Cytotechnology (2010) 62:157–165
DOI 10.1007/s10616-010-9272-2

ORIGINAL RESEARCH

Boric acid: a potential chemoprotective agent against aflatoxin b_1 toxicity in human blood

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Received: 11 December 2009 / Accepted: 7 April 2010 / Published online: 30 April 2010

Abstract Aflatoxin B_1 is the most potent pulmonary and hepatic carcinogen. Since the eradication of Aflatoxin B_1 contamination in agricultural products has been difficult, the use of natural or synthetic free radical scavengers could be a potential chemopreventive strategy. Boric acid is the major component of industry and its antioxidant role has recently been reported. The present study assessed, for the first time, the effectiveness of boric acid following exposure to Aflatoxin B_1 on human whole blood cultures. The biochemical characterizations of glutathione and some enzymes have been carried out in erythrocytes. Alterations in malondialdehyde level were determined as an index of oxidative stress. The sister-chromatid exchange and micronucleus tests were performed to assess DNA damages in lymphocytes. Aflatoxin B_1 treatment significantly reduced the activities of antioxidants by increasing malondialdehyde level (30.53 and 51.43%) of blood, whereas, the boric acid led to an increased resistance of DNA to oxidative damage induced by Aflatoxin B_1 in comparison with control values ($P < 0.05$). In conclusion, the support of boric acid was especially useful in Aflatoxin-toxicated blood. Thus the risk on tissue targeting of Aflatoxin B_1 could be reduced ensuring early recovery from its toxicity.

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Keywords Aflatoxin B_1 · Boric acid · Genotoxicity · Antioxidant enzymes · Human blood cultures

Introduction

Boron is an ubiquitous constituent of man's external environment and it is essential for the growth of many plants. Boron deficiency and supplementation exert measurable biological effects in human and animal tissues (in osteoporosis, arthritis, plasma lipid profiles and brain function) (Devirian and Volpe 2003). Boron mineral is released in the form of boric acid (BA) to environment. BA is a component of pharmaceuticals and is also used in numerous industrial processes (NTP 1987; Avino-Martinez et al. 2008). Additionally, it is used as a pesticide and food preservative against plant fungicides (Turkoglu 2007). BA having antimicrobial activity (De Seta et al. 2009), is able to strengthen the tissue antioxidant defenses via a yet unidentified mechanism that may involve changes in oxidative metabolism (Hunt and Idso 1999; Pawa and Ali 2006). BA also shows minimal potential for genotoxicity in bacteria and cultured mammalian cells (Moore and Expert Scientific Comittee 1997).

Aflatoxins are contaminants of improperly stored foods. They not only contaminate our food stuffs but

are also found in edible tissues, milk and eggs after consumption of contaminated feed by farm animals (Bennett and Klich 2003). Among various aflatoxins, Aflatoxin B_1 (AFB_1) is the most potent carcinogen ever tested (Umarani et al. 2008). AFB_1 is produced by some strains of Aspergillus and ingestion of aflatoxin-contaminated food and feed is known to cause hepatotoxicity, teratogenicity, immunotoxicity and even death in farm animals and humans (Guindon et al. 2007). The mechanism of cellular damage caused by AFB_1 has not been fully elucidated (Rastogi et al. 2001). Reactive oxygen species (ROS) and lipid peroxidation (LPO) have been considered to be main mechanisms in the toxicity of AFB_1 (Shon et al. 2004). Finally, AFB_1 causes micronucleus (MN), sister chromatid exchanges (SCEs), unscheduled DNA synthesis, and chromosomal strand breaks as well as forms adducts in rodent and human cells (Groopman and Kensler 1999). In 2005, Geyikoglu and Turkez reported similar findings in human lymphocytes in an in vitro model for the first time. They have suggested that blood is a valuable tissue for monitoring the genotoxicity of AFB_1. Virtually, blood offers several advantages as a test system (Lerda et al. 2005). The first is easy availability of large numbers of human blood cells. The second is that lymphocytes can be stimulated by mitogens to undergo mitosis in culture. The third is that the blood cells are potential vulnerable cells. Because, the distinct external entrances with adverse effects of environmental agents are from blood into the other tissues. So blood tissue is specially important for evaluating the risk on target tissues (Laakso et al. 2001). On the other hand, the knowledge of the vascular changes involved in boron compounds presents significant contributions to the effective pharmacokinetic researches (Erica et al. 2001). At this context, genetic and oxidative studies in blood tissue are pivotal. Such studies will serve to evaluate AFB_1 toxicity and improve the therapeutic gain of BA.

So far, antioxidants have attracted much interest with respect to their protective effect against damage by free radical that may be the cause for many diseases including cancer (Shon et al. 2004). Since the complete avoidance of exposure to AFB_1-producing mould is very difficult, chemoprevention is an attractive strategy for protecting humans and animals from the risk of cancer caused by exposure to this mycotoxin. Oxidative stress can lead to some cancers, artherosclerosis, and adverse effects of aging (Ma et al. 2008). However, the compounds with antioxidant properties contribute to protection of cells and tissues (Ramos et al. 2008). Thus, this study investigated the efficacy of BA against AFB_1-induced DNA damages. Firstly, some oxidative parameters including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione (GSH) and malondialdehyde (MDA) which are recently used to monitor the development and extent of damage due to oxidative stress in human blood were investigated. In addition, The sister-chromatid exchange (SCE) and micronucleus (MN) tests covering a wide range of induced genetic damage as primary DNA damage were performed on peripheral lymphocytes.

Materials and methods

Experimental design

Blood samples were obtained by veinpuncture from five healthy non-smoking donors. AFB_1 ($C_{17}H_{12}O_6$, CAS No. 1162-65-8, 98% purity, Sigma Chemical Co., St Louis, MO. USA; in concentrations of 1.56 and 3.12 ppm) and BA (H_3BO_3, CAS No. 10043-35-3, 99% purity, Sigma, St Louis; in concentrations of 0.25, 0.5, 1, 2, 5, 10 and 20 ppm) were dissolved in distilled water. These compounds were added to the cultures just before incubation for biochemical and cytogenetic analysis. Experiments were conform to the guidelines of the World Medical Assembly (Declaration of Helsinki). The concentrations were selected according to the previous studies (Yamashoji and Isshiki 2001; Geyikoglu and Turkez 2005; Turkez et al. 2007). After supplementation of BA and AFB_1, the blood was incubated for 1 h at 37 °C to adjust body conditions, except for testing SCE and MN (see below). Each individual whole blood culture without BA or AFB_1 was studied as a control group.

Erythrocytes

Erythrocytes were obtained from heparinized blood samples by centrifugation (2500*g*, for 20 min) at 4 °C. The red cells were then washed three times with 5 volumes of phosphate buffered saline (PBS; 150 mmol L^{-1}·NaCl, 1.9 mmol L^{-1}·NaH_2PO_4, 8.1 mmol L^{-1}·Na_2HPO_4, pH 7.4), and with a ratio of 1:1 divided into

appropriate aliquots and stored at −80 °C until further analysis.

Biochemical methods

SOD activity

SOD activity was determined by the method of Misra and Fridovich (1972), which is based on the ability of SOD to inhibit the process of epinephrine self-oxidation in alkaline medium. In the reaction of colored adrenochrome formation, the superoxide anion-radical is formed as an intermediate product. Erythrocyte SOD activity was measured by monitoring the increase in the absorbance at 480 nm.

CAT activity

CAT was determined in erythrocytes by the method of Aebi (1984). To 3 ml·H_2O_2 (54 mM·H_2O_2 in 50 mM phosphate buffer, pH 7.0), 5 µL of a catalase solution was added and the decrease in H_2O_2 was measured spectrophotometrically (Beckman DU 500, USA) at 240 nm for 60 s at 25 °C. In the erythrocyte preparations, haemolysates were centrifuged (9000 g) and estimation of activity was made with 1% haemolysates. One unit of catalase activity was defined as the activity required for degrade 1 µmol hydrogen peroxide in 60 s.

GSH-Px activity

GSH-Px activity of erythrocytes was measured using hydrogen peroxide as substrate (Carlberg and Mannervik 1972). Potassium azide was added to inhibit catalase. Potassium ferricyanide was added to inhibit the pseudo-peroxidase activity of hemoglobin. Conversion of NADPH was monitored continuously in a spectrophotometer at 340 nm for 3 min at 25 °C.

GSH level

The level of GSH in erythrocytes was determined by the method of Elman (1959). Erythrocyte suspension was deproteinated by addition of trichloroacetic acid (TCA). DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] was added to supernatants cleared by centrifugation. The formation of 5-thio-2-nitrobenzoic acid, which is proportional to total glutathione concentration, was monitored at 412 nm at 25 °C against reagent blank.

MDA level

The content of MDA was measured in plasma preparations by the thiobarbutiric acid (TBA) method which was modified from methods of Satoh (1978) and Yagi (1984). Peroxidation was determined as the production of MDA which, in combination with TBA, forms a pink chromogen compound whose absorbance at 532 nm was measured.

Cytogenetic methods

SCE assay

Cultures were set up according to a slight modification of the protocol described by Evans and O'Riordan (1975). A 0.5 mL aliquot of heparinized blood was cultured in 6 mL of culture medium (Chromosome Medium B, Biochrom, Berlin) with 5 µg/mL of phytohemagglutinin (Biochrom). Above-mentioned doses of test compounds were added into the culture tubes. With the aim of providing successive visualization of SCEs, 5-bromo-2′-deoxyuridine (Sigma) was added at culture initation. The cultures were incubated in complete darkness for 72 h at 37 °C. Exactly 70 h and 30 min after beginning of the incubations, demecolcine (N-Diacetyl-N-methylcolchicine, Sigma) was added to the cultures. After hypotonic treatment (0.075 M KCl), followed by three repetitive cycles of fixation in methanol/acetic acid solution (3:1, v/v), centrifugation, and resuspension, the cell suspension was dropped onto chilled, grease-free microscopic slides, air-dried, aged for 3 days, and then differentially stained for the inspection of the SCE rate according to the fluorescence plus Giemsa (FPG) procedure as reported by Perry and Wolff (1974). For each treatment condition, well-spread twenty-five second division metaphases containing 42–46 chromosomes per cell were scored, and the values obtained were calculated as SCEs per cell.

MN assay

The MN test was performed by adding cytochalasin B (Sigma) after 44 h of culture. At the end of the 72-h incubation period, the lymphocytes were fixed with

ice-cold methanol/acetic acid (1:1, v/v). The fixed cells were put directly on slides using a cytospin, and stained with Giemsa solution. All slides were coded before scoring. The criteria for scoring MN were as described by Fenech (1993). At least 1,000 binucleated lymphocytes were examined per concentration for the presence of one, two or more MN.

Statistical analysis

Statistical analysis was performed using SPSS Software (version 12.0, SPSS, Chicago, IL, USA). The two-tailed Student's *t*-test was used to compare SCE frequencies between treated and control groups. The statistical analysis of MN frequencies was also performed by use of the χ^2 test. For statistical analysis of biochemical data Fisher's Least Significant Difference (LSD) test was used. Statistical decisions were made with a significance level of 0.05.

Results

Table 1 reveals the abnormal activities of antioxidant enzymes in human blood treated with AFB_1. The activities of erythrocyte SOD, CAT and GSH-Px were decreased by 37.55, 21.11 and 21.70%, respectively in low dose AFB_1 when compared to controls. Activities of these marker enzymes were significantly ($P < 0.05$) decreased in erythrocytes of blood cultures treated with 3.12 ppm AFB_1, whereas, all BA doses (except for 0.25 ppm) caused increases of all enzyme activities. It was noteworthy that the cultures treated with BA against AFB_1 restored these enzyme levels (especially at 0.5, 1, 2 and 5 ppm) to nearly that of control values indicating the protective role of BA.

Table 2 shows the effects of BA on AFB_1-induced MDA and antioxidant substrate. BA alone was able to increase significantly the amount of GSH without affecting the MDA level of blood. Moreover, 30.53 and 51.43% rise in MDA seen in the low and high doses of AFB_1, respectively, were maintained at normal levels in the presence of BA. The significant decreases ($P < 0.05$) in the levels of GSH were also seen for both AFB_1 doses. On the contrary, BA treatment restored the levels of this nonenzymic antioxidant to control level, thereby indicating that BA protects blood against oxidative stress-induced depletion of antioxidant. However, the lowest BA concentration (0.25 ppm) was not found to be effective.

Figure 1 shows the results of SCE and MN analyzed in human peripheral lymphocytes treated for 72 h with different concentrations of AFB_1 in the presence or absence of BA. Frequencies of SCE and MN observed in cultures treated with AFB_1 (1.56 and 3.12 ppm) were significantly higher than control values. However, BA did not lead to genetic damages for all tested concentrations. Moreover, it was efficient against AFB_1-induced genotoxicity in blood cells. The co-application of 1.56 ppm AFB_1 and BA caused the significant decreases in SCEs/cell (at 1, 2 and 5 ppm) and MN/1000 cell (except for 20 ppm) values compared to AFB_1 treated alone. On the other hand, the negative effects of 3.12 ppm AFB_1 were moderated with all tested BA concentrations (except for 0.25 ppm) but the values were still different from the control levels.

Discussion

Oxidative stress develops when the levels of antioxidants are lowered. In the present study, the toxic effects of increasing doses of AFB_1 involved decreased antioxidant enzyme levels and increased MDA content. Decline in the activities of antioxidant enzymes after AFB_1 administration might be due to the inactivation of these enzymes. Antioxidant enzymes like SOD, CAT and GSH-Px form the first line of defense against ROS and a decrease in their activities is observed after AFB_1 administration (Rastogi et al. 2001; Verma and Nair 2001). The antioxidant enzymes can be induced or inhibited in the blood cells exposed to different toxicants and these enzymes play a main role in the defence of mammalian blood (Prasad et al. 2006). Souza et al. (1999) reported that the oxidative stress is the principle manifestation of AFB_1-induced toxicity which could be mitigated by antioxidants. Our previous study (Turkez et al. 2007) and the current study well establish that BA alone does not lead to the induction of oxidative stress. On the contrary, it modifies the oxidative metabolism as a result of the supporting antioxidant capacity. BA is able to increase significantly the activity of antioxidant enzymes without affecting the MDA level of blood. The increase in the MDA level is an important sign of

Table 1 Activities of SOD, CAT and GSHPx in human erythrocytes incubated with different concentrations of AFB_1 and BA

Groups	SOD activity	CAT activity	GSH-Px activity
Control	90.16 ± 10.25	270.33 ± 29.32	9127.36 ± 679.58
AFB_1 (L)	51.30 ± 7.65*	203.26 ± 23.64*	7102.28 ± 635.42*
AFB_1 (H)	39.73 ± 6.18*	181.71 ± 21.36*	6284.41 ± 585.95*
BA1	91.12 ± 11.45	266.22 ± 24.33	9080.25 ± 663.14
BA2	115.32 ± 19.43**	268.17 ± 26.92	9089.18 ± 438.35
BA3	109.40 ± 13.71**	289.19 ± 30.17**	9171.65 ± 587.40
BA4	108.11 ± 12.96**	291.75 ± 27.83**	9537.11 ± 757.18**
BA5	111.61 ± 14.71**	282.62 ± 24.42**	9976.75 ± 683.34**
BA6	118.19 ± 13.66**	291.48 ± 27.74**	10192.24 ± 711.27**
BA7	102.52 ± 10.27	277.71 ± 22.90	9134.63 ± 626.68
AFB_1 (L) + BA1	54.41 ± 8.23*	212.27 ± 24.14*	7211.27 ± 557.63*
AFB_1 (L) + BA2	96.43 ± 8.69	226.65 ± 23.13*	8977.52 ± 589.17
AFB_1 (L) + BA3	89.91 ± 7.13	247.82 ± 26.67*	9218.20 ± 587.44
AFB_1 (L) + BA4	87.41 ± 9.05	268.41 ± 21.91	9074.44 ± 603.76
AFB_1 (L) + BA5	86.36 ± 8.84	266.12 ± 25.82	8796.30 ± 563.22*
AFB_1 (L) + BA6	83.30 ± 8.77*	246.18 ± 26.68*	8615.53 ± 497.16*
AFB_1 (L) + BA7	72.90 ± 8.65*	244.33 ± 21.67*	8207.75 ± 514.29*
AFB_1 (H) + BA1	40.24 ± 6.22*	184.86 ± 17.76*	6332.34 ± 421.72*
AFB_1 (H) + BA2	75.63 ± 9.44*	221.67 ± 19.41*	7147.23 ± 427.63*
AFB_1 (H) + BA3	83.68 ± 7.14*	246.75 ± 22.81*	7996.65 ± 493.45*
AFB_1 (H) + BA4	74.18 ± 8.47*	257.55 ± 26.25*	8047.93 ± 622.41*
AFB_1 (H) + BA5	83.79 ± 9.96*	255.62 ± 23.74*	8068.22 ± 587.19*
AFB_1 (H) + BA6	70.38 ± 7.14*	251.18 ± 21.98*	7338.39 ± 496.23*
AFB_1 (H) + BA7	67.13 ± 6.92*	250.63 ± 24.33*	7142.61 ± 505.11*

Units: SOD: U/mL; CAT: U/g Hb; GSH-Px: U/L. Values are expressed as mean ± SD for five cultures in each group. * Statistically significant decreases, ** Statistically significant increases at $P < 0.05$ level from the control group. AFB_1 (L): 1.56 ppm, AFB_1 (H): 3.12 ppm, BA1: 0.25 ppm, BA2: 0.5 ppm, BA3: 1 ppm, BA4: 2 ppm, BA5: 5 ppm, BA6: 10 ppm, BA7: 20 ppm

oxidative stress (Kim et al. 2006). The MDA levels are determined as an index of LPO and LPO is one of the main manifestations of the toxicity and carcinogenesis of many carcinogens (Grammatikos et al. 1994; Rastogi et al. 2001; Siu et al. 2008). The marked increases in MDA content after AFB_1-exposure could contribute to the effect of AFB_1 on antioxidant defense system. GSH is a tripeptide containing cysteine and AFB_1 easily conjugates with GSH (Janssen et al. 1993). GSH is also a major component of red blood cells that plays a central role in the antioxidant defenses of cells (Meister 1983; Ray 1984). Whereas, the significant decrease in the levels of GSH observed in erythrocytes after AFB_1 exposure causes decreased degradation of lipid peroxides leading to their accumulation thus amplifying the toxicity of AFB_1. GSH has a critical role in the protection of tissues from the deleterious effects of activated AFB_1 and consequently the consumption of GSH causes significant decreases in the activity of GSH-Px (Larsson et al. 1994). The reduction in the activity of GSH-Px on AFB_1 administration may be due to decrease in the availability of the substrate (GSH) and also due to ROS induced alterations in its protein structure (Janssen et al. 1993). It is predicted that the induction of GSH-Px following exposure to AFB_1 might contribute to reduction in covalent binding of AFB_1 to macromolecules as reported in liver (Loury and Hsieh 1984). Likewise, supplementation of BA in blood tissue increases the GSH status resulting in the increase in GSH-Px activity thereby preventing the deleterious effects of peroxidation products. In our

Table 2 The GSH and MDA levels in human blood cultures treated with of AFB_1 and BA

Groups	GSH level	MDA level
Control	51.23 ± 4.83	358.30 ± 29.96
AFB_1 (L)	40.46 ± 4.27*	447.70 ± 39.41**
AFB_1 (H)	31.25 ± 4.10*	522.60 ± 44.83**
BA1	51.60 ± 4.55	353.30 ± 26.80
BA2	51.47 ± 4.69	337.10 ± 27.75
BA3	57.62 ± 5.03**	339.40 ± 26.61
BA4	53.20 ± 4.92	321.10 ± 21.90
BA5	58.71 ± 5.39**	335.20 ± 23.35
BA6	56.91 ± 5.13**	346.60 ± 30.72
BA7	52.12 ± 4.97	338.30 ± 26.97
AFB_1 (L) + BA1	42.28 ± 4.17*	433.60 ± 38.93*
AFB_1 (L) + BA2	46.39 ± 3.98*	367.70 ± 34.57
AFB_1 (L) + BA3	49.61 ± 4.46	354.40 ± 29.13
AFB_1 (L) + BA4	50.49 ± 5.65	369.80 ± 37.41
AFB_1 (L) + BA5	49.91 ± 4.51	364.10 ± 34.22
AFB_1 (L) + BA6	47.02 ± 4.88*	401.20 ± 36.25*
AFB_1 (L) + BA7	45.41 ± 4.32*	394.60 ± 37.74*
AFB_1 (H) + BA1	32.44 ± 3.87*	496.80 ± 41.62*
AFB_1 (H) + BA2	43.20 ± 3.72*	363.20 ± 31.10
AFB_1 (H) + BA3	44.65 ± 3.95*	358.70 ± 32.82
AFB_1 (H) + BA4	43.45 ± 4.29*	356.60 ± 27.96
AFB_1 (H) + BA5	43.62 ± 4.23*	361.70 ± 28.33
AFB_1 (H) + BA6	39.40 ± 3.78*	419.80 ± 39.45*
AFB_1 (H) + BA7	37.76 ± 3.69*	427.80 ± 41.43*

Units: MDA: μM/L; GSH: M/L. Abbreviations and symbols are as in Table 1

study, BA also showed significant antioxidant actions at concentrations higher than 0.25 ppm against AFB_1 toxicity. BA's protective role derives from its stimulatory effect on SOD, CAT, GSH-Px and GSH and its inhibitory action on MDA. SOD catalyses the dismutation of superoxide to hydrogen peroxide and oxygen which are deleterious to polyunsaturated fatty acids and structural proteins of plasma membrane (Johnson and Giulivi 2005). Endogenous H_2O_2 is converted to H_2O by catalase (Svistunenko 2005). The CAT activity protects lipids and proteins against peroxide radicals in erythrocyte membranes (Hunt and Idso 1999). The rising of activities of antioxidant enzymes after BA administration indicates that this action of BA may be mediated via a nuclear BA receptor with a subsequent action on gene expression. Studies on antioxidants in animal cells have revealed that many known anti-oxidants act through the stimulation of transcription factors that regulate the expression of genes involved in ROS scavenging such as SOD, CAT and GSH-Px (Harschman et al. 1988).

The enzyme inactivations induced by AFB_1 also corroborate by the genotoxic findings. The results obtained by us indicate a significant increase in the ratios of the SCEs and MNs in lymphocytes, which is in accordance with previous reports (Groopman and Kensler 1999; Geyikoglu and Turkez 2005). The SCEs are formed by toxic oxygen metabolites in cultured human leukocytes and other mammalian cells (Weitberg et al. 1983). AFB_1 toxicity is also related to LPO and oxidation of DNA in vivo and in vitro (Shen et al. 1996). Thus, the genome formation leading to carcinogenesis could be occured by this xenobiotic (Amici et al. 2007). Lipid peroxides enter the nucleus where they react with Fe^{+2} to generate the alkoxyl radical which attacks DNA (Fraga and Tappel 1988). Also, intracellular calcium levels increase as a result of oxidative damage to cell membranes, calcium then enters the nucleus where it can activate nucleases which cause DNA strand breaks (McConkey et al. 1989). DNA damages and defective DNA repairs cause SCEs (Bozkurt et al. 2003). On the other hand, MNs are the results of acentric fragments or lagging chromosomes that fail to incorporate into either of the daughter nuclei during telophase of the mitotic cells (Albertini et al. 2000). It was established that BA alone was non-genotoxic. Moreover, the treatment with BA significantly decreased the ratios of the SCEs and MNs compared with those of the AFB_1 treated groups. The biologically fundamental macromolecules in mammalian cells such as nucleic acids and proteins are protected by antioxidants (Kedziora-Kornatowska et al. 2004). Thus, our results reveal that BA exhibits antigenotoxic and antioxidant properties at concentrations ranging from 0.25 to 20 ppm. We suggest that it may be a chemopharmaceutical molecule of interest against AFB_1. BA is beneficial in cases of aflatoxicosis to inhibit blood cell damage by correcting the disturbance of oxidant/antioxidant balance system and ensures early recovery from AFB_1 toxicity and thereby decreases incidences of DNA damages. It also appears that biochemical and genotoxic tests dealing with BA reveal safe results for a food preservative. In conclusion, the present study is the first report describing BA's ability to

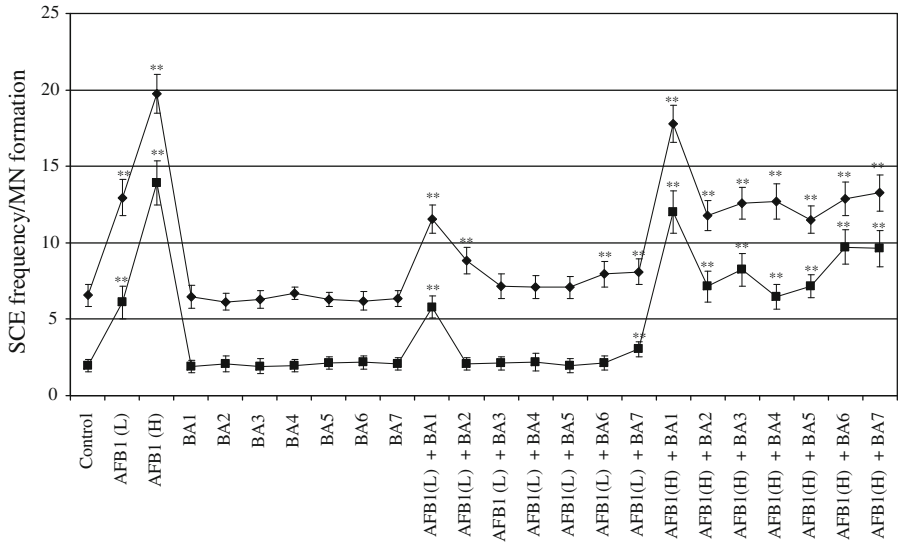

Fig. 1 The frequencies of SCEs and MNs (‰) in human blood cultures treated with different concentrations of AFB_1 and BA. Values are expressed as mean ± SD for five cultures in each group. ** Statistically significant increases at $P < 0.05$ level from the control group. (*filled diamond*) symbol represents mean SCE/cell and (*filled square*) symbol represents mean MN/1000 cell. Abbreviations are as in Table 1

protect nuclear DNA from oxidative damage against AFB_1 and it is speculated that BA protective effects are a consequence of its ability to reduce MDA formation and increase associated antioxidants.

Acknowledgments This investigation was supported by Atatürk University (BAP-2004/172 and 2008/76). We are greatful to volunteers for the blood samples. The author is greatful to Savaş Yeşilyurt for his proofreading the article.

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