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Dermatocarpon intestiniforme (A Lichen) Modulates Aflatoxin B_1 Induced Genetic and Oxidative Damage *In Vitro*

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ABSTRACT

Aflatoxin B_1 (AFB_1), a worldwide toxic contaminant of foods produced by *Aspergillus* species, exhibits oxidative stress mediated genotoxic damage although, the mechanism of cellular damage caused by AFB_1 has not been fully elucidated. Different antioxidant molecules such as ascorbic acid, beta-carotene and tocopherol have been shown to possess anti-carcinogenic and anti-mutagenic properties against AFB_1 toxicity. On the other hand, lichens have long been investigated popularly for biological roles; mainly anti-tumor, anti-microbial and anti-oxidant activities. Also, the influence of lichenic substances on DNA binding of AFB_1, in mammalian cells, is still unknown. Therefore, in this study, we aimed to determine whether *Dermatocarpon intestiniforme* extracts conferred a protection against AFB_1-induced genotoxic and oxidative damage *in vitro.* For this aim, we determined sister chromatid exchange (SCE) rates and main antioxidant enzyme activities including superoxide dismutase (SOD) and catalase (CAT) in AFB_1 (10 µM) and lichen (1, 5, 10, 25, 50 and 100 µM) treated human whole blood cultures (n=3) for 72h. The lichen extracts at tested concentrations did not exhibit any negative effects on above studied parameters in culture tubes. Moreover, the results of the present study indicated that the increases of SCE frequencies and the decreases of antioxidant enzyme activities by AFB_1 were minimized by the application of the lichen extracts (at 25 and 50 µM). Our results firstly suggest that *D. intestiniforme* augments the antioxidants defense against AFB_1 induced toxicity. Again, these results demonstrate that dose controlled *D. intestiniforme* lichen diet may play a protective role in the process of AFB_1 mutagenesis and/or carcinogenesis.

Key Words: Dermotocarpon intestiniforme, aflatoxin b_1, human erythrocytes, human lymphocytes, in vitro, oxidative stress

INTRODUCTION

Lichens are symbiotic organisms which are composed of fungi and algae (Nash, 1996). Lichens have been used for many fields. Especially, they are used for sources of natural drugs, and pharmaceutical industry and food supplement (Gulcin et al. 2002). Also, lichens was effective in treatment of bronchitis, tuberculosis, and hemorrhoids (Dülger et al. 1998). Chemical composition of lichens and their biological activities have long been investigated for anti-tumor, anti-viral, anti-herbivore and they are enzyme inhibitory until today (Huneck 1999). Moreover, pharmaceutical studies were also carried out about antioxidant and anti-inflammatory activities of aqueous extracts of lichens (Gulcin et al. 2002; Halıcı et al. 2005; Odabasoglu et al. 2006)

Among various aflatoxins, AFB_1 the most potent carcinogen ever tested (Umarani et al. 2008). The ingestion on AFB_1-contaminated feed is known to cause teratogenicity. Also, it causes immunotoxicity and even death in humans and animals (Guindon et al. 2007). But, the mechanism of cellular damage caused by Aflatoxin B_1 has not fully elucidated (Rastogi et al. 2001). Besides, to main mechanism in the toxicity Aflatoxin B_1, reactive oxygen species (ROS) and lipid peroxidation (LPO) have been considered (Shon et al. 2004). Therefore, Aflatoxin B_1 causes sister chromatid exchanges (SCEs) and chromosomal strand breaks as well as forms adducts in human cell and rodent. (Groopman and Kensler 1999).

The antioxidants have attracted with respect to their protective effect against free radical damage. So, that might be cause for many diseases including cancer (Shon et al. 2004). So as to the complete avoidance of exposure to AFB_1-producing mould is very difficult, chemoprevention is common and attractive strategy for protecting humans and animals from the risk of cancer caused by exposure to this mycotoxin. Yet, the anti-mutagenic activity of lichen extracts is a fairly new subject with few studies in the literature (Geyikoglu et al. 2007; Zeytinoglu et al. 2004, 2008). For this reason, we investigated the effects of *D. intestiniforme* extracts against AFB_1-induced DNA damages for improving the therapeutic gain of the lichens. Moreover, SCE test is providing sensitive and rapid monitoring of induced genetic damage was performed on peripheral lymphocytes.

In light of the foregoing, this study was designed to describe for the first time dose depended oxidative and genetic effects of the aqueous lichen extracts in human blood. Therefore, we determined SCE rates and main antioxidant enzyme activities including superoxide dismutase (SOD) and catalase (CAT) in Aflatoxin B_1 (10 µM) and lichen extracts (1, 5, 10, 25, 50 and 100 µM) treated blood cultures.

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MATERIAL AND METHODS

Experimental design

D. intestiniforme samples were collected from the vicinity of Inci village, Oltu district, Erzurum, Turkey. Besides, these samples identified using various flora books and papers (Poelt 1974; Purvis et al. 1992; Aslan 2000; Dobson 2000; Brodo et al. 2001; Aslan et al. 2002). Identified samples were air dried and kept in the herbarium Kazım Karabekir Education Faculty, until they were used. 20 gr lichen sample was mixed with 400 ml distilled and boiling water using magnetic stirrer for 15 min for water extraction of lichens. And then, we were filtered the extract over Whatman No. 1 paper. Also, we used different concentrations (1, 5, 10, 25, 50 and 100 µM). We used whole heparinized blood from three healthy male non- smoking donors between 24 and 30 with no history of exposure to any toxic agent in our experiments. AFB1 ($C_{17}H_{12}O_6$, CAS No. 1162-65-8, 98% purity, Sigma Chemical Co., St Louis, MO. USA; in concentration of 10 µM) was dissolved in distilled water. Experiments were conform to the guidelines of the World Medical Assembly (Declaration of Helsinki). After supplementation of extracts and AFB1, the blood was incubated for 1 h at 37 ºC to adjust body conditions, except for testing SCE (see below). Each individual whole blood culture without extract or AFB1 was studied as a control group.

Enzymatic activities

SOD activity was determined by the method of Misra and Fridovich (1972). This method is based on the superoxide dismutase to inhibit the process of epinephrine self-oxidation in alkaline medium. When in the reaction of coloured adrenochrome formation, the superoxide radical anion is formed as an intermediate product. Moreover, SOD activity was measured by the increase in the absorbance at 480 nm. CAT was determined by the method of Aebi (1984). To 3ml H_2O_2 (54 Mm H_2O_2 in 50Mm phosphate buffer, pH 7.0), 5 µM of catalase solution were added. Also, the decrease in H_2O_2 was measured spectrophotometrically (Beckman DU 500, USA) at 25°C. Besides, in the erythrocyte preparations, haemolysates were centrifuged (900 X g). And estimation of activity was made with %1 haemolysates. And then, one unit of catalase activity was defined as the activity required degrading 1 µmol of H_2O_2 in 60s.

Cytogenetic analysis

SCE Assay

With the providing a beter visualition of SCEs, 5-bromo-2'-deoxyuridene (Sigma, final concentration 20 µM) was added after culture initation. And the cultures were incubated in complete darkness for 72 h at 37^0 C. After 70 h and 30 min we added colcemid to the cultures to achieve a final concentration of 0.5 µg/L. Later, hypotonic treatment (0.075 M KCl) followed by three repetitive methanol/acetic acid solution (3:1,v/v), centrifugation and resuspension. The cell suspension was dropped onto chilled and Grease-free microscopic slides, air dried, aged, and then differentially stained for inspection of the SCE rate according to the fluorescence plus Giemsa (FPG) procedure (Perry and Wolff 1974). For each treatment condition, 25 well-spread second division metaphases were scored. The values obtained were calculated as SCEs per cell.

Statistics

We used S.P.S.S software (version 12.0) for the statistical analyses. SCE test was performed by Student's *t*-test. And statically decision was made with a significance level of 0.05.

RESULTS

All the lichen extracts at tested concentrations did not induce significant ($p>0.05$) number of SCEs (Table). Furthermore, the lichen extracts caused significant increases of the antioxidant enzyme activities (SOD and CAT) in erythrocytes at the concentrations of 25, 50 and 100 µM. However, lichen extracts were not found to be effective at 1, 5 and 10 µM. It was noteworthy that the cultures treated with extracts against AFB_1 restored these enzyme levels (especially at 25, 50 and 100 µM) to nearly that of control values indicating the protective role of *D. intestiniforme*. Likewise, the co-application of AFB1 and extracts (at 25, 50 and 100 µM) caused the significant decreases in SCEs/cell values compared to AFB_1 treated alone.

Table 1. SOD and CAT enzyme activities in human erythrocytes and SCE rates in human lymphocytes exposed to AFB_1 and *D. intestiniforme* extracts

Treatments	SOD (U/mL)	CAT (U/g Hb)	SCE/cell
Control	97.6 ± 7.1	276.8 ± 18.2	6.4 ± 0.3
AFB_1 (10 µM)	83.1 ± 6.4*	241.2 ± 17.3*	14.2 ± 0.9*
Di (1 µM)	97.4 ± 7.7	279.6 ± 15.4	6.5 ± 0.4
Di (5 µM)	97.7 ± 7.1	279.7 ± 19.3	6.5 ± 0.5
Di (10 µM)	98.1 ± 5.3	278.4 ± 22.3	6.5 ± 0.4
Di (25 µM)	105.4 ± 10.4*	281.7 ± 19.1	6.7 ± 0.6
Di (50 µM)	107.6 ± 8.6*	298.6 ± 26.7*	6.5 ± 0.3
Di (100 µM)	106.8 ± 7.2*	296.5 ± 22.7*	6.7 ± 0.7
AFB_1 + (1 µM)	84.5 ± 7.9*	243.7 ± 26.8*	13.8 ± 1.4*
AFB_1 + (5 µM)	85.1 ± 8.7*	244.6 ± 24.6*	12.9 ± 1.0*
AFB_1 + (10 µM)	88.9 ± 7.6*	252.8 ± 17.9*	9.8 ± 0.8*
AFB_1 + (25 µM)	94.2 ± 8.5	267.5 ± 23.4	7.3 ± 0.9
AFB_1 + (50 µM)	95.9 ± 7.7	273.8 ± 21.6	7.0 ± 1.2
AFB_1 + (100 µM)	96.6 ± 8.3	275.7 ± 20.5	6.8 ± 0.9

AFB_1= Aflatoxin B1 (10 µM); Di= *Aqueous extract of Dermatocarpon intestiniforme*,
* symbol represents statistically significant differences from control group ($P < 0.05$). Values are means±standard deviation.

DISCUSSION

Oxidative stress develops when the levels of antioxidants are lowered. In current study, the toxic effects of AFB_1 involved decreased antioxidant capacity. Also, it increased genetic damage. Decline in the levels of antioxidant capacity after AFB_1 treatment might be due to the inactivation of antioxidant enzymes such as SOD and CAT form the first line of defense against ROS. Moreover, a decrease in their activities is observed with AFB_1 administration (Rastogi et al. 2001; Verma and Nair 2001). Besides, Souza et al. (1999) reported that the oxidative stress is principle manifestation of AFB_1-induced toxicity. And, it could be mitigated by antioxidants. Especially, the enzyme inactivation induced by AFB_1 also corroborate by genotoxic findings. Our results obtained by us indicate a significant increase in the ratios of the SCEs in lymphocytes. And, AFB_1 toxicity is also related LPO and oxidation of DNA *in vivo* and *in vitro* (Shen et al. 1996). Thus, this xenobiotic could contribute to the formation of the genome leading to carcinogenesis (Amici et al. 2007). And then, lipid peroxides enter the nucleus. They react with Fe^2 to generate the alkoxyl radical that attacks DNA (Frega and Tappel 1988). Moreover, intraceluler calcium levels increase as a result of oxidative damage to cell membranes. Later, calcium enters the nucleus where it can activate nucleases that cause DNA strand breaks (McConkey et al. 1989). DNA damage and defective DNA repairs cause SCEs (Bozkurt et al. 2003)

The results of the current study revealed that treatment with *D. intestiniforme* extracts provide anti-genotoxic effects by AFB_1 at different degree. The lichen presents positive effects with increasing concentrations without leading to any genetic damage on human blood cells. *D. intestiniforme* extracts alone were non genotoxic. At this study, *D. intestiniforme* could support the antioxidant defense mechanism against AFB_1. We evidence that *D. intestiniforme* has significant antioxidant activity. Antioxidant enzymes play a main important role in difference of mammalian blood. Also, these enzymes can be induced or inhibited in the blood cells exposed to different toxicants (Afaq et al. 1998; Prasad et al. 2006). In this study, there is considerable evidence that *D. intestiniforme* influence the antioxidant enzyme activities as related to the dose not leading to any genetic damage in human blood.

It was pointed out that lichen extracts can change the oxidative metabolism in animal systems. The current study clearly proved that low doses of various lichens extracts increased antioxidant capacity (25, 50,

100 µM). When the levels of antioxidants are lowered, oxidative stress develops. So the activities of antioxidant enzymes (SOD, CAT) have importance in the cell defence (Tapiero et al. 2004). And, SOD has a central role against oxidative damage. This enzyme catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen (Kakarla et al. 2005). In our study, SOD activity decreased after the application with AFB_1 alone. And, the extracts (at 25, 50, 100 µM) ameliorated the decreases of SOD activity by AFB_1. These lichen extracts also induced CAT activity. The activity of this enzyme protects proteins and lipids in erythrocyte membranes against peroxide radicals (Hunt and Idso 1999).

The results of this study may be, at least in part, attributed to antioxidant activity of the lichen extracts, as AFB_1 is known to induce mutagenic damage through oxidative stress. The findings this investigation clearly indicated that *D. intestiniforme* modulated AFB_1 toxicity. So, the tested lichen extracts reduced genetic damage in human blood cultures due to its antioxidant and detoxifying nature. Therefore, this lichen can be a new resource of therapeutics as recognized in this study against genetic and oxidative damages.

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