Short Communication

Cytotoxic effects of aflatoxin B1 on human brain microvascular endothelial cells of the blood-brain barrier

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Abstract

Aflatoxins are mycotoxins produced by Aspergillus spp. Although AFB1 is implicated as a carcinogen in hepatocellular carcinoma, brain autopsies in affected areas have revealed its presence in 81% of cases. Given its haematogenous spread, here we determined the cytotoxic effects of AFB1 on primary human brain microvascular endothelial cells (HBMEC), which constitute the blood-brain barrier, human umbilical vein endothelial cells (HUVEC) as well as immortalized epithelial cells of human hepatocellular carcinoma (Huh7). The cell types were exposed to AFB1 (3–32 nM) for 24 h and release of lactate dehydrogenase was measured as cell cytotoxicity marker. Furthermore, DNA was collected from both cell types and DNA adduct formation was determined by immunoblot using anti-AFB1-DNA adduct antibody. At 32 nM, AFB1 killed >85% HBMEC, while controls showed minimal effects (P < .05). Similar concentrations of AFB1 showed 22% cell death of HUVEC, while the same concentration did not kill Huh7. At low concentrations, in other words, 3.2 nM, AFB1 produced DNA adduct formation in HBMEC, while high concentration (32 nM) did not form DNA adducts. For HUVEC, 16 nM and 32 nM exhibited DNA adduct formation. For Huh7, 3.2 nM did not form DNA adducts, while 32 nM exhibited DNA adduct formation. For the first time, we report that AFB1 affected the viability of primary endothelial cells but not immortalized Huh7 cells. Cytotoxicity of brain endothelial cells suggests extra-hepatic complications post-AFB1 exposure.

Key words: Aflatoxin B1 (AFB1), Aspergillus flavus, blood-brain barrier, endothelial cells, Huh7.

Introduction

Aflatoxins are mycotoxins generally produced by the fungus Aspergillus flavus which is a common contaminant of food items such as corn, spices, rice, nuts, and flour [1]. Given the opportunity and access, Aspergillus flavus can produce aspergillosis affecting various body organs, while its toxin, Aflatoxin B1 (AFB1) has been implicated as a...
carcinogen in hepatocellular carcinoma (HCC) [2]. Hepatocellular carcinoma is the second leading cause of all cancer deaths worldwide [3]. In the United States alone, the overall incidence rate of HCC has been estimated at 2.99 per 100,000 [4,5]; however, for Asian and African countries, figures are alarming, in other words, about 50–150 cases per 100,000 [6,7].

According to a recent study in 2010, there are 550,000–600,000 new HCC cases reported, out of which 25,200–155,000 may be attributed to aflatoxin exposure [8]. The regions where most of these cases occur are sub-Saharan Africa, China, and Southeast Asia, areas where the prevalence of viral hepatitis is high and there is poor processing of aflatoxin-contaminated foods [8]. On a global scale, approximately 4.5 billion people living in developing countries are frequently exposed to unchecked amounts of the toxin [9], while indirect exposure may also occur through animals that have ingested contaminated feed. As aflatoxins are highly liposoluble, they are readily absorbed through the gastrointestinal tract and enter the bloodstream [1]. There are many types of aflatoxins (B1, B2, G1, G2, M1), but AFB1 has been classified as a Group 1 carcinogen (definitely carcinogenic to humans). Once converted to its active epoxide form by the cytochrome P450 group of liver enzymes, AFB1 has the ability to bind to serum albumin forming albumin adducts [10]. It can also bind with guanine residues in DNA resulting in AFB1-N7-guanine adducts resulting in guanine to thiamine transversion mutations [11]. The ability to modulate DNA activity by creating such lesions in the DNA may play a major role in AFB1-induced carcinogenesis [12]. AFB1-treated rat liver tissue has shown abnormal expression of cancer-related miRNAs, which may also contribute to the development of liver cancer [13].

Aflatoxin exposure not only damages the liver but other body organs including lungs and the brain [14,15]. Brain autopsies of children living in areas with high aflatoxin exposure revealed presence of aflatoxins in 81% of the cases studied [15] and known to deregulate signal transduction activity of neuroblastoma cells [16]. Ingestion of AFB1 is shown to produce brain injury in vivo using Sprague-Dawley rats [17]. Human brain microvascular endothelial cells (HBMEC) are primary cells derived from the brain that constitute the blood-brain barrier. Given the haematoencephalogenous spread of AFB1, the purpose of this study was to determine its effects on the HBMEC. Using primary HBMEC, here we determined the cytopathogenic effects and DNA adduct formation post-AFB1 exposure. Additionally, primary human umbilical vein endothelial cells (HUVEC) were used for comparison.

### Materials and methods

#### Human brain microvascular endothelial cells (HBMEC)

Primary HBMEC were grown in tissue culture flasks in Roswell Park Memorial Institute Medium (RPMI-1640) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM Na-pyruvate, 100U penicillin per ml, 100 μg streptomycin per ml, non-essential amino acids and vitamins as previously described [18]. For assays, HBMECs were seeded in 24-well plates at a density of $2 \times 10^5$ per ml in each well and plates incubated at 37°C in a 5% CO₂ incubator, which resulted in the formation of complete monolayers within 48 h. Primary HBMEC originated from small fragments of cerebral cortex derived from individuals who had undergone surgical resections for seizure disorder at Johns Hopkins University School of Medicine (USA) were used for HBMEC isolation, after approval from the Institutional Medical Ethics Committee. The primary HBMEC were purified by fluorescent activated cell sorting, and their purity tested using endothelial markers such as expression of F-VIII, carbonic anhydrase IV and uptake of acetylated low density lipoprotein (DiI-AcLDL) as previously described [18,19] and resulted in >99% pure endothelial cultures [20].

#### Human hepatocellular carcinoma cells (Huh7)

Cells were purchased from Cell Lines Service GmbH (Germany) grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 100U penicillin per ml, 100 μg streptomycin per ml and 2 μg ciprofloxacin per ml. For assays, Huh7 cells were seeded in 24-well plates at a density of $0.05 \times 10^6$ per ml in each well and plates incubated at 37°C in a 5% CO₂ incubator, which resulted in the formation of complete monolayers within 48 h [21].

#### Human umbilical vein endothelial cells (HUVEC)

Primary HUVEC (American type culture collection, ATCC PCS-100-013) were grown in tissue culture flasks in Roswell Park Memorial Institute Medium (RPMI-1640) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM Na-pyruvate, 100U penicillin per ml, 100 μg streptomycin per ml, nonessential amino acids, vitamins and heparin sulfate (0.75 units per ml), hydrocortisone hemisuccinate (1 μg per ml) and ascorbic acid (50 μg per ml). For assays, HUVEC were grown to confluency as described for HBMEC.
Purified Aflatoxin B1

Purified aflatoxin B1 (AFB1) was obtained from Meryer Chemical Technology Co., Ltd., Shanghai, China. Using LC-MS/MS, purity of AFB1 was found to be >99% and it was dissolved in 100% methanol at a concentration of 1 mg per ml (3.2 mM) and stored in dark at -20°C until tested.

Cytotoxicity assays

Cytotoxicity assays were performed as previously described [18]. Briefly, HBMEC, HUVEC and Huh7 were grown in 24-well plates, and cells were exposed to purified AFB1 at different concentrations (3.2 nM, 8 nM, 16 nM and 32 nM) dissolved in 100% methanol, and the final volume adjusted to 0.5 ml. The control solution was diluted so the solvent (methanol) amount remains the same (5 μl of methanol) in all concentrations tested. The solvent alone was used as a control (5 μl of methanol). Plates were incubated at 37°C in a 5% CO₂ incubator for 24 h. Next, supernatants were collected by centrifugation at 3000 x g for 5 min to remove cellular debris. Cytotoxic effects were determined by estimating the amount of lactate dehydrogenase released from the cells using Cytotoxicity Detection kit (Roche Applied Sciences, Mannheim, Germany). The percent cytotoxicity was calculated as follows: % cytotoxicity = (sample value − control value) / (total LDH release − control value) x 100. Control values were determined by incubating HBMEC, HUVEC, or Huh7 monolayers with RPMI-1640 or DMEM, respectively. Total LDH release was obtained by completely lysing the HBMEC, HUVEC and Huh7 by treating them with 1% Triton X-100 for 30 min.

DNA extraction

The HBMEC, HUVEC, and Huh7 cells were grown to confluency in 24-well plates. Cells were exposed to purified AFB1 at different concentrations (3.2 nM, 8 nM, 16 nM, and 32 nM). The solvent alone was used as a control. Plates were incubated at 37°C in a 5% CO₂ incubator for 24 h. Cell pellets were harvested after trypsinization, and resuspended in 1 ml of distilled water. DNA extraction was performed using 180 μl of Proteinase K (10 mg/ml) and 180 μl of 10% SDS, then mixed gently by inversion and incubated in water bath for 15 min at 55°C. Next, 1125 μl of 5M sodium chloride was added, and tube was centrifuged at 14000 x g for 20 min. Supernatants were collected and 400 μl of 3M sodium acetate was added. DNA was precipitated using absolute isopropanol, up to 70% of the volume and mixed by inversion. Tube was centrifuged at 14000 x g and DNA pellet was washed with 70% ethanol and dissolved in Tris-EDTA buffer (10 mM Tris HCl pH 8, 1 mM EDTA). DNA was quantified using Nanodrop ND-1000 Spectrophotometer (Thermo Scientific).

Detection of AFB1 DNA adducts

Extracted DNA was denatured by boiling for 5 min followed by immediate cooling on ice. The zeta-probe membrane (Bio-Rad, California, USA) was activated by soaking in deionized water for 5 min. The denatured DNA was blotted onto the zeta-probe membranes at 10μg concentration for each tested concentration (3.2 nM, 8 nM, 16 nM, and 32 nM) by using Bio-Dot Microfiltration Apparatus (Bio-Rad, California, USA). The immune-dot-blot assay was performed as described by Besaratinia et al. [22]. Following this, the membranes were blocked using phosphate-buffered saline (PBS) plus 0.2% Tween-20 (PBST) containing 5% nonfat milk overnight at 4°C with gentle shaking. The membranes were washed with PBST and incubated with anti-AFB1 DNA adduct antibody (mouse monoclonal AFB1 antibody, 6A10, Novus Biologicals, Colorado, USA) at a dilution of 1:10,000 for 2 h. The membranes were washed three times, 10 min each with PBST and incubated with secondary antibody (rabbit polyclonal rabbit anti-mouse immunoglobulin G [IgG] antibody-linked with horse radish peroxidase, Novus Biologicals, Colorado, USA) diluted 1:10,000 for 1 h. The membranes were washed three times, 10 min each with PBST and peroxidase activity revealed using Enhanced Chemiluminescence (ECL) reagent (Amersham) and reactions detected by exposure on an X-ray film. The intensity of luminescence was determined using the software ImageJ from National Institute of Health, USA. The percent relative intensity was calculated as follows: % relative intensity = (sample value - negative control value)/(positive control value - negative control value) x 100.

Positive and negative controls for AFB1 DNA adducts

Huh7 cells were grown in T-75 flasks and pellet collected following trypsinization. Pellet was resuspended in 500 μl of lysis buffer (50 mM Tris pH 8, 5 mM EDTA, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride). Cells were sonicated on ice, thrice for 30 seconds each, at 70% duty cycles at maximum limits of microtip using Branson 450 sonifier. Following this, lysates were centrifuged at 14000 x g for 20 min, and clear supernatant were collected. For positive controls, 1.4 mg of calf thymus DNA (Sigma) dissolved in water, was incubated with 50 μg of AFB1 in 500 μl of Huh7 cell lysate [to provide a system to convert AFB1 to its reactive form, since Huh7 contain...
metabolically active cytochrome P450 that can efficiently convert AFB1 to its reactive epoxide form [23] and incubated at 37°C overnight. Next, DNA was extracted and immunoblots performed as described above. For negative controls, same procedure was followed except 50 µg of AFB1 was not added.

**Results**

**AFB1 produced cytotoxic effects on primary HBMEC but limited effects on HUVEC and no effects on immortalized Huh7 cells**

In order to determine the effects of AFB1 on human cells, primary HBMEC, HUVEC and immortalized Huh7 cells were used. Both cells were exposed to varying concentrations of purified toxin and cytotoxicity assessed. As low as 3.2 nM exhibited 52.1% ± 6.8 HBMEC cytotoxicity (P < .05 using Student’s t-test) (Fig. 1). At 8 nM, AFB1 produced 63.1% ± 2.3 HBMEC cytotoxicity (P < .05). At 32 nM, AFB1 produced 87.2% ± 2.2 HBMEC cytotoxicity (P < .05). For HUVEC cells, 3.2 nM and 8 nM exhibited minimal cytotoxicity, 6.5% ± 1.1 and 12% ± 2.1. High concentration of 32 nM of AFB1 showed up to 28.5% ± 3.5) HUVEC cytotoxicity. For Huh7 cells, 3.2 nM and 8 nM exhibited only 1.5% ± 1.4 and 3.8% ± 5.1 Huh7 cytotoxicity. Even high concentration of 32 nM of AFB1 showed no effect (6.7% ± 4.9) on Huh7 cytotoxicity (P > .05). Controls were solvent alone (methanol), and their effects were minimal on HBMEC (7.6% ± 1.9), HUVEC (3.5% ± 0.6), and Huh7 (4.8% ± 0.9).

**DNA adduct formation in primary HBMEC, HUVEC and immortalized Huh7 following AFB1 exposure**

To determine whether AFB1 exposure results in DNA adduct formation, assays were developed. As a positive control, calf thymus DNA incorporated with AFB1 DNA adducts was used and a positive reaction (dense dot) was observed on the immunoblot (Fig. 2). This value was taken as 100%. For negative control, the calf thymus DNA without AFB1 DNA adducts was used and this value was subtracted from all other values. For primary HBMEC, lower concentrations of 3.2 nM and 16 nM exhibited DNA adduct formation (73% ± 3.9 and 102% ± 3.6, respectively), while 32 nM did not show DNA adduct formation (1.5% ± 0.2). For primary HUVEC, lower concentrations of 3.2 nM did not show DNA adduct formation, while 16 nM and 32 nM exhibited DNA adduct formation (45% ± 4.6 and 75% ± 6.9, respectively). For Huh7, lower concentration of 3.2 nM did not result in DNA adduct formation (1% ± 0.2), while 16 nM and 32 nM exhibited DNA adduct formation 67.2% ± 4.3 and 118.5% ± 7.7, respectively.

**Discussion**

Association of AFB1 with hepatocellular carcinoma has been well documented [2]. For the first time, we report the effects of AFB1 on primary cells of the brain origin and compared these effects to HUVEC and cancer cells of the liver. Once AFB1 is ingested, it is absorbed through the gut via passive diffusion. The toxin enters the liver where liver biotransformation enzymes convert it to its active AFB1 epoxide form [1]. This reactive epoxide form affects primary site, in other words, liver but as it reaches systemic circulation, it affects other cells and organs of the body including lymphocytes, lungs and the brain [14,15,24]. Since brain autopsies of children living in areas with high aflatoxin exposure revealed presence of aflatoxins in 81% of the cases [15], we determined whether aflatoxins could pass the blood-brain barrier by affecting its integrity. This was tested using primary human brain microvascular endothelial cells (HBMEC), which constitute the blood-brain barrier. Even though many studies have utilized cancer cell lines like HepG2 [25] and neuroblastoma cells [16] for studies on toxicity, we chose to use primary cells because they more closely reflect the metabolism and response in the *in vivo* state of the organism.

Our data show that the primary HBMEC, are more susceptible to the cytotoxic effects of AFB1, followed by HUVEC, while cancer cells, Huh7 showed minimal susceptibility at concentrations tested in this study. With increasing concentration of the toxin, there is a marked increase in the percentage of cytotoxicity of primary cells. The high concentration had minimal toxic effect (6.7%) on immortalized Huh7 cells. A likely explanation is that due to neoplastic transformation in cancer cells, biotransformation enzymatic activity may be compromised. For example, low level of expression of phase 1 biotransformation enzymes in HepG2 cells as compared to primary hepatocytes obtained from patients undergoing hepatic resections has been reported [26]. When these cells were exposed to different food promutagens like benzo[a]pyrene, dimethylnitrosamine (DMN), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), the primary hepatocytes showed more sensitivity to the toxins as they expressed significantly higher levels of biotransformation enzymes [26]. This phenomenon of primary cells being affected at lower concentrations of AFB1 as compared to cancer cell lines has also been reported by Iwaki et al. [27]. They observed DNA, RNA, and protein synthesis inhibition in chicken primary hepatocytes at 0.1 µg/ml, while established cell lines only showed DNA synthesis.
Figure 1. Cytotoxicity of purified AFB1 on human brain microvascular endothelial cells (HBMEC), human umbilical vein endothelial cells (HUVEC), and human hepatocellular carcinoma cells (Huh7). Cells were exposed to purified toxin and LDH assay was performed to assess cytotoxicity. With each concentration, the toxic effects of AFB1 were enhanced and HBMEC showed increased death as compared to controls \((P < 0.05\) using Student’s t-test\) (A). AFB1 toxin showed moderate cytotoxic effects on HUVEC at higher concentrations (B). AFB1 toxin showed minimal effects on Huh7 cells (C). Equal volume of methanol was used as control. The results are presented as the mean ± standard error of three independent experiments performed in duplicate.
inhibition at 1 μg/ml. The HBMEC are representative of the blood-brain barrier, and cytotoxicity of AFB1 at low concentrations suggests that AFB1 exposure compromises the blood-brain barrier. Previous studies suggest that lipophilic nature of aflatoxins allow their storage in the brain tissue [15]. Similarly, in chicken fibroblast cells most incorporated AFB1 was isolated from lipid fractions as opposed to DNA, RNA and protein incorporation in chicken primary hepatocytes [27].

Our data show AFB1-N7-guanine adducts (AFB1 DNA adduct) formation in all cell types tested. The presence of adducts indicates that the toxin was converted to its active AFB1 epoxide form in both cell types. It is plausible that there was variation in biotransformation enzyme activity in the cell types but presence of 67.7% AFB1 DNA adducts at 16 nM of AFB1 in Huh7 means there was sufficient quantity of active AFB1 epoxide. In HBMEC or HUVEC, 16 nM showed increasing adduct formation but 32 nM did not show any adduct formation in HBMEC. Is it because of 87% HBMEC cytotoxicity seen at that concentration and that the cells were dying before adduct formation could occur? If so, this would suggest that perhaps cytotoxicity was independent of AFB1 DNA adduct formation much like in acute toxicity of AFB1 where hepatic necrosis results from over exposure to the toxin [28].

In Huh7, no adduct formation was seen at 3.2 nM of toxin. It is possible that due to low activity of biotransformation enzymes there was less active form of AFB1 at this concentration. At 16 nM and 32 nM, there was steady increase in the adduct formation and it was almost two fold more with each increasing concentration, suggesting enough active AFB1 epoxide form.

Mechanisms of cytotoxicity may vary from cell to cell. Primary rat liver cells, hepatocytes (parenchymal cells) exhibited AFB1 concentration-related cytotoxicity as opposed to non-parenchymal cells, yet both formed AFB1 DNA adducts [29]. Certain studies have revealed that HepG2 exposed to AFB1 show failure to activate p53 and did not
undergo cell cycle arrest or apoptosis, even though DNA adducts were present [30]. In contrast, lung cells undergo apoptosis via mitochondrial pro-apoptotic proteins with increasing concentration of the toxin [14]. Another mechanism hints towards deregulation of signal transduction; i.e., when mouse neuroblastoma cells were exposed to AFB1, they exhibited effective alteration of cyclic nucleotide phosphodiesterase activity [16].

Further studies are needed to elucidate the underlying molecular mechanisms of cytotoxicity in HBMEC. Additionally, studies related to liver cancer need to be conducted on primary cells, as cancer cells almost always give altered results. Studies where neutralizing effects of antioxidants like β-carotene and lycopene against AFB1-induced toxicity were determined, the cell lines used were cancer cell lines [31]. Since lower concentrations of the toxin give adverse effects on primary cell lines, the effect of neutralizing agents may not be enough. Interaction of the toxin with the cellular machinery in causing toxicity has merit for further analysis and suggests that AFB1 exposure may have extra-hepatic complications.

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Declaration of interest

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

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