The effects of dietary iron overload on fumonisin B₁-induced cancer promotion in the rat liver

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Abstract

The present study was performed to determine whether excess hepatic iron modulates the cancer-initiating and promoting properties of FB₁. Thirty-eight male F344 rats were divided into four dietary treatment groups: (i) control diet (AIN, n = 8); (ii) FB₁ 250 mg/kg diet (FB₁, n = 10); (iii) 1–2% carbonyl iron (CI, n = 10); or (iv) FB₁ plus iron loading (FB₁/CI, n = 10) for 5 weeks (2 × 2 factorial design). Hepatic iron concentrations in iron-loaded animals at 5 weeks were 444 ± 56 (CI) and 479 ± 80 μmol/g dry weight (FB₁/CI) (mean ± SEM). All the FB₁-fed rats, in the presence or absence of CI, developed a toxic hepatitis with a 4-fold rise in serum alanine transaminase (ALT) levels. FB₁ appeared to augment iron-induced hepatic lipid peroxidation, as measured by the generation of thiobarbituric acid reacting substances (TBARS) in liver homogenates (P < 0.0001). Morphometric analysis showed that FB₁ caused a significantly greater mean ± SEM number of ‘enzyme-altered’ foci and nodules per cm² (5.34 ± 1.42 vs. 1.50 ± 0.52, P < 0.05), as well as a greater area (%) of liver occupied by foci and nodules (0.33 ± 0.12% vs. 0.05 ± 0.03%, P < 0.001), compared with FB₁/CI. The addition of FB₁ to dietary iron loading caused a shift in distribution of iron from hepatocytes to Kupffer cells, probably due to phagocytosis of necrotic iron-loaded hepatocytes. In conclusion, (i) FB₁ appears to cause toxicity in the liver independently from effects on lipid peroxidation; (ii) FB₁ has a potentiating effect on iron-induced lipid peroxidation; and (iii) dietary iron loading appears to protect against the cancer promoting properties of FB₁, possibly due to a stimulatory effect of iron on hepatocyte regeneration. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Fumonisin B₁; Iron overload; Rat liver; Hepatic lipid peroxidation; Cancer initiation and promotion

1. Introduction

Fumonisin B₁ (FB₁), a food-borne mycotoxin produced by the fungus Fusarium moniliforme [1], causes a variety of naturally occurring toxicoses in animals, including fatal illnesses in horses and pigs [2,3]. Human dietary consumption of Fusarium-
contaminated corn products has been linked epidemiologically to increased rates of esophageal cancer [4,5] and, perhaps, hepatocellular carcinoma (HCC) [6], in regions of the world in which corn is the staple grain, such as South Africa and China.

FB1 is hepatotoxic and hepatocarcinogenic in rats. Short-term feeding with FB1 causes zone 3 liver injury, marked apoptosis, oval cell proliferation, and hepatic fibrosis [7]. Chronic feeding with FB1 leads to chronic toxic hepatitis, cirrhosis, and HCC [8]. FB1 acts as a strong promoter (and possibly weak initiator) of 'enzyme-altered' hepatic foci and nodules in short-term cancer studies [9,10]. The mechanisms of FB1-induced toxicity and cancer induction in the liver are unclear, but there is some evidence that peroxidation of lipid membranes [11,12] and oxidative DNA damage [13] might play a role.

Dietary iron overload is common in sub-Saharan Africa, and hepatic iron concentrations rival those occurring in genetic hemochromatosis. Recent studies suggest that dietary iron overload may be a risk factor for HCC in black Africans [14,15]. The development of HCC in humans with iron overload (genetic or dietary) usually occurs in the setting of iron- or alcohol-induced cirrhosis [16], and it is not clear whether iron plays a direct role in the induction of liver tumors or whether the increased cancer risk arises solely from the cirrhotic process. Although mechanisms of iron-induced hepatotoxicity are incompletely understood, free radical-mediated peroxidative damage to cellular lipids, proteins, and DNA is likely [17,18]. Experimentally, the potentiation of cancer induction due to polyhalogenated hydrocarbons by iron overload in rodents [19] would suggest that iron may act as a promoter of initiated hepatocytes. Studies carried out to test this hypothesis have, however, shown conflicting results, with both promoting and inhibiting effects on cancer induction by excess hepatic iron [20,21].

Both FB1 and excess hepatic iron may thus cause peroxidation of membrane lipids and oxidative liver injury [11,17]. Furthermore, both agents may, either directly or indirectly, affect the induction of liver tumors. The aims of this study were to determine whether dietary iron loading modulates the cancer initiating and promoting properties of FB1.

2. Materials and methods

2.1. Chemicals

FB1 was purified from corn cultures of Fusarium moniliforme strain MRC 826 according to the method described by Cawood et al. [22] to a purity of 92–95%. Carbonyl iron (CI), an extremely pure form of elemental iron (>98% iron with < 0.8% carbon, < 0.3% oxygen and < 0.9% nitrogen), was purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Animals and diet

The study was approved by the Ethics and Research Committee of the University of Cape Town, and the experiments were conducted in accordance with the laws and regulations controlling experiments on live animals in South Africa. Thirty-eight male Fischer 344 rats were fed a modified AIN diet [23], which has an iron content of 50 mg/kg feed [24]. The diets containing FB1 250 mg/kg were prepared as described previously [23] and stored under nitrogen at −20°C for the duration of the study. Animals that were randomized to receive dietary iron loading, were initially fed 2% (w/w) CI when weaned, but this had to be temporarily discontinued after 1 week (for 1 week) due to growth retardation, whereafter iron supplementation was resumed with 1% (w/w) carbonyl iron until the completion of the study.

2.3. Experimental design

The 5-week FB1-feeding experiment (250 mg/kg diet) was commenced when the rats weighed approximately 155–165 g (group 1, 160.9 ± 5.0 g; group 2, 165.2 ± 1.9 g; group 3, 153.2 ± 4.6 g; group 4, 155.7 ± 4.8 g). The 38 animals were divided into four treatment groups according to a 2 × 2 factorial design, i.e. controls (group 1, n = 8); FB1 250 mg/kg diet (group 2, n = 10); dietary CI 1–2% (group 3, n = 10); and FB1 plus CI (group 4, n = 10). The quantities of feeds in the different groups were adjusted to match the average intake of animals in group 4 (FB1/CI). Two animals from each treatment group and one animal from the control group were sacrificed at weeks 3 and 4 for analysis of hepatic histopathology, and the remainder of the animals (n = 6, each group) were sacrificed at week 5. The
rats were weighed daily and feed intake and wastage were carefully determined. At sacrifice, animals were anaesthetized by the intraperitoneal injection of a sodium pentobarbitone solution (6% m/v). Blood was drawn by cardiac puncture for measurement of alanine transaminase (ALT) levels for biochemical assessment of liver injury, and animals were terminated by exanguination. The livers were harvested and weighed. A slice of liver was taken from the left, right, and median lobes of each animal, and these were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin for light microscopy. The remaining liver was snap frozen in liquid nitrogen and stored at −70°C.

2.4. Light microscopy and immunohistochemistry

Liver sections (5 μm) were stained with hematoxylin and eosin (HE) for routine light microscopy, and with sirius red for collagen. Perl’s Prussian blue stain for trivalent iron was used to assess hepatic iron content [25]. Stainable iron in hepatocytes was graded 0–4, using a modification of the scale devised by Scheuer et al. [26]. Staining with rabbit polyclonal GST pi (Novacastra, Newcastle-Upon-Tyne, UK) was performed for ‘enzyme-altered’ hepatic foci and nodules. After sequential layering with swine anti-rabbit, (Dako, Copenhagen, Denmark) link antibody (1:250 dilution), peroxidase-conjugated with streptavidin-biotin (Dako, Copenhagen, Denmark) 1:500, was applied for 30 min at room temperature.

2.5. Morphometric analysis of hepatic foci and nodules

A total of 18 liver sections (three sections per rat in six rats sacrificed at week 5) each from rats in group 2 (FB1) and group 4 (FB1/CI) were stained with GST pi for determination of the number and size of ‘enzyme-altered’ foci and ‘premalignant’ nodules. One section from each of the left, right, and median lobes was examined for each rat. The number of GST pi-positive hepatic lesions (foci and nodules) per cm² were counted, and were considered an estimate of the cancer initiating effects of FB1 with or without iron loading [27,28]. For the purposes of this study, a group of GST pi-positive cells was classified as a ‘focus’ if it had an area of less than 100 μm², and a ‘nodule’ if it had an area of 100 μm² or greater. The percentage area of liver occupied by GST pi-positive foci and nodules was determined using video image analysis (Optimas, Bothell, WA), and was considered a measurement of the cancer promoting effects of FB1 with or without iron loading [27,28].

2.6. Hepatic iron concentration

Liver tissue for determination of iron content was dried for 24 h at 105°C, weighed, digested in 50% nitric acid at 70°C for 1 h, and diluted in 0.2 M sodium acetate buffer (pH 4.5) [29]. All glassware was rendered iron-free and rinsed with iron-free water [29]. Iron concentration was determined using a Roche Unimate 5 Iron Kit (Roche Diagnostic Systems, Basel, Switzerland), on a Roche Cobas Fara II Centrifugal Analyser.

2.7. Assessment of oxidative damage

Malondialdehyde (MDA) is formed when polyunsaturated fatty acids of membrane phospholipids undergo peroxidation. Lipid peroxidation was measured by the thiobarbituric acid (TBA) assay for MDA concentration on samples of liver homogenate, as described by Esterbauer and Cheeseman [30]. Related substances such as sucrose, non-ferrous metal ions and whole tissue homogenates may also react with TBA or influence the assay procedure, and the term thiobarbituric acid reacting substances (TBARS) more accurately describes the product of this assay [30]. Addition of EDTA to the liver homogenate and butylated hydroxytoluene (BHT) to the TBA reagent prevent further oxidative changes during the assay procedure. Lipid peroxidation was expressed as nmol MDA equivalents per mg protein, using a molar extinction coefficient of 1.56 × 10⁵ M⁻¹ cm⁻¹ at 532 nm for MDA [31].

2.8. Statistics

The data are presented as the mean ± SEM. Outcome measurements between the treatment groups were compared by one way analysis of variance (ANOVA). Individual comparisons were made using Scheffé’s test. FB1-induced GST pi-positive hepatic lesions in groups 2 and 4 were compared using the Mann–Whitney U-test for non-parametric data. Significance was set at a P-value of 0.05.
3. Results

3.1. Body weight gain and liver weight/body weight ratio

Total feed intake was 222.35 ± 14.26 g/100 g body wt., and equivalent total FB1 intake was calculated as 55.59 ± 3.56 mg/100 g body wt. Daily feed intake was 6.57 ± 0.40 g/100 body wt. and daily FB1 intake was 1.64 ± 0.10 mg/100 g body wt. Despite control of feed intake there were significant differences in weight gain in the treatment groups, presumably reflecting differential toxic effects (Fig. 1). The liver weight/body weight ratio was reduced only in rats from group 2 (FB1; \( P < 0.0001 \)) (Fig. 2).

3.2. Liver injury analysis

Serum levels of ALT in animals that received control AIN diet was 42 ± 2 units/l at 5 weeks. There was a marked increase in week 5 ALT levels in rats fed FB1 (group 2, 200 ± 28 units/l) and FB1/CI (group 4, 191 ± 10 units/l), reflecting significant hepatotoxicity of FB1-containing regimens. Serum ALT levels in animals given CI (group 3) were mildly raised (66 ± 7 units/l) at 5 weeks, indicating minimal hepatocellular injury caused by dietary iron loading alone.

3.3. Liver histopathology

Pathological changes in rat liver caused by feeding of FB1 250 mg/kg diet for 5 weeks have been described elsewhere [7], and include severe zone 3 injury with collapse of the reticulin framework, frequent hepatocyte mitoses, and apoptotic bodies, seen after 1 week. These initial changes are followed by the development of ‘enzyme-altered’ foci and nodules, marked oval cell proliferation, and hepatic fibrosis by week 3. Sequential liver sections from weeks 3–5 in group 2 animals (FB1) from the present study showed milder liver injury, with cell loss and collapse in zone 3, mitoses, minimal fibrosis, and multiple small GST pi-positive foci and nodules. Even though the FB1 content of the diet was the same compared with the earlier study [7], total intake of FB1 in the present study was reduced because of averaged feeding. Similarly, liver sections of group 4 rats (FB1/CI) showed evidence of mild liver injury histologically, and there appeared to be fewer hepatic GST pi-positive foci in these animals. Liver sections from control animals and group 3 rats (CI) showed no evidence of liver injury.

Perls’ Prussian blue staining of sequential liver sections showed progressive hepatic iron loading from weeks 3–5 in animals that received iron supplementation (groups 3 and 4). There was a striking difference in the pattern of iron distribution between animals in group 3 (CI) and group 4 (FB1/CI). At week 5, livers from group 3 rats (CI) showed grade 3–4 parenchymal iron loading and a zonal gradient of iron deposition, with maximum deposition in zone 1. Lesser amounts of iron was detected in Kupffer cells (Fig. 3A). Livers from group 4 rats (FB1/CI) showed evidence of marked iron deposition mainly in the Kupffer cells in zone 3, occurring in association with hepatocyte death in this region. (Fig. 3B).

Fig. 1. Body weight gain (g) of rats over 35 days according to treatment group.

Fig. 2. Liver weight to body weight (LW/BW) ratio of rats according to treatment group.
Fig. 3. Stainable iron in livers from rats in groups 3 and 4. (A) Liver section from a rat in group 3 (CI), showing grade 3 parenchymal iron deposition with maximal deposition in zone 1. (B) Liver section from a rat in group 4 (FB/CI), showing loss of hepatocytes in zone 3 and abundant iron in Kupffer cells, with lesser amounts of parenchymal iron deposition. Perls’ stain for iron, objective ×10. Straight arrows indicate central veins; curved arrows indicate portal tracts.
3.4. Morphometric analysis of GST pi-positive foci and nodules

Rats given dietary iron loading alone (CI) did not develop any GST pi-positive hepatic foci or nodules. At 5 weeks, 4/6 rats treated with FB1 (group 2) had developed hepatic foci and 6/6 rats from this group had developed hepatic nodules. In contrast, 3/6 rats treated with FB1/CI (group 4) had developed foci by 5 weeks, and 4/6 rats from this group had developed nodules. At week 5, corresponding liver sections (3 sections/rat from 6 rats in each group) from rats in group 2 contained an average of $5.34 \pm 1.42$ GST pi-positive lesions per cm$^2$, as compared with $1.50 \pm 0.52$ GST pi-positive lesions per cm$^2$ in livers of rats from group 4 ($P < 0.05$; Table 1). Furthermore, the area (%) of lesions in rats from group 2 was $0.33 \pm 0.12$, as compared with $0.05 \pm 0.03$ in sections of rats from group 4 ($P < 0.05$; Table 1). These data indicate that there were more, and larger, lesions in the livers of rats that received FB1 only (group 2).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>FB1 (group 2)</th>
<th>FB1/CI (group 4)</th>
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<tbody>
<tr>
<td>No. of rats with foci*</td>
<td>4/6</td>
<td>3/6</td>
</tr>
<tr>
<td>No. of rats with nodules</td>
<td>6/6</td>
<td>4/6</td>
</tr>
<tr>
<td>No. of lesions per cm$^2$b</td>
<td>$5.34 \pm 1.42$</td>
<td>$1.50 \pm 0.52$</td>
</tr>
<tr>
<td>Area (%) of lesions$^b$</td>
<td>$0.33 \pm 0.12$</td>
<td>$0.05 \pm 0.03$</td>
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* A GST pi-positive lesion was classified as a ‘focus’ if it had an area of less than 100 $\mu$m$^2$, and as a ‘nodule’ if it had an area of 100 $\mu$m$^2$ or greater.

b Data presented as mean $\pm$ SEM.

$^c P < 0.05.$

3.5. Hepatic iron concentration

Hepatic iron concentration ($\mu$mol/g dry weight of liver) in the livers of rats treated with CI (group 3) and FB1/CI (group 4) was $444 \pm 56$ and $479 \pm 80$ $\mu$mol/g dry weight, respectively. In contrast, liver iron concentration in control animals (group 1) and rats treated FB1 was $48 \pm 4$ and $48 \pm 5$ $\mu$mol/g dry weight, respectively.

3.6. Hepatic lipid peroxidation

Generation of TBARS in liver homogenates differed between the treatment groups (Fig. 4), and these differences persisted when using differences in weight gain as the covariant ($F$ ratio $= 5.24$, $P < 0.01$). Although treatment with FB1 (group 2) slightly increased TBARS generation above control levels, these changes were not significant ($P < 0.3$). Treatment with CI (group 3), however, caused a significant increase in TBARS generation ($P < 0.001$). Contrast coefficient analysis revealed that hepatic TBARS generation due to FB1/CI (group 4) was significantly more than the sum of TBARS generation by CI (group 3) plus FB1 (group 2) ($P < 0.0001$), indicating a potentiating effect of FB1 on iron-induced lipid peroxidation (Fig. 4).

4. Discussion

FB1-induced hepatotoxicity may be caused, at least in part, by lipid peroxidation and oxidative damage to hepatocytes [11–13]. Abel and Gelderblom [11] recently showed that FB1 caused a dose-dependent increase in the level of TBARS in rat liver in vivo and in primary rat hepatocytes in vitro. The in vitro effect was further potentiated by the addition of cumene hydroperoxide (CMHP), a potent oxidizing agent. The authors suggested that lipid peroxidation with generation of TBARS appeared to be an end result rather than a cause of FB1-induced hepatic injury [11]. Sahu et al. [13] found no effect of metals (iron or copper) on FB1-induced peroxidation of lipid.

![Fig. 4](image-url)

Fig. 4. Generation of thiobarbituric acid reacting substances (TBARS) from liver homogenates according to treatment group ($n = 6$, each).
membranes and oxidative DNA damage of isolated rat nuclei. The present in vivo study, however, shows that excess liver iron (mean content 479 μmol/g dry weight) has a significant potentiating effect on FB₁-induced lipid peroxidation, measured as generation of TBARS, in liver homogenates. In contrast to the study by Abel and Gelderblom [11], FB₁ 250 mg/kg diet alone did not cause a significant increase in hepatic TBARS generation, although the level appeared to be slightly higher than in controls. This might have been due to the lower total dose of FB₁ administered in the present study due to averaged feeding of the animals. FB₁-induced hepatotoxicity was reflected by a reduction in the liver/body weight ratio and a four-fold rise in serum ALT levels, and was confirmed histologically.

Agents may increase the risk of cancer by causing DNA damage (genotoxicity) and/or by causing increased proliferation (increased DNA replications) in a pluripotential cell population of the tissue [32]. To increase the number of DNA replications, an agent can either increase cell births (direct mitogenesis or toxicity and regenerative proliferation) and/or decrease cells deaths (inhibition of apoptosis) [33]. FB₁ appears to be a unique carcinogen which causes marked TGF-β1-induced apoptosis accompanied by regeneration of hepatocytes and proliferation of oval cells [7], putative precursor cells for liver tumors [34]. Although FB₁ was found to be non-genotoxic in the Ames mutagenicity test [35,36], recent studies have reported the DNA damaging potential of FB₁ [13,37,38]. FB₁-induced lipid peroxidation might result in oxidative damage to DNA and errors in replication. These effects might be enhanced by oxidative injury due to iron loading. However, in the present study, iron overload significantly enhanced lipid peroxidation in the absence of hepatotoxic injury, whereas FB₁-induced hepatocyte injury was only associated with mild changes in lipid peroxidation. These findings are in accordance with the hypothesis of Abel and Gelderblom [11] that lipid peroxidation is secondary to FB₁-induced liver injury. With respect to cancer induction, this study showed that moderately severe hepatic iron overload (9-fold increase in liver iron content) decreased the number and size of GST pi-positive foci and nodules in FB₁-fed rats. A similar protective effect of hepatic iron loading (6- to 13-fold increase) was reported by Stål et al. [20], who added or substituted dietary iron loading for the initiating and promoting events in the Solt Faber model of chemical hepatocarcinogenesis. They showed a mild mitostimulatory effect of iron on normal hepatocytes, which may have protected against the promotion of resistant hepatocytes. Although hepatocyte proliferation was not measured in this study (e.g. BrdU labeling, PCNA), the liver weight/body weight ratio was maintained in animals treated with FB₁/CI, suggesting that iron may augment the hepatic regenerative response to FB₁-induced loss of hepatocytes and hence may counteract the selection process (mitoinhibition of normal hepatocytes) that effects the outgrowth of initiated cells into GST pi-positive foci and nodules. Under the present conditions, where cancer promotion was apparently impaired, the effect of excessive iron on FB₁-induced cancer initiation could not be determined.

There was a striking difference in the pattern of distribution of liver storage iron between group 3 (CI) and group 4 (FB₁/CI). Dietary supplementation with CI is known to cause predominantly parenchymal (zone 1) hepatic iron deposition, with a zonal gradient, similar to that seen in genetic hemochromatosis [39,40]. Addition of FB₁ feeding to iron loading resulted in a shift of liver iron from parenchymal cells to Kupffer cells, presumably due to ingestion of dead hepatocytes by liver macrophages. A similar shift in the distribution of iron from hepatocytes to reticuloendothelial cells is seen in alcohol–iron–CCl₄-treated rats [41], and also occurs in genetic hemochromatosis with episodes of alcoholic hepatitis [42]. Although African dietary iron overload is also characterized by massive deposition of reticuloendothelial iron [43], the pathogenesis of the hepatic siderosis is different, and appears to relate to associated ascorbic acid deficiency with impaired release of iron from Kupffer cells [44,45].

In conclusion, FB₁ had a potentiating effect on iron-induced lipid peroxidation in the liver. However, the effects on cancer induction by the fumonisins mycotoxins still need to be elucidated, particularly as dietary iron overload appears to protect against the promotion of GST pi-positive hepatic lesions by FB₁, probably due to a stimulatory effect on hepatocyte regeneration. These exciting findings provide new avenues of research regarding the mechanisms involved in FB₁-induced carcinogenesis and the
modulating role of environmental factors, such as iron overload.

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