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Protective role of vitamins A, C, and E against the genotoxic damage induced by aflatoxin B_1 in cultured human lymphocytes

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In this study, we aimed to evaluate the effect of vitamins A, C, and E against aflatoxin B_1 (AFB_1) on blood cultures in relation to induction of sister chromatid exchange (SCE). The results indicated genotoxic and mutagenic damage in cultured human lymphocytes exposed to AFB_1. The results showed that 5 μM concentration of AFB_1 increased SCE. When vitamins A, C, and E were added to AFB_1, the frequency of SCE decreased. These results suggest that vitamins A, C, and E could effectively inhibit AFB_1-induced SCE, which may partially responsible for its mutagenic effect of AFB_1. Besides, the protective effect of vitamins A, C, and E against AFB_1 was increased in a dose-dependent manner (i.e., as the doses increased, their protective effects also increased). There was a significant decrease in the SCE frequency in AFB_1-treated group compared with the groups receiving AFB_1 and also vitamins A, C, and E. The most effective concentration was 100 microM vitamin C, and the lowest effective concentration was 0.5 microM vitamin A. Vitamin C has the most effective concentration of 100 μM, and vitamin A has the lowest effective concentration of 0.5 μM. The order of the decreasing effect of the SCE frequency of vitamins was as follows: vitamin C > vitamin E > vitamin A. *Toxicology and Industrial Health* 2009; **25**: 183–188.

Key words: *aflatoxin B_1; genotoxicity; sister chromatid exchange; vitamins A; C; and E*

Introduction

Aflatoxins are secondary toxic fungal metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. They not only contaminate our food stuffs but are also found in edible tissues, milk, and eggs of farm animals by the consumption of contaminated feed. In utero exposure of aflatoxin through mother's blood has also been reported in human beings (Pohland, 1993; Fink, 1999). Aflatoxins are well known for their hepatotoxic and hepatocarcinogenic effects (Wogan, 1999). Aflatoxin B_1 (AFB_1) ($C_{17}H_{12}O_6$) is activated to AFB_1-8,9-oxide and forms adduct primarily at N7 position of guanine and is responsible for its mutagenic and carcinogenic effects (Wang and Groopman, 1999; Denissenko, *et al.*, 1999). AFB_1, a human carcinogen and the most potent genotoxic agent, is mutagenic in many model systems and produces chromosomal aberrations (CA), micronuclei (MN), sister chromatid exchange (SCE), unscheduled DNA synthesis, and chromosomal strand breaks as well as forms adducts in rodent and human cells (Denissenko, *et al.*, 1999). In animal studies, selenium has been shown to inhibit aflatoxin hepatocarcinogenesis (Shi, *et al.*, 1995). These inhibitory effects are supported by many diverse mechanisms,

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including inhibition of carcinogen formation, modulation of carcinogen metabolism, inhibition of mutagenesis and genotoxicity, and inhibition of cell proliferation (Lu, *et al.*, 1996).

Nevertheless, some antioxidants (vitamins A, C, E, selenium, and coenzyme Q10) may help to optimize the antioxidant defense system (Morris and Carson, 2003).

Selenium and vitamin E are recognized as essential nutrients for humans and animals. The protective effects of both antioxidants were found in experimental animals fed a diet supplemented with vitamin E and/or selenium (Fischer and Whanger, 1997) under various pathological conditions. Vitamin E is important in giving protection against cardiovascular disease and neurodegeneration (Mueller and Goss-Sampson, 1990). Vitamin C can regenerate vitamin E from the tocopherol radical formed from reaction with other radicals. Reduced glutathione in the respiratory tract scavenges oxidative toxins such as NO_2, ozone, and free radicals in cigarette smoke (Slade, *et al.*, 1993). Vitamin C also helps in this instance, as does vitamin A. Urate, the end product of purine metabolism, is an important scavenger of free radicals (Kaur and Halliwell, 1990). Plant-derived phenolic compounds, such as flavonoids, inhibit lipid peroxidation (Laughton, *et al.*, 1991). The levels of CA and SCEs were lowered, suggesting a protective role of vitamins against genotoxic damage. Administration of vitamins C and E combination appeared to be more effective in preventing chromosomal damage than a separate administration (Ahmad, *et al.*, 2002). Also SCE frequency is reduced by red orange complex that contains vitamins C and E (Boninaa, *et al.*, 2008).

Retinoids (retinol, retinoic acid, retinol acetate, retinol palmitate, and retinal) and β-carotene inhibit mutagenicity (Huang, *et al.*, 1982; Bhattacharya, *et al.*, 1984, 1987; Qin and Huang, 1985, 1986; Hill and Shih, 1974; Whong, *et al.*, 1988; Busk and Ahlborg, 1980; Qin, *et al.*, 1985), production of CA (Qin. and Huang, 1985), and DNA adduct formation by AFB_1 (Bhattacharya, *et al.*, 1984). Retinoids inhibit the formation of reactive metabolites from polycyclic aromatic hydrocarbons, and the formation of AFB_1-DNA adducts in a dose-dependent manner (Yu, *et al.*, 1994).

Both vitamin A and β-carotene significantly increased benzo(a)pyrene-induced unscheduled DNA synthesis and decreased benzo(a)pyrene-DNA adducts in hamster tracheal epithelium (Wolterbeek, *et al.*, 1995). It was found that SCE level was significantly increased by high doses of vitamin A (10^{-5}–10^{-4} M) (Alija, *et al.*, 2006), and the enhancement of SCE was restored to its original level by addition of vitamin E (final concentration 2 μg/mL). This may be due to the latter inhibiting the oxidation of vitamin A and reducing the amount of oxidized carotene, which is toxic for cultured cells. Combination of vitamins A and E at low doses inhibited SCE induced by methylnitronitrosoguanidine, but no protective activity was observed when used separately. It was also found that vitamin A (2×10^{-7} M) and retinol (16 μg/mL) inhibited SCE induced by AFB_1, which is activated by S-9 mixture (Deng, *et al.*, 1988).

However, no study has been carried out to evaluate the effect of vitamins A, E, and C against AFB_1 on blood cultures in terms of genotoxicity. Therefore, we aimed to investigate this condition in this study.

Methods

The chemicals (AFB_1, vitamins A, E, and C) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Peripheral blood lymphocytes were taken from four (two men and two women) nonsmoking healthy individuals. Lymphocyte cultures were set up by adding 0.5 mL of heparinized whole blood to RPMI-1640 chromosome medium supplemented with 15% heat-inactivated fetal calf serum, 100 IU/mL streptomycin, 100 IU/mL penicillin, and 1% L-glutamine. Lymphocytes were stimulated to divide by 1% phytohemaglutinin. AFB_1 (in concentration of 5 μM), vitamin A (in concentrations of 0.5, 1, and 1.5 μM), vitamin C (in concentrations of 25, 50, and 100 μM), and vitamin E (in concentrations of 40, 100, and 200 μM) were added to the cultures just before incubation. The experiments were performed on 11 groups as follows:

- Group 1: control
- Group 2: 5 μM AFB_1
- Group 3: 5 μM AFB_1 + 0.5 μM vitamin A
- Group 4: 5 μM AFB_1 + 1 μM vitamin A

- Group 5: 5 μM AFB_1 + 1.5 μM vitamin A
- Group 6: 5 μM AFB_1 + 25 μM vitamin C
- Group 7: 5 μM AFB_1 + 50 μM vitamin C
- Group 8: 5 μM AFB_1 + 100 μM vitamin C
- Group 9: 5 μM AFB_1 + 50 μM vitamin E
- Group 10: 5 μM AFB_1 + 100 μM vitamin E
- Group 11: 5 μM AFB_1 + 200 μM vitamin E

For SCE demonstration, the cultures were incubated at 37 °C for 72 h, and 5-bromo 2-deoxyuridine at 8 mg/mL was added at the initiation of cultures. All cultures were maintained in darkness. Next, 0.1 mg/mL of colcemide was added 3 h before harvesting to arrest the cells at metaphase. Cells were harvested and treated for 30 min with hypotonic solution (0.075 M KCl) and fixed in a 1:3 mixture of acetic acid/methanol (vol/vol). Bromodeoxyuridine-incorporated metaphase chromosomes were stained with fluorescence plus Giemsa technique as described by Perry and Evans (1975). In SCE study, by selecting 30 satisfactory metaphases, the results of SCE were recorded on the evaluation table. One hundred metaphases per subject were also scored to determine the proportion of cells that undergo first, second, and third divisions. For each treatment condition, well-spread second division metaphases containing 42–46 chromosomes in each cell were scored, and the values obtained were calculated as SCEs per cell.

Statistical analysis

For statistical analysis, *t*-test was used. A $P < 0.05$ was accepted as statistically significant.

Results

SCE frequencies (as mean ± SD) of the experimental groups are given in Table 1. SCE frequency in AFB_1-treated group was higher than the frequency in the control group ($P < 0.001$) Figure 2.

There was a significant decrease in the SCE frequency in AFB_1-treated group when compared with the groups receiving AFB_1 and vitamins A, C, and E. The decrease in the SCE frequency was high in vitamin C and low in vitamin A-treated group. This decrease in SCE frequency correlated with the increase in vitamin concentration. The most effective concentration was 100 μM vitamin C, and the lowest effective concentration was 0.5 μM vitamin A. The order of the decrease in the SCE frequency in relation to vitamin concentration was as follows: vitamin C > vitamin E > vitamin A (Figure 1).

Discussion

AFB_1 is activated to AFB_1-8,9-oxide and forms an adduct primarily at N7 position of guanine and is responsible for its mutagenic and carcinogenic effects (Wang and Groopman, 1999; Denissenko, *et al.*, 1999). Selenium has been shown in animal studies to inhibit aflatoxin hepatocarcinogenesis (Shi, *et al.*, 1995). These inhibitory effects are supported by many diverse mechanisms, including inhibition of carcinogen formation, modulation of carcinogen metabolism, inhibition of mutagenesis

Table 1. The effects on the number of SCEs of AFB_1 and vitamins A,C, and E in human peripheral lymphocytes

Culture types	Number of samples	Range of SCEs	SCEs/cell
C (DMSO)	4	0–9	6.17 ± 0.12
5 μM AFB_1	4	5–17	9.89 ± 0.17[a]
5 μM AFB_1 + 0.5 μM VA	4	4–17	9.01 ± 0.24[a,b]
5 μM AFB_1 + 1 μM VA	4	2–14	8.48 ± 0.25[c,d]
5 μM AFB_1 + 1.5 μM VA	4	3–14	6.86 ± 0.38[b]
5 μM AFB_1 + 25 μM VC	4	2–13	7.76 ± 0.37[c,d]
5 μM AFB_1 + 50 μM VC	4	2–13	6.86 ± 0.31[b,c]
5 μM AFB_1 + 100 μM VC	4	2–13	6.47 ± 0.29[b]
5 μM AFB_1 + 50 μM VE	4	1–18	8.49 ± 0.27[c,d]
5 μM AFB_1 + 100 μM VE	4	3–14	7.55 ± 0.46[c,d]
5 μM AFB_1 + 200 μM VE	4	2–15	6.83 ± 0.32[b]

AFB_1, aflatoxin B_1; VA, vitamin A; VC, vitamin C; VE, vitamin E.
[a]$P < 0.001$ compared with control.
[b]$P < 0.001$ compared with AFB_1group.
[c]$P < 0.05$ compared with control.
[d]$P < 0.05$ compared with AFB_1group.

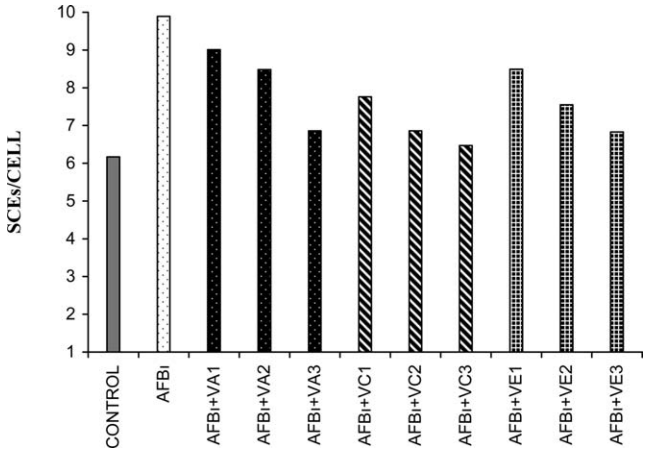

Figure 1 The values for SCEs/cell in the cultures of human peripheral lymphocytes in the study groups.

Figure 2 Representative example of AFB_1-treatment a cell with spontaneous sister chromatid exchanges.

and genotoxicity, and inhibition of cell proliferation (Lu, *et al.*, 1996). AFB_1 administration to human lymphocytes in culture was shown to cause increased SCE (Ahmad, *et al.*, 2002; Türkez and Sişman, 2007). In addition, lipid peroxidation and oxidative DNA damage are also the manifestations of AFB_1-induced toxicity (Wang and Groopman, 1999; Denissenko, *et al.*, 1999). Souza, *et al.* (1999) have reported significant rise in lipid peroxidation in the liver of rats 72 h after a single intraperitoneal dose of AFB_1 (Souza, *et al.*, 1999). AFB_1, human carcinogen and the most potent genotoxic agent, is mutagenic in many model systems and produces CA, MN, SCE, unscheduled DNA synthesis, and chromosomal strand breaks as well as forms adducts in rodent and human cells (Wang and Groopman, 1999). Our findings also support this view. It enhances SCE in AFB_1-induced genotoxicity in human lymphocytes in-vitro (Figure 2).

Curcumin, carotenoid, flavonoid, and vitamin C are able to protect the genotoxic damage in human lymphocyte cultures. Vitamin C and curcumin are more effective in comparison to carotenoid and flavonoid. The order of activity was noted as follows: vitamin C > curcumin > carotenoid > flavonoid (Ahmad and Afzal, 2004). A mechanism of antimutagenesis proposed for β-carotene is through its antioxidant and free radical scavenging activity (Flora, 1990). Plasma level of 0.4 μM vitamin A has been correlated with the reduced risk of various cancers (Rice-Evans, 1995). Another study showed that vitamin A decreases the CA and SCE and increases replication index (RI), but the effect is poor (Ahmad and Afzal, 2004). Also SCE frequency is reduced against arsenic by vitamin A (Avani and Rao, 2007). Vitamin E and Se in the cultures treated concurrently with CCl_4 and both antioxidants were found to decrease the frequency of SCE (Sivikova, *et al.*, 2001). Another study showed that vitamin E decreases SCE (Chin, *et al.*, 2008).

Research efforts are being made to investigate therapeutic agents such as vitamins, sulfhydryl substances, and plant products are capable of minimizing the genotoxicity of various natural and man-made mutagens in our daily life. (Edenharder, *et al.*, 1999; Hour, *et al.*, 1999). The anticarcinogenic, anticlastogenic, and even antimutagenic roles of vitamin C have been tested in a variety of in-vivo and in-vitro systems exposed to radiation, pesticides, and estrogenic drug diethylstilbestrol (Hoda and Sinha, 1993; Sweetman, *et al.*, 1997; Castillo, *et al.*, 2000; Shadab, *et al.*, 2006).Vitamin C is a water-soluble dietary antioxidant that plays an important role in controlling the oxidative stress (Panayiotidis and Collins, 1997). It can also protect DNA against damages induced by various chemicals (Duthie, *et al.*, 1996 Blasiak and Kowalik, 1999). However, some reports indicate that vitamin C can be genotoxic (Shamberger, 1984; Blasiak and Kowalik, 2000). In these studies, different doses of allicin and vitamin C were tested against the genotoxic damage induced by chlormadinone acetate (CMA; 40 μM) using CAs and SCEs as the

parameters. Treatment with allicin and vitamin C resulted in reduction of CAs and SCEs. The results suggested that a protective role of allicin and vitamin C against CMA induced genotoxic damage (Siddique and Afzal, 2005).

Our study showed that these agents have ameliorative effects against AFB_1 on human lymphocyte cultures. Administration of vitamin E appeared to be more effective in preventing SCEs than vitamin A. Administration of vitamin C appeared to be most effective in SCEs. Vitamin C may act as free radical scavenger and therefore, might react with mutagenic metabolites (such as OH radical) before they reach the DNA and damage occurs. Vitamin E has more antioxidant capacity than vitamin A that decreases the SCE, but the effect is poor (Ahmad and Afzal, 2004).

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