Dietary Butylated Hydroxytoluene Protects against Aflatoxicosis in Turkeys

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Turkeys are among the most sensitive species to the toxic effects of the mycotoxin aflatoxin B1 (AFB1). In mammals, dietary antioxidants, such as butylated hydroxytoluene (BHT), have been shown to lessen the toxic effects of AFB1 by various mechanisms. To test whether BHT protects against aflatoxicosis in turkeys, we supplemented the feed of 10-day-old male white turkeys with low (1000 ppm) and high (4000 ppm) BHT for 20 days. AFB1 (1 ppm) was then added to the diets and continued for another 10 days. Birds in the AFB1-alone group had a lower weight gain, a condition that had returned to near normal in groups fed diets containing AFB1 + BHT. Significant elevations in serum aspartate transaminase, alanine aminotransferase, and lactate dehydrogenase, which were evident in the AFB1 group, were reversed in the AFB1 + BHT groups. Histopathology revealed hepatic substantive necrotic lesions and biliary hyperplasia, the severity of which was lessened in the AFB1 + BHT-treated birds. Hepatocellular hydropic degeneration was observed in the BHT-alone group, but not in the AFB1 + BHT groups. This condition associated with BHT treatment was found in a separate study to be reversible and without any long-term adverse effects. These results indicate that BHT counteracts many of the deleterious effects caused by AFB1 and that this antioxidant may prove to be a viable feed additive for the reduction of aflatoxicosis in turkeys. © 2002 Elsevier Science (USA)

Aflatoxin B1 (AFB1), a mycotoxin produced by the ubiquitous fungi Aspergillus flavus and A. parasiticus, is a nearly universal contaminant of poultry feeds (Dalvi, 1986; Coulombe, 1993; Bhatnagar et al., 1994; Cespedes and Diaz, 1997). Avoidance of contaminated feeds is rarely possible, and feed that contains relatively low concentrations of AFB1 may still have deleterious effects on sensitive species, such as poultry (Carmaghan, 1965; Doerr et al., 1983; Giambrone et al., 1985a). In poultry, even small amounts of AFB1 cause a reduction in growth rate, feed efficiency, hatchability, increased susceptibility to bacterial and viral diseases, and severe hepatotoxicosis (Edds, 1973; Pier et al., 1980; Giambrone et al., 1985b; Kubena et al., 1995). Turkeys exhibit greater sensitivity to this toxin compared to other poultry species, particularly chickens (Hamilton et al., 1972; Giambrone et al., 1985b).

In order to exert its toxic effects, AFB1 must be activated to the exo-AFB1-8,9-epoxide (AFBO) by cytochrome P450s (CYPs) (Guengerich et al., 1992a, 1998), which in most animals is detoxified largely by glutathione S-transferase (GST)-mediated conjugation with glutathione (GSH) (Guengerich et al., 1992b, 1998; Raney et al., 1992; Johnson et al., 1997). The resistance of mice to AFB1 has been shown to result from the high affinity a-class GST, specifically the Yc2 isoform, toward AFBO (O'Brien et al., 1983; Monroe and Eaton, 1987; Buettler and Eaton, 1992; Hayes et al., 1992; Raney et al., 1992). Murine liver exhibits a 12- to 50-fold greater AFBO-GSH-conjugating activity compared to rat liver, while at the same time possessing a threefold greater AFB1 activation (Monroe and Eaton, 1987; Neal et al., 1987). Thus, the affinity of phase II enzymes, such as GSTs, toward AFBO can determine a species' resistance or susceptibility to this toxin (O’Brien et al., 1983; Ramsdell and Eaton, 1990; Hayes et al., 1991).

Several compounds have been shown to possess chemoprotective properties against AFB1 in rodent models. Such protection is often mediated by biochemical modulation of either phase I or phase II enzymes or an increase in nucleophilic trapping of activated AFB1 intermediates (Kensler et al., 1994). In chickens, dietary butylated hydroxytoluene (BHT) results in phase II enzyme induction and a resultant decrease in AFB1 toxicity (Larsen et al., 1985; Ehrlich et al., 1986, 1988). Dietary BHT also protects against AFB1-related growth depression in chickens (Larsen et al., 1985).

Compounds such as butylated hydroxyanisole (BHT) (Ch’ih et al., 1989), octilpraz (Langouet et al., 1995, 1997; Kulman et al., 2000), 2-(allylthio)pyrazine, gestodene (Ha and Kim, 1998; Kim and Kim, 1999), and indole-3-carbinol (Fong et al., 1990; Stresser et al., 1995; Takahashi et al., 1995) all directly inhibit AFB1 activation in vitro. Octilpraz, for example, inhibits AFB1-relevant enzymes CYP1A, 3A4, and CYP2B in rat liver mi-
BHT chemoprevention:

![Diagram of BHT chemoprevention](image)

**Long-term BHT:**

![Diagram of long-term BHT](image)


cromosomes, in recombinant human CYP microsomes from yeast, and in cultured rat and human hepatocytes (Langouet et al., 1995, 1997), which may be an important mechanism in oltipraz-induced chemoprotection (Jacobson et al., 1997; Wang et al., 1999). At present, the role that inhibition might play in chemoprotection in poultry has not been investigated.

We previously have shown that turkeys are extremely sensitive to the effects of AFB1, likely because of a combination of extremely efficient CYP-mediated activation and deficient GST-mediated detoxification of this mycotoxin (Klein et al., 2000). Given the considerable database demonstrating the chemoprotective properties of BHT toward AFB1 in rodents and chickens, ostensibly by induction of protective phase II enzymes (Ehrich et al., 1981; Kensler et al., 1985; McLellan et al., 1994; Sun et al., 1996; Allameh, 1997), it seemed appropriate to investigate the potential protective effect of this antioxidant in turkeys, especially in light of their extreme sensitivity and because AFB1 is an unavoidable contaminant of poultry feeds. We report that dietary BHT exerts a chemoprotective effect as measured by a reduction in many endpoints normally associated with AFB1 hepatotoxicosis.

**MATERIALS AND METHODS**

**Chemicals and reagents.** AFB1, BHT, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

**Animals and treatments.** Day-old male white turkeys were obtained from Moroni Feed Co. (Moroni, UT) and maintained on a corn-based commercial diet (Moroni Feed Co.). Preliminary studies demonstrated that 1 ppm AFB1 produced a variety of toxic endpoints, but not mortality in the time frame of this experiment. Thus, 1 ppm AFB1 was chosen as the challenge dose to test possible BHT protection. At 10 days of age, turkeys (ca. 100 g) were randomly assigned to one of six treatment groups: control (no treatment), AFB1 only (1 ppm), low (1000 ppm) and high (4000 ppm) BHT only, and AFB1 + low BHT and AFB1 + high BHT (Fig. 1). After 10 days of BHT pretreatment, 1 ppm AFB1 was added to the diets of two groups of BHT-treated pouls; these AFB1 + BHT diets were continued for 10 more days (n = 7 in all groups: Fig. 1).

At the end of the 10-day study, the birds were euthanized by CO2 asphyxiation and the livers were removed, examined, weighed, sampled, and fixed in neutral buffered formalin for histological examination. Blood was collected in
FIG. 2. Protective effect of dietary BHT on AFB1-induced reductions in weight gain in turkeys. Male turkeys at 10 days of age were placed on the following treatments: control and low (1000 ppm) and high (4000 ppm) BHT. After 10 days of BHT pretreatment, 1 ppm AFB1 was added to the diets of two groups of BHT-treated birds and these BHT + AFB1 diets were continued for 10 more days. Nontreated controls and birds receiving AFB1 alone and BHT alone were also included. Each bar represents the mean ± SD (n = 7). Different superscripts indicate a significant difference among groups (p < 0.05).

sterile, no-additive vials (Becton Dickinson Vacutainer, Franklin Lakes, NJ). Within 1 h of collection, the blood was clotted and the serum was removed. The serum was then analyzed by a Synchro CX 5 Clinical System (Beckman, Fullerton, CA) for alanine aminotransferase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), creatinine phosphokinase (CK), total bilirubin, and direct bilirubin.

Another experiment was conducted to examine the effect of long-term dietary treatment of BHT in turkeys. In this 90-day study, 10-day-old turkeys were randomly assigned into a control group that received untreated feed, a group that received BHT (4000 ppm) for 40 days, and a group that received BHT (4000 ppm) for 10 days, which was then replaced with control feed; these birds were kept on control feed for an additional 30 days (Fig. 1). Three birds from each group were euthanized at 10-day intervals and samples were taken and treated identically to those in the AFB1, chemoprotection experiment (Fig. 1).

Liver tissue analysis. A section of each liver was fixed in neutral buffered formalin immediately after removal. This section was then embedded in paraffin using a Model TP1050 Embedding Station (Leica Microsystems, Deerfield, IL), thin sectioned (RM 2145 Microtome, Leica Microsystems), and stained with hematoxylin and eosin (H & E) (Jung Autostainer XL, Leica Microsystems). The tissue was then fixed to a slide for histological evaluation. Frozen sections were then sectioned (Model CTD International-Harris Crystal, International Equipment Co., Needham Heights, MA) and stained with Oil red O stain for lipid (McKinney and Riley, 1967). Slides were evaluated histologically for hydropic degeneration, hepatocellular necrosis, and biliary hyperplasia. Histologic evaluations were scored 1 to 5 for severity as follows: hepatocellular necrosis, based on percent of viewed cells affected, 1 = <5%, 2 = 5 to 30%, 3 = 30 to 60%, 4 = >60 to 80%, or 5 = >80%; biliary hyperplasia, 1 = normal, 2 = mild proliferation without parenchymal displacement, 3 = moderate proliferation with some mild parenchymal displacement, 4 = moderate to severe proliferation with moderate parenchymal displacement, or 5 = diffuse proliferation with severe parenchymal displacement; hydropic degeneration, 1 = none present, 2 = <40% of the cells with small vacuoles and few large vacuoles, 3 = >40 to 75% of the cells with small vacuoles and/or 20 to 50% with large vacuoles, 4 = >75 with small vacuoles and/or >50 to 80% with large vacuoles, or 5 = >80% with large vacuoles.

Statistical analysis. Groups were compared, for differences, using one-way ANOVA and post-hoc Tukey test (Sigma Stat Software). A level of p < 0.05 was chosen as statistically significant.

RESULTS

For the duration of the first experiment, there was no mortality in any of the treatment groups. As observed in the preliminary dose-finding study, 1 ppm dietary AFB1 resulted in profound hepatotoxic toxic effects in turkeys. In many cases, BHT exerted a strong protective effect against end points of aflatoxinsis. For example, birds receiving only AFB1 had significantly lower weight gain (Fig. 2) as well as significantly lower liver weights (Fig. 3) compared to controls. Weight gain in the AFB1 + BHT groups were significantly higher than that in the AFB1-only group, and weight gain in the AFB1 + high BHT group was not statistically different from control (Fig. 2). The protective effect of high BHT was also seen when liver weights were considered (Fig. 3). Body weight gain and liver

FIG. 3. Protective effect of dietary BHT on AFB1-induced reduction of liver weights in turkeys. Male turkeys at 10 days of age were placed on the following treatments: control and low (1000 ppm) and high (4000 ppm) BHT. After 10 days of BHT pretreatment, 1 ppm AFB1 was added to the diets of two groups of BHT-treated birds and these BHT + AFB1 diets were continued for 10 more days. Nontreated controls and birds receiving AFB1 alone and BHT alone were also included. Each bar represents the mean ± SD (n = 7). Different superscripts indicate a significant difference among groups (p < 0.05).
weights in birds given BHT alone were not statistically different from control.

The principal target organ for AFB₁ is the liver. As a means of assessing hepatic damage resulting from AFB₁ treatments, an analysis of serum chemistries was performed. Serum ALT, AST, LDH, and CK, marker enzymes for hepatocellular necrosis, were significantly elevated in turkeys receiving only AFB₁ compared to control (Fig. 4). These parameters were reduced in both AFB₁ + BHT groups in a dose-related fashion (Fig. 4). There was no statistically significant trend associated with bilirubin values (data not shown).

Gross postmortem examinations of livers identified hepatic changes, such as increased tissue firmness and hemorrhaging, associated with AFB₁ treatment whereas these changes were reduced or absent in AFB₁ + BHT-treated groups and completely absent in all other groups. Histopathological examination of liver tissue revealed biliary hyperplasia and hepatocellular necrosis in the AFB₁-only birds (Fig. 5B). These lesions
were not observed in control birds. Hepatic pathology was significantly reduced in turkeys treated with AFB$_1$ + BHT (Fig. 5D). Based on lesion scoring, BHT treatment significantly decreased the hepatocellular necrosis and biliary hyperplasia that are associated with AFB$_1$ toxicosis in a dose-responsive manner (Fig. 6).

Compared to controls, turkeys receiving dietary BHT alone had no significant changes in any of the endpoints of toxicity measured—body weights, liver weights, and serum enzymes—compared to control (Figs. 2–4). While there were noticeable reductions in biliary hyperplasia and hepatocellular necrosis in the AFB$_1$ + BHT groups compared to the AFB$_1$-only group, livers of birds receiving BHT alone revealed hydropic degeneration, as characterized by a vacuolar ballooning of the cells (Fig. 5C). Oil red O staining further confirmed that this condition was not associated with lipid accumulation. This pathology was noticeably absent or reduced in other groups, including those that received a diet including AFB$_1$ + low BHT (Figs. 5D and 6). A separate experiment was then conducted to determine the severity and reversibility of BHT-induced hepatic hydropic degeneration.

Animals receiving long-term BHT had no statistically significant changes in weight gain, liver weight, liver-to-body weight ratios, or spleen-to-body weight ratios, serum ALT, AST, LDH, bile acid concentrations, or total protein compared to control birds (data not shown). Hepatic hydropic degeneration was observed starting at day 10 of the BHT treatment and remained at the same severity through the entire 40 days of treatment (data not shown). However, in animals receiving BHT that was removed after 10 days, signs of hydropic degeneration resolved.
The protective effects of BHT on decreases in weight gain due to AFB₁ have been noted in chickens (Larsen et al., 1985; Dalvi, 1986; Ehrich et al., 1986, 1988). Importantly, BHT alone did not cause a reduction in weight gain compared to controls.

Serum endpoints of AFB₁ hepatotoxicity also were reversed by BHT treatment. In most cases, 4000 ppm was more protective of AFB₁-induced changes than 1000 ppm. Histopathological examination of liver tissue provided compelling data supporting a strong protective effect of BHT. In sensitive species, such as poultry and rats, biliary hyperplasia and hepatocellular necrosis are common signs of aflatoxicosis (Larsen et al., 1985; Dalvi, 1986; Ehrich et al., 1986, 1988; Coulombe, 1993; Cullen and Newberne, 1994; Eaton and Gallagher, 1994). The severity of these hepatic lesions found in the AFB₁-only group was significantly reduced in the AFB₁ + BHT birds.

Livers in the BHT-only groups showed hydropic degeneration, which was later shown to be reversible once animals were taken off the BHT-containing diet. This lesion did not apparently have any other effects, as all other measurements—body weights, liver weights, and serum enzymes—were unchanged compared to control birds. Hydropic degeneration is a well-documented hepatic lesion caused by subnecrogenic doses of compounds such as hexachloro-1,3-butadiene and CCI₃ (Lock et al., 1985; Zhou et al., 1996). In rodents, BHT has been associated with similar lesions (Powell and Connolly, 1991; Safer and al-Nughamish, 1999). Interestingly, hydropic degeneration was minimal or absent in the AFB₁ + BHT groups. The lack of any other adverse effect of BHT indicates that this compound may be safely used as an adjunct in an overall poultry management scheme.

A wide variety of structurally diverse compounds has been shown, in various animal models, to protect against AFB₁ hepatocarcinogenesis. These compounds include β-naphthoflavone (Swenson et al., 1977), α-hexachlorocyclohexane (Angsukthakorn et al., 1978), γ-hexachlorocyclohexane (Angsukthakorn et al., 1989), ethoxyquin (Cabral and Neal, 1983), phenobarbital (McLean and Marshall, 1971), BHA (Williams et al., 1986), BHT (Williams et al., 1986; Kessler et al., 1994), and oltipraz (Roebuck et al., 1991). Many of these compounds modulate AFB₁ toxicity or carcinogenesis by modulation of the metabolism—detoxification pathways.

The mechanism of protection exerted by BHT in turkeys has not been determined with certainty. Induction of protective GSTs has been posited as a possible chemoprotective mechanism by BHT and other phenolic antioxidants (Hayes et al., 1996). In mammals, GSTs are critical AFB₁-detoxifying enzymes (Eaton and Gallagher, 1994). Inasmuch as turkeys are deficient in GST-mediated detoxification of AFB₁ (Klein et al., 2000), it seems plausible that BHT may exert some protection by inducing critical phase II enzymes. It is interesting to note that AFB₁ possesses some antioxidant activity per se (Moon et al., 1998). We have also recently shown that dietary BHT increased the activity and expression of hepatic GST isoforms.
as much as fourfold in turkeys (Klein et al., 2002). The possible mechanisms of chemoprotection by BHT will be the subject of a report in the near future.

The protective concentrations of BHT (1000 and 4000 ppm) we observed in the present study are in line with the concentration of other chemoprotective protocols. For example, 4000 ppm chlorophyllin exerted strong protection against AFB<sub>1</sub> hepatocarcinogenesis in rainbow trout (Breinholt et al. 1999); ethoxyquin and BHT at 1000 and 3000 ppm counteracted many of the adverse effects of dietary AFB<sub>1</sub> on weight gain and feed efficiency in chickens (Ehrich et al., 1986). While dose and safety considerations eliminate some compounds from further consideration as chemoprotectants, butylated hydroxytoluene (BHT) is a food additive that is considered generally recognized as safe (GRAS) by the U.S. Food and Drug Administration with a long history of safe use in human foods. This antioxidant may hold future promise as a protective feed additive that can be used as part of an overall management
strategy to improve animal health and provide a safer food for consumers.

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