Involvement of oxidative stress in subacute toxicity induced by fumonisin B₁ in broiler chicks

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ABSTRACT

Fumonisin B₁ (FB₁) is a mycotoxin produced by Fusarium spp. It has been reported to be a potential cause of liver cancer in rats and esophageal cancer in humans. The underlying mechanisms of FB₁ toxicity are thought to be related to the inhibition of ceramide synthase, causing an accumulation of sphingosine (SO) and sphinganine (SA), which in turn may cause tissue functional impairment and the development of oxidative stress. Therefore, in this study, we investigate the effects of an FB₁-contaminated diet on markers of oxidative stress in chick liver. A total of 24 male broiler chicks (Cobb 500) were fed a standard control diet or a diet contaminated with FB₁ (100 mg/kg) for 21 days, starting on postnatal day one. The feed and animals were weighed on days 0, 7, 14 and 21 to estimate the feed conversion ratio, and at 21 days, the liver weight and liver relative weight were determined. At the end of the experiment, samples of blood and liver were collected. The blood was used to quantify the SA/SO ratio, and the liver was used to determine the activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST); ascorbic acid levels (Vit C), non-protein thiol (NPSH) levels and TBARS content were also determined. The FB₁ diet increased the liver weight, liver relative weight, feed conversion and SA/SO ratio. Furthermore, hepatic TBARS levels, Vit C content and CAT activity were also increased. Conversely, the activities of SOD, GST and NPSH levels, in the liver were not altered by the mycotoxin-contaminated diet. In summary, we showed that subacute exposure of broiler chicks to FB₁ induced liver oxidative stress concomitantly with SA/SO accumulation.

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1. Introduction

Fumonisins are mycotoxins produced by Fusarium spp. mold that contaminates maize and maize-based food worldwide. These mycotoxins were first described and characterized in 1988 (Gelderblom et al., 1988), and FB₁ is
the most abundant and toxic of the mycotoxins (Seo and Lee, 1999).

The presence of mycotoxins in feeds and food represent public health and economic concerns in many countries. In this context, the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) and Expert Committee on Food Additives (JECFA) estimated the prevalence of dietary exposure to fumonisins in several countries. Studies in Brazil based on 208 samples of corn-based products revealed high fumonisins contamination levels between 1680 and 2040 μg/kg and estimated that the total exposure to fumonisins (FB1 + FB2) from the consumption of corn-based products in Brazil was between 0.5 and 7.1 μg/kg bw per day in the total population. Thus, the rates of contamination of food and feed by fumonisins are high compared to the maximum tolerable daily intake (MTDI) dose for FB1, FB2, and FB3, alone or in combination, of 2 μg/kg bw, increasing the risk of the development of toxic effects caused by such exposure (Bulder et al., 2012).

Studies on the toxicity of FB1 showed that ingestion of foods and feeds contaminated with this mycotoxin have been associated with the following: leukoencephalomalacia in equines (Marasas et al., 1988) and rabbits (Bucci et al., 1996); pulmonary edema and hydrothorax in swine (Harrison et al., 1990); and hepatotoxic carcinogenic effects (Gelderblom et al., 1988) and apoptosis in liver of rats (Pozzi et al., 2001). In humans, the presence of FB1 is correlated with a high incidence of esophageal cancer in regions of Transkei (South Africa), China, and northeast Italy (Peraica et al., 1999). Therefore, the International Agency for Research on Cancer (IARC) has evaluated the cancer risk of fumonisins in humans and classified them as group 2B (possibly carcinogenic). All species appear to be sensitive to the toxin, but the target tissue for its toxicity differs from one species to another.

The cellular mechanisms behind FB1-induced toxicity are complex (Voss et al., 2007) and include the inhibition of ceramide synthase, due to the structural similarity between fumonisins and the sphingoid bases sphinganine (SA) and sphingosine (SO). Their ability to disrupt sphingolipid metabolism (Riley et al., 2001) leads to intracellular accumulation of sphingoid bases, which mediate several key biological processes, such as cell proliferation and DNA replication (Riley et al., 2001; Voss et al., 2007) and oxidative stress (Torres-Sanchez and Lopez-Carrillo, 2010). In addition, it has been demonstrated that FB1 inhibits macromolecule biosynthesis (Abad-Becognee et al., 1998; He et al., 2006) and induces lipid peroxidation in rat liver and rat spleen mononuclear cells (Abel and Gelderblom, 1998; Theumer et al., 2010). Thus, susceptibility to damage by mycotoxins, especially FB1, varies according to age, sex and animal species (Johnson and Sharma, 2001).

In this context, studies have demonstrated that chickens intoxicated with FB1 exhibit reduced productive performance, diarrhea, lack of appetite, oral lesions, increased liver weight and high mortality (Ledoux et al., 1992), leading to an important economic impact to producers. However, there are no studies on the oxidative stress markers after exposure to FB1 in chicks, and this study aims to evaluate the effects of a diet contaminated with FB1 on nutritional parameters, the SA/SO ratio, and the markers of oxidative stress in the liver of broiler chicks.

2. Materials and methods

2.1. Animals

Twenty-four one-day-old male broiler chicks (Cobb 500) were housed in an experimental shed and were randomly divided into 2 groups; one group received a control diet for 21 days and the other received a diet contaminated with FB1 (100 mg/kg of feed) for 21 days, both starting on postnatal day one. The animals were maintained and used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources.

2.2. Mycotoxin and feed preparation

A corn and soybean meal-based diet was formulated based on the standard nutritional requirements of broilers in the initial phase (NRC 1994). To achieve the desired FB1 contamination in the feed (100 mg/kg of feed), a Fusarium verticillioides (strain MRC 826) culture material containing 4850 mg/kg of FB1 was incorporated into the diet (Rauber et al., 2012). Feed and water were provided ad libitum. Before the experiment, feedstuffs and diets were screened for aflatoxins [B1, B2, G1, and G2], fumonisins [B1 and B2], zearalenone and trichothecenes [deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, diacetoxyscirpenol, nivalenol, fusarenone-X, T-2 toxin and HT-2 toxin]. The assayed levels of these mycotoxins were below the detection limits of the technique used (Vishwanath et al., 2009).

2.3. Sampling

At the end of the treatment (21 days), chicks were killed using CO2 for stunning followed by bisection of the cervical vessels. Blood was collected into tubes, which were centrifuged at 3000 rpm × 10 min, and the serum was used for the subsequent SA/SO analyses. The livers were removed, weighed, and homogenized in Tris–HCl (50 mM, pH 7.4) buffer; the resulting homogenate was centrifuged at 10,000 rpm × 10 min at 4 °C for the determination of enzymatic and non-enzymatic indicators of oxidative stress.

2.4. Liver weight, relative liver weight and feed conversion

To evaluate the influence of FB1 on broiler performance, animals and feed were weighed on days 0, 7, 14 and 21. Feed conversion was estimated as the rate between feed intake and body weight gain weekly. The livers of the control and FB1-treated animals were weighed on day 21, and the relative liver weight was calculated.
2.5. Thiobarbituric acid reactive substance (TBARS) determination

Lipid peroxidation was estimated by measuring TBARS and was expressed in terms of malondialdehyde (MDA) content, according to the method described by Boeira et al. (2012). In this method, MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. TBARS content was estimated in a medium containing the supernatant fraction of the liver, 0.015 mL of 8.1% SDS, 0.06 mL of acetic acid buffer (2.5 M, pH 3.4), and 0.115 mL of 0.81% thiobarbituric acid (TBA). The mixture was finally made up to 0.3 mL with type I ultrapure water and heated at 95 °C for 120 min in a water bath using a glass ball as a condenser. After cooling to room temperature, absorbance was measured in the supernatant at 532 nm. The results were calculated as μmol MDA/mg of protein.

2.6. Ascorbic acid (Vit C) determination

Liver ascorbic acid determination was performed as described by Boeira et al. (2012). Protein was precipitated in 10 V of a cold 5% trichloroacetic acid solution. An aliquot of the sample (300 μL) in a final volume of 575 μL of the solution was incubated with TCA 13.3% and a color reagent containing dinitrophenyl hydrazine, thiourea and CuSO₄ at 37 °C for 3 h; then, 500 μL of H₂SO₄·H₂O (65:35, v/v) was added to the medium. The reaction product was determined spectrophotometrically at 520 nm as μg ascorbic acid/mg of protein.

2.7. Non protein thiol (NPSH) determination

Hepatic NPSH levels were determined according to the method described by Boeira et al. (2012) with some modifications. Samples were precipitated with TCA (10%) and subsequently centrifuged at 3000 × g for 10 min. After the centrifugation, the supernatant fraction (60 μL) was added to a reaction medium containing potassium phosphate buffer (1 M, pH 7.4) and DTNB (10 mM). The NPSH levels were measured spectrophotometrically at 412 nm. The results were calculated using a standard curve constructed with reduced glutathione (GSH) and corrected based on protein content. The results were calculated as nmol NPSH/mg of protein.

2.8. Enzyme assays

2.8.1. Catalase (CAT) activity

CAT activity was determined by following the decomposition of 30 mM hydrogen peroxide in 50 mM potassium phosphate buffer (pH 7.0) at 240 nm for 120 s in a thermostatized (37 °C) spectrophotometer, according to the method described by Boeira et al. (2012). CAT specific activity was expressed as the first-order rate constant k, per mg of protein. Appropriate controls for non-enzymatic decomposition of hydrogen peroxide were included in the assays.

2.8.2. Superoxide dismutase (SOD) activity

Liver SOD activity was determined according to the method described by Boeira et al. (2012). This method is based on the ability of SOD to inhibit autoxidation of adrenaline to adenochrome. Briefly, the supernatant fraction (20–60 μL) was added to a medium containing glycine buffer (50 mM; pH 11) and adrenaline (1 mM). The kinetic analysis of SOD was started after adrenaline addition, at 38 °C, and the color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 38 °C, and the results were expressed as units (U)/mg of protein.

2.8.3. Glutathione S-transferase (GST) activity

GST activity was assayed spectrophotometrically at 340 nm at 30 °C using the method described by Boeira et al. (2012). The reaction mixture contained an aliquot of liver supernatant, 0.1 M potassium phosphate buffer (pH 7.4), 100 mM GSH and 100 mM CDNB, which was used as the substrate. The enzymatic activity was expressed as nmol CDNB/min/mg of protein.

2.9. Protein determination

Protein content was measured colorimetrically using the Bradford method (Boeira et al., 2012), and bovine serum albumin (1 mg/mL) was used as the standard.

2.10. Free sphinganine and sphingosine analysis for SA/SO ratio calculation

Extraction was performed as follows: 0.5 mL of the serum sample was mixed with 1.5 mL of 0.8% potassium chloride solution, 0.05 mL of potassium hydroxide solution and 4.0 mL of ethyl acetate for 20 min with gentle shaking; then, the mixture was centrifuged for 10 min at 1800 rpm, after which the supernatant was collected. The extract was then dried and suspended in 0.5 mL of methanol. The methanol-suspended extract was analyzed using HPLC tandem mass spectrometry (LC/MS-MS, API 5000, Applied Biosystems, Concord, Ontario, Canada) according to Rauber et al. (2012).

2.11. Statistical analysis

GraphPad prism 5 software was used for statistical analysis and for plotting graphs. Statistical analyses were carried out by Student’s t test, and P < 0.05 was considered significant. All data are reported as the mean and S.E.M.

3. Results

Liver toxicity induced by FB₁ exposure to broiler chicks was analyzed based on liver weights, liver relative weights and feed conversion. Statistical analyses revealed that the FB₁ diet increased liver weight (t(18) = 2.280, P = 0.0350; Table 1). Furthermore, the mycotoxin treatment augmented liver relative weight (t(22) = 2.480, P = 0.0213) and feed conversion ratio at 7,
Increased liver levels in statistical analyses revealed that hepatic ascorbic acid levels were increased in animals that fed a diet contaminated with FB1 \((t(16) = 3.085, P = 0.0071)\). In addition, the statistical analyses revealed that hepatic ascorbic acid levels were increased in FB1-treated chicks \((t(18) = 4.50, P = 0.0003)\; \text{Table 2}\). Conversely, the levels of non-protein thiols were not altered by the mycotoxin-contaminated diet \((t(18) = 1.266, P = 0.2218)\; \text{Table 2}\).

Enzymatic markers of oxidative stress were also determined after treatment with FB1. Statistical analyses showed that FB1 increased liver CAT activity \((t(15) = 2.686, P = 0.016)\; \text{Table 3}\). However, the SOD and GST liver activities were not altered by the subacute treatment with FB1 \((t(17) = 0.3736, P = 0.7133)\; \text{Table 3}\). and \((t(17) = 0.2759, P = 0.7859)\; \text{Table 3}\).

In addition to the role of oxidative stress in FB1 toxicity, we also evaluated the contribution of ceramide synthase in this toxicity, determining the SA/SO ratio in plasma samples from broiler chicks. Statistical analyses revealed that the SA/SO ratio \((t(17) = 15.18, P < 0.0001)\; \text{Table 4}\) was increased, confirming the role of this enzyme in the toxic actions of FB1.

### 4. Discussion

FB1 is a mycotoxin that is being produced mainly by fungi of Fusarium species. This fusarotoxin is found worldwide in cereals, animal food and forages and adversely affect mainly the liver and kidneys. While corn is the main component of the poultry diet, these animals present different signs of intoxication depending on age, sex, species and the toxin involved. The present study shows that subacute exposure to an FB1-contaminated diet in male Cobb broiler chicks increased the liver weights, liver relative weights and feed conversion ratio. Additionally, the TBARS and the ascorbic acid content were increased, as well as the liver CAT activity. Conversely, the activities of SOD, GST and NPSH in the liver were not altered by ingestion of the mycotoxin-contaminated diet. Furthermore, FB1 increased the SA/SO ratio indicating that both oxidative stress and ceramide synthase inhibition are involved in the toxic action of FB1 in broiler chicks.

Performance parameters, such as organ weight/relative weight and feed conversion, are frequently used as an index to determine the development of farm animals or as markers of toxicity (Bagherzadeh Kasmani et al., 2012). Accordingly, liver weight and its relative weight increase are used as markers of toxicity because this organ is the major site of detoxification of many toxicants including FB1 (Voss et al., 2007). In addition, increased liver weight and liver relative weight after FB1 exposure have been reported in other broiler chick studies (Ledoux et al., 1992). We observed an increased feed conversion rate, indicating that chicks need to consume more feed to produce the same weight gain as controls, whereas Rauber et al. (2012) reported a similar result in Cobb chicks.

In addition to the nutritional parameters commonly used as markers of toxicity, the increase in the SA/SO ratio can also be used as a toxicity indicator of FB1 exposure in diverse experimental animal models (Domijan, 2012). The increased SA/SO ratio in broiler chicks was expected because FB1 is similar to sphingosine and sphinganine and...
therefore, it can cause the competitive inhibition of a key enzyme involved in sphingolipid metabolism (Direito et al., 2009). The inhibition of ceramidase synthase leads to an increase in free sphinganine and sphingosine to a lesser extent leading to an increased SA/SO ratio in poultry treated with FB1. In addition, the accumulation of these sphingolipid bases induced an inhibition of complex I of the respiratory chain and therefore, stimulated the production of reactive oxygen species (ROS) (Domijan and Abramov, 2011). Diverse ROS are generated by the respiratory chain, including the superoxide radical and hydrogen peroxide (Rodrigues and Gomes, 2012). In this context, enzymes such as CAT and SOD are important for the controlling the development of oxidative stress because SOD is responsible for dismutation of the superoxide radical in hydrogen peroxide, and CAT decomposes hydrogen peroxide to water and oxygen. The combined action of these two enzyme activities may neutralize the ROS generated by respiratory chain inhibition and therefore may minimize the subsequent oxidative damage to biomolecules (Mary et al., 2012). Moreover, it has been suggested that FB1 increases the susceptibility of cells to lipoperoxidation because reduction in complex sphingolipids contributes to membrane dysfunction resulting in cellular damage (Abel and Gelderblom, 1998). Therefore, the increased ROS generation and subsequent oxidative stress has been suggested as a possibly mechanism of FB1 toxicity in many animal species/tissues (Mary et al., 2012; Klaric et al., 2007; Mobio et al., 2000; Stockmann-Juvala et al., 2004).

In this context, evidence that FB1 may induce lipid peroxidation has been found in studies performed in rat liver (Abdel-Wahhab et al., 2010), intestinal cell lines (Kouadio et al., 2005) and mononuclear cell suspensions (Mary et al., 2012; Theumer et al., 2010). Altogether, such results agree with the findings in this study because we also showed increased liver TBARS content.

Additional evidence for the involvement of oxidative stress in the toxicity of FB1 is the increased liver CAT activity because this increase could be a consequence of high ROS production. The liver has high CAT expression, and CAT activity was possibly increased as a response to augmented ROS production. In addition, it has been demonstrated that hydrogen peroxide could lead to lipoperoxidation (Mary et al., 2012). Moreover, Marnewick et al. (2009) reported that SOD activity was not altered in rat liver, confirming our results in the liver of broiler chicks after FB1 exposure. In this context, the elevated levels of hepatic ascorbic acid can contribute to a pro-oxidant effect due to the ability of ascorbate to reduce Fe3+ to Fe2+, leaving Fe2+ available for reaction with H2O2 and thus stimulating ROS generation, mainly the hydroxyl (OH•) radical (Colpo et al., 2008). Moreover, ascorbate is an antioxidant vitamin that acts as a scavenger of many deleterious reactive species, such as the superoxide radical, alkoxy radical, peroxyl radical, hydroxyl radical and peroxynitrite (Halliwell, 2001). Therefore, at present, we cannot conclude whether the increased levels of ascorbic acid reported in this study contributed negatively or positively to FB1 toxicity.

Glutathione S-transferases are a group of multifunctional proteins encoded by a multigene family. This family of enzymes is responsible for detoxifying electrophilic compounds to protect against oxidative damage. In this study, GST activity did not change in the liver of chicks exposed to FB1. Because FB1 is a highly water soluble mycotoxin, it could be excreted in the original form, dispensing conjugation catalyzed by GST for its elimination.

In summary, to the best of our knowledge, this is the first study investigating the parameters of oxidative stress in broiler chicks. We showed evidence for ROS participation in the toxicity mechanisms induced by FB1, concomitantly with inhibition of ceramidase synthase, indicating that oxidative stress subsequent to inhibition of ceramidase synthase underlies at least in part the toxic action of FB1. The present data are of fundamental importance for preventing or reducing the impact caused by FB1 in the economy and health of animals and humans. In conclusion, we showed that subacute treatment with FB1 has a significant negative effect on broiler performance, on oxidative stress parameters and on the sphingoid base ratios in the liver of broiler chicks.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References


