFB_1. P, plasmid; CP, control plasmid.

1993, Madhusudhanan et al. 2006). The toxic effects of these substances are due to their ready reaction with macromolecules such as nucleoproteins and nucleic acids, which blocks the synthesis of proteins and disrupts cell integrity (Hsieh et al. 1977, Nyandieka et al. 1989).

We found that all embryos in the 40 ng/egg group were dead by day 21. This may be due to changes to DNA caused by AFB_1 and accumulation of mutations during exposure. Our findings are consistent with reports that AFB_1 causes liver cancer (Hsu 1988, Iwaki et al. 1990, Bressac et al. 1991, Leeson et al. 1995, Oğuz and Kurtoğlu 2000). Mutagenic effects of AFB_1 are thought to be caused by inhibition of the activity of DNA polymerase enzymes by the epoxide derivative of AFB_1 during synthesis of new DNA in the cell (Dietert et al. 1985, Ellis et al. 1991).

Banlunara et al. (2005), reported that in ducks, addition of 100 ppb AFB_1 to the feed increased significantly the proliferation index in liver epithelial cells compared to controls. Similarly, Oznurlu et al. (2012) reported that animals treated *in ovo* with 15 and 40 ng/egg AFB_1 exhibited a significant increase the proliferating cell nuclear antigen (PCNA) index of tibial growth plate cells. Hamid et al. (2013) reported that AFB_1 caused cancer by inducing DNA adducts that caused genetic changes in the target cells, which then caused DNA strand breakage, DNA base damage and mutations that may ultimately led to tumors.

We found transversion mutations rather than transmission mutations by sequence analysis. A large proportion of transversion mutations was G→A and G→T. Similarly, Smela et al. (2001) reported that AFB_1 increased GC→TA transversions in the p53 gene. It also has been reported that AFB_1 increased activation of proto-oncogenes and caused G→T, G→A transversion mutations of codon 12 of the Ki ras gene (Al-Terehi 2012). Consistent with our study, El-Amir et al. (2012) tested DNA damage caused by AFB_1 in rat liver using Comet analysis and reported that AFB_1 increased DNA damage two fold 1 h after application and four fold after 24 h. It also has been reported that AFB_1 damaged the DNA six fold 3 days after the application (Neldon-Ortiz and Qureshi 1992).

We found that treatment with AFB_1 *in ovo* causes AFB_1 binding to the DNA of embryos, which at high doses causes DNA damage and increases base changes depending on the dose and time. We also determined using plasmid DNA and restriction enzymes that AFB_1 binds to adenine and guanine bases.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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