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Original Article

Aflatoxin B1 damages peripheral blood lymphocytes in patients with primary hepatocellular carcinoma by inducing peroxidation

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Abstract: Objective: The aim of this study was to examine the effect of aflatoxin B1 (AFB1) on lymphocytes in patients with primary hepatocellular carcinoma (HCC) and the underlying mechanism. Methods: Peripheral blood from 20 HCC patients (liver cancer group) and 10 healthy individuals (control group) were collected for isolating peripheral blood lymphocytes. The peripheral blood lymphocytes of each patient were cultured *in vitro* and divided into four subgroups; blank control subgroup and subgroups treated with AFB1 doses of 0.2 μg/mL (low toxicity), 2 μg/mL (mild toxicity), and 20 μg/mL (high toxicity). After incubation for 24 h, the level of the DNA damage marker, 8-hydroxyguanine (8-oxoG), was detected in the cells using flow cytometry and the activity of oxidative damage markers, hydroxy radical (OH^-) and oxygen free radical, were detected using Fenton method and Xanthine oxidase method, respectively. Results: The content of 8-oxoG in liver cancer group was significantly higher than that in the control group ($P < 0.05$). It increased in an AFB1-dose-dependent manner in both groups. The same trend was also found with respect to the OH^- content. However, the total superoxide dismutase (T-SOD) activity decreased with increasing dose of AFB1. Partial correlation analysis showed that the content of 8-oxoG was negatively correlated with the T-SOD activity ($r = 0.5539$, $P = 0.001$), but positively correlated with the content of OH^- ($r = 0.3812$, $P = 0.038$). Conclusion: AFB1 causes more oxidative damage in lymphocytes of patients with HCC, by inducing peroxidation, than in lymphocytes of healthy individuals.

Keywords: Aflatoxin B1 (AFB1), lymphocytes, oxidative damage, hepatocellular carcinoma (HCC)

Introduction

Hepatocellular carcinoma (HCC), the most common type of liver cancer worldwide, is a major health problem in Asia [1], especially among the people of developing countries [2], including the Chinese [3]. A recent study revealed that HCC causes the highest mortality rate in China. HCC is caused mainly by viruses (such as hepatitis B virus (HBV) and hepatitis C virus (HCV)), alcohol consumption, aflatoxin exposure, metabolic disorders, and immune-related factors [4]. Besides HBV infection [5] and an increased susceptibility to HCC due to ethnic variation [6], exposure to food contaminated with Aflatoxin B1 (AFB1) is also an important reason for the incidence of HCC [7].

Epoxide produced during the metabolism of AFB1 in liver can react strongly with DNA and form adducts at the N7 position of guanine [8], which leads to cancerous transformation of cells. It has been reported that individuals exposed to AFB1 have a high likelihood to develop HCC [9] and immune suppression [10].

Owing to the technical and ethical difficulties associated with the preparation of normal human liver cells, the relative weight of spleen was used to evaluate the toxicity of AFB1. It has been found that the relative weight of spleen in the AFB1 group was significantly lower than that in the control group [11]. A recent study showed that human lymphocytes exposed to AFB1 (100 μM) *in vitro,* exhibit impairments in cellular respiration and caspase is activated, leading to necrosis [12], indicating that the cytotoxicity of AFB1 is mediated by apoptosis and necrosis. Apoptosis has already been detected in some studies [13]. DNA damage index in peripheral

blood lymphocytes can reflect the degree of carcinogen-induced damage to liver DNA [14]. Therefore, we isolated peripheral blood lymphocytes from blood of healthy individuals and patients with HCC to establish an *in vitro* model of AFB1-induced oxidative damage to cells. Since the average dose of exposure to AFB1 in the population in Eastern China is less than 0.5 mmol per day [15], different concentrations of AFB1 were used in this study to determine its toxicity to lymphocytic DNA and its effect on the antioxidant capacity of lymphocytes.

Materials and methods

Study subjects

Twenty patients with HCC, hospitalized in the First Affiliated Hospital of Guangxi Medical University in 2013, were enrolled in the liver cancer group, and 10 healthy individuals from the medical center of the same hospital were enrolled in the control group. Lymphocytes were separated from 20 mL anticoagulant-treated peripheral blood of each subject, using lymphocyte separating medium (TBDLTS1077, from Tianjin Haoyang Biological manufacture co., Ltd, Tianjin, China) and cultured *in vitro*. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was obtained from all participants.

Experimental groups

Lymphocytes of each subject were divided into four subgroups: lymphocytes treated with saline (blank control), lymphocytes treated with 0.2 μg/mL AFB1 (Sigma-Aldrich, USA) (low toxicity subgroup), 2 μg/mL AFB1 (mild toxicity subgroup), and 20 μg/mL AFB1 (high toxicity subgroup).

Flow cytometry

After incubation for 24 hours, cells were harvested for the detection of lymphocytic DNA damage marker, 8-hydroxyguanine (8-oxoG), by flow cytometry (Beckman Coulter, USA). The cell suspension was introduced into the microfluidic channel and hydrodynamically focused by sheath flow, ensuring that the cells travel in the center of the fluidic channel at a uniform velocity. Fluorescence and backscattered light from the sample were detected by two individual photomultiplier tubes (PMTs) in a wide-field fluorescence microscope. The microfluidic channel into which the cells were injected was made of soft-molded PDMS bonded to a glass substrate. To accommodate the geometry of the microfluidic device, a laser beam was introduced to the optical interrogation site in the fluidic channel by a miniature 45-degree dichroic mirror positioned in front of a 50X objective lens (NA = 0.55, working distance = 13 mm). The 45-degree dichroic mirror was small enough to allow the backscattered light (147° to 168° with respect to the normal incident light) to pass the dichroic mirror and enter the objective lens. Both the fluorescent and backscattered light from a travelling cell are collected by the objective lens and passed through the filter before reaching their respective PMT detectors. Another dichroic mirror splits the light to route the desired emission bands to the appropriate PMTs. Finally, the output of each PMT is sent to a computer and processed to generate images of cells using fluorescence and backscattering data.

Activity of total superoxide dismutase (T-SOD)

The activity of T-SOD in the cell supernatants was determined using xanthine oxidase method. The hydroxyl radical ($OH^{\cdot}$) content was determined using Fenton method.

Xanthine oxidase method: The substrate PG electrode was first polished using rough and fine sand papers. It was then polished to mirror smoothness with aluminum oxide (particle size of about 0.05 μm)/water slurry on silk. Finally, the electrode was thoroughly washed by ultrasonication for 5 min each in double distilled-water and ethanol. A mixture of 10 μL 3.5 U/mL XOD and 10 μL 1.0 mg/mL salmon sperm DNA solution were evenly spread on the surface of the PG electrode. The modified electrode was dried overnight in dark, at room temperature, and was thoroughly rinsed with double-distilled water before use [16].

Fenton method: Fenton oxidation is commonly used to degrade the organic components and kill microorganisms during wastewater treatment. Fenton oxidation destroys the sludge structure, releasing water and organics. This reagent also oxidizes odorous substances, kills

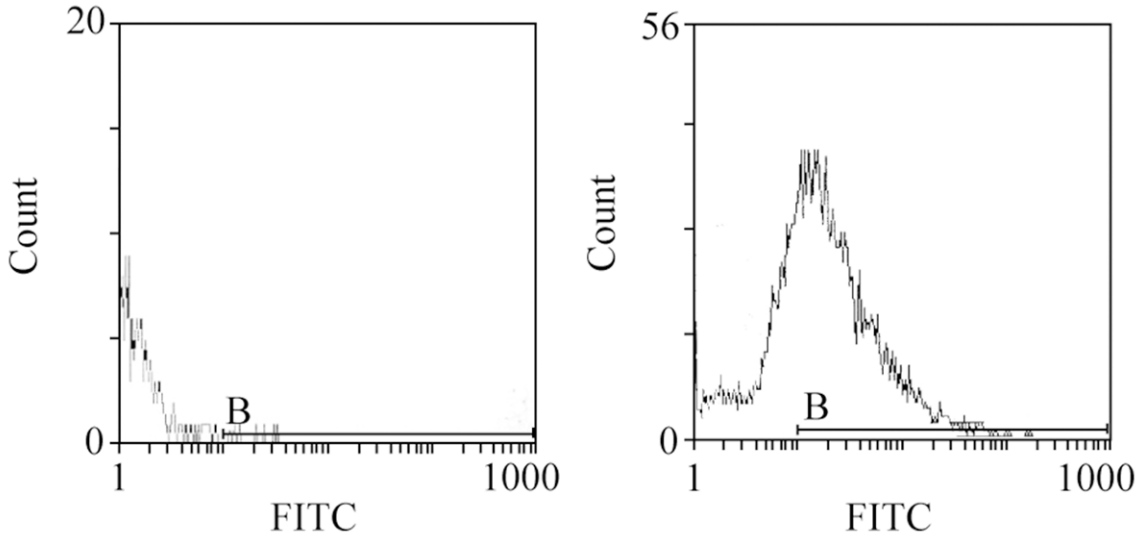

Figure 1. The 8-oxoG-positive lymphocytes increased in the liver cancer group after the treatment with AFB1.

Table 1. The positive rates of 8-oxoG$^+$ lymphocytes in the liver cancer group and the control group

Group	No. of cases	8-oxoGP (%)			
		0 μg/mL	0.2 μg/mL	2 μg/mL	20 μg/mL
Liver cancer	20	33.69±8.01	37.41±9.55	44.78±11.74**	60.83±14.78**
Control	10	27.11±7.58	26.77±5.74	27.91±5.08	30.22±7.57
P		0.040*	0.001**	< 0.001**	< 0.001**

Data are shown as mean ± SD. **P < 0.01, compared with blank control subgroup using ANOVA. *P < 0.05 and **P < 0.01, compared with control group using *t*-test.

Table 2. The fluorescence intensity of 8-oxoG$^+$ lymphocytes in the liver cancer group and the control group

Group	No. of cases	8-oxoGI (RFI)			
		0 μg/mL	0.2 μg/mL	2 μg/mL	20 μg/mL
Liver cancer	20	30.31±7.24	33.91±7.39	36.33±7.84*	40.51±8.17**
Control	10	23.22±3.36	23.16±3.58	23.62±3.61	24.24±3.48
P		< 0.001**	< 0.001**	< 0.001**	< 0.001**

Data are shown as mean ± SD. *P < 0.05 and **P < 0.01, compared with blank control subgroup using ANOVA. **P < 0.01, compared with control group using *t*-test.

pathogenic bacteria, and stabilizes the sludge. Terephthalic acid (TA) traps OH-. The concentration of TA was adjusted to 2 mM in sludge samples and the samples were then treated with U, F, or U+F. The amount of OH^- in the supernatant was measured after centrifugation (8000 rpm for 10 min) [17].

Statistical analysis

All data were processed using SPSS software v10.0. Results are shown as mean ± standard deviation (SD) and compared using One-Way ANOVA or *t*-test. P < 0.05 is considered statistically significant.

Results

AFB1 elevated significantly the expression of lymphocytic 8-oxoG in liver cancer group

The expression of 8-oxoG in lymphocytes of both groups was detected using flow cytometry (FCM). The percentages of 8-oxoG$^+$ lymphocytes in the liver cancer group and the control group, before treatment with AFB1, ranged from 21.5% to 86.9% and 17.1% to 42.7%, respectively. The fluorescence intensities of 8-oxoG (8-oxoGI) of the two groups ranged from 20.1 to 54.2 and 18.4 to 29.5, respectively. After treatment with AFB1 for 24 hours, both the proportion of 8-oxoG-positive cells and the fluorescence intensity of lymphocytes increased sharply in the liver cancer group, but slowly in the control group (**Figure 1**; **Tables 1** and **2**). Moreover, the proportion of 8-oxoG-positive cells and the fluorescence intensity of lymphocytes increased with increasing concentration of AFB1 in the liver cancer group (**Table 3**).

Table 3. The pearson correlation analysis among the concentration of AFB1 and variables

Variables	AFB1			
	Liver cancer		Control	
	R	*P* value	R	*P* value
The positive rates of 8-oxoG$^+$	0.655	< 0.001	0.205	0.204
The fluorescence intensity of 8-oxoG$^+$	0.368	0.001	0.121	0.456
T-SOD	-0.227	0.042	-0.185	0.254
OH^-	0.532	< 0.001	0.673	< 0.001

Table 4. The activity of T-SOD in the liver cancer group and the control group

Group	No. of cases	AFB1 concentration (μg/mL)			
		0	0.2	2	20
Liver cancer	20	5.54±2.34	5.14±2.19	4.81±2.20	4.09±1.65*
Control	10	8.74±2.48	8.15±2.43	7.75±2.60	7.21±2.61
P		0.002**	0.002**	0.003**	< 0.001**

Data are shown as mean ± SD. $^*P < 0.05$ and $^{**}P < 0.01$, compared with blank control subgroup using ANOVA. $^*P < 0.05$ and $^{**}P < 0.01$, compared with control group using *t*-test.

AFB1 reduces the total superoxide dismutase activity (T-SOD)

In order to investigate the effect of AFB1 on the antioxidant capacity of lymphocytes, the T-SOD activity in the supernatant of cultured lymphocytes was examined by xanthine oxidase method. T-SOD activity in the supernatant decreased with increasing concentration of AFB1 (**Table 3**). When the concentration of AFB1 reached 20 μg/mL, the activity of T-SOD in the supernatant was markedly lower in the liver cancer group, compared with that in the blank control subgroup ($P < 0.05$, **Table 4**), whereas it was only mildly reduced in the control group. There was no significant difference among the control subgroups (**Table 4**). However, comparison of the liver cancer group and the control group showed statistically significant difference at each dose ($P < 0.01$, **Table 4**).

OH^- content increased with increasing concentrations of AFB1

OH^- content in the supernatant, another parameter of the antioxidant capacity of lymphocytes, was determined by Fenton method. AFB1 increased the OH^- content in the liver cancer group, in a dose-dependent manner. There was significant difference between the OH^- content in the liver cancer group and that of blank group at each AFB1 dose tested ($P < 0.01$). However, compared with that in each subgroup of the control group, the content of OH^- was significantly higher in the corresponding dose subgroup of the liver cancer group, especially at 0.2 and 20 μg/mL doses ($P < 0.05$, **Tables 3** and **5**).

DNA damage in the lymphocytes correlated with OH^- content

Since we hypothesized that DNA damage in the lymphocytes was associated with the antioxidant capacity of lymphocytes, we analyzed the relationship between the 8-oxoG content in the lymphocytes and the OH^- and T-SOD contents in the supernatant of lymphocytes treated with 20 μg/mL AFB1 for 2 h. Partial correlation analysis showed that 8-oxoG content was positively correlated with the OH^- content ($r = 0.3812$, $P = 0.0388$) and negatively correlated with the T-SOD activity ($r = 0.5539$, $P = 0.001$).

Discussion

Hepatocellular carcinoma is one of the most common malignant cancers in China. Many factors contribute to the occurrence of HCC, including chronic HBV infection and prolonged exposure to aflatoxin; especially the latter [4]. Studies have shown that AFB1 binds readily to the N7 position on the guanosine residues of DNA chain, leading to many types of DNA damage, including the formation of DNA adduct and base damage, generation of no purine or no pyrimidine loci, breakage in single-stranded or double-stranded DNA, oxidative damage, increased frequency of sister chromosome exchange, and mismatch of DNA bases [14, 18, 19].

Hearse and others showed as early as in 1973 that excess production of oxygen free radicals is very harmful to the body [20]. Present day researchers believe that oxidation and antioxidation are in a dynamic balance *in vivo,* under normal conditions [21]. The presence of a certain amount of free radicals in the body is nec-

Table 5. The content of OH^- in the liver cancer group and the control group

Group	No. of cases	AFB1 concentration (μg/mL)			
		0	0.2	2	20
Liver cancer	20	412.38±168.14	892.38±212.66**	1029.84±304.76**	1369.69±164.26**
Control	10	229.30±150.23	652.65±332.64**	773.21±378.93**	1071.61±403.91**
P		0.072	0.048*	0.074	0.034*

Data are shown as mean ± SD. *$P < 0.05$ and **$P < 0.01$, compared with blank control subgroup using ANOVA. *$P < 0.05$, compared with control group using *t*-test.

essary for maintaining normal physiological functions, but excess free radicals are harmful to the system. The mechanism by which free radicals damage the various systems in the body is quite complex, which can be summarized as follows. Free radicals can damage the structure and function of cell membrane, leading to cell death [22], cell senescence, and apoptosis [23, 24]. Secondly, ischemia reperfusion injury in tissues induces gene mutation or canceration, playing a role in the development of inflammation [25]. Third, the excess free radicals can attack almost all the biological macromolecules, including DNA, protein, lipid and carbohydrate, producing a variety of different adverse effects [26].

Among the oxidative damages to DNA, 8-oxoG formation, which has the strongest mutagenic ability, occurs with the highest frequency and is closely associated with the occurrence and development of tumors [27]. 8-oxoG is the biomarker of the damage to nuclear and mitochondrial DNA caused by oxygen free radicals. Thus, 8-oxoG is often used to estimate the degree of oxidative damage in DNA. 8-oxoG is also one of the potential indices for clinical diagnosis of precancerous condition. Its content had a strong positive correlation to the incidence of cancer [28]. The presence and content of 8-oxoG in nuclear DNA in the cells can reliably reflect the oxidative damage to DNA caused by environmental chemicals, and is helpful in exploring the mechanism by which environmental chemicals cause tissue lesions and tumor formation. It may even enable the early detection of tumors in susceptible individuals. AFB1 has oxidative activity and its action on DNA produces 8-oxoG. Thus, 8-oxoG is an important indicator of DNA oxidative damage. Other studies also show that 8-oxoG is associated with the process of AFB1-induced gene mutations. In this study, we detected at molecular level, oxidative damage to lymphocyte DNA, using FCM, a method used by Peng [29]. The results showed that both 8-oxoGI and 8-oxoGP contents in liver cancer group were higher than those in the control group and the difference was statistically significant ($P < 0.05$), indicating a state of high oxidative stress in the lymphocytes of patients with HCC, which causes the accumulation of intracellular DNA oxidative damage products, including an excess of 8-oxoG. In this study, treatment with moderate and high levels of AFB1 caused significantly higher production of 8-oxoGI and 8-oxoGP in liver cancer group, while it caused no significant change in the control group, suggesting that lymphocytes in patients with HCC are more sensitive to AFB1. The production of 8-oxoGP and 8-oxoGI in lymphocytes in patients with HCC increased with increasing concentration of AFB1 and further aggravated DNA damage. In contrast, lymphocytes in healthy people could tolerate exposure to a low concentration of AFB1, demonstrating that the repair mechanism in patients with HCC is compromised, compared to that in healthy individuals or that there is a defect in the DNA repair machinery in patients with HCC.

Approximately, 1.0×10^{11} free oxygen radicals are produced by cells every day during the course of cellular metabolism. These free oxygen radicals have an extremely short half-life of about 10^{-3} second, because of the presence of a variety of enzymatic and non-enzymatic systems *in vivo,* which neutralize the free radicals and maintain physiological balance, protecting the body from damage. Therefore, the concentration of active free radicals in the body at any given time is very low. As one of the key enzymes of free radical neutralization, superoxide dismutase (SOD) occurs widely in various tissues and cells. The main function of SOD is the disproportionation of O_2 into H_2O_2. H_2O_2 is further decomposed by hydrogen peroxidase to generate H_2O.

This study established a lymphocyte oxidative damage model and studied the effect of treating the cells with different concentrations of AFB1. T-SOD activity of lymphocytes treated with different concentrations of AFB1 was measured to evaluate the effect of AFB1 on the antioxidant levels in lymphocytes. The results showed that AFB1 treatment reduced the T-SOD activity of lymphocytes in patients with HCC in a concentration-dependent manner ($P < 0.05$). Lymphocytes in liver cancer group showed significantly lower T-SOD activity, compared to that in the control group ($P < 0.01$), indicating lymphocytes in patients with HCC had weaker oxidative damage resistance than those in the healthy individuals, especially when they were influenced by other oxidative damage factors.

OH^-, the worst of the active free radicals in cells, may induce chemical modification of DNA bases. The OH^- radical reacts with DNA at C-4, C-5 and C-8 positions of purine residues leading to the formation of 8-oxoG [30]. The presence of 8-oxoG may cause mismatches during DNA replication and miscoding during transcription, resulting in genetic mutations. Treatment with AFB1 increased the content of free hydroxyl radical significantly, in a concentration-dependent manner ($P < 0.01$), indicating that the oxidative damage is mainly associated with AFB1 exposure.

In this study, we found that there was a strong positive correlation between the OH^- and 8-oxoG contents with a correlation coefficient of 0.3813, suggesting that excess production of OH^- may lead to DNA damage and exhaust SOD. This result also confirmed that the free radical with the most harmful activity in cells is OH^-. AFB1 exposure can result in the lymphocytes producing excessive OH^- leading to serious damage.

Conclusion

This study established an *in vitro* model for AFB-1-induced oxidative damage by using human peripheral blood lymphocytes. Our results showed that the peripheral blood lymphocytes in patients with HCC exhibited more severe oxidative damage and higher 8-oxoG levels than those in healthy people. This damage may have resulted from AFB1-induced peroxidation.

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Disclosure of conflict of interest

None.

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