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CLINICAL & BASIC RESEARCH

The Cytotoxicity of Aflatoxin B_1 in Human Lymphocytes

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سمية الأفلاتوكسين في الخلايا البشرية اللمفاوية

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ABSTRACT: ***Objectives:*** Aflatoxin B_1 (AFB_1) is a naturally occurring carcinogenic and immunosuppressive compound. This study was designed to measure its toxic effects on human peripheral blood mononuclear cells (PBMC). ***Methods***: The study recruited 7 healthy volunteers. PBMC were isolated and cellular respiration was monitored using a phosphorescence oxygen analyser. The intracellular caspase activity was measured by the caspase-3 substrate N-acetyl-asp-glu-val-asp-7-amino-4-methylcoumarin. Phosphatidylserine exposure and membrane permeability to propidium iodide (PI) were measured by flow cytometry. ***Results***: Cellular oxygen consumption was inhibited by 2.5 µM and 25 µM of AFB_1. Intracellular caspase activity was noted after two hours of incubation with 100 µM of AFB_1. The number of Annexin V-positive cells increased as a function of AFB_1 concentration and incubation time. At 50 µM, a significant number of cells became necrotic after 24 hours (Annexin V-positive and PI-positive). ***Conclusion***: The results show AFB_1 is toxic to human lymphocytes and that its cytotoxicity is mediated by apoptosis and necrosis.

Keywords: Aflatoxin B_1; Oxygen Analyzer; Cellular Respiration; Mitochondria; Caspases; Leukocytes, Mononuclear.

الملخص: **الهدف**: الأفلاتوكسين هو مركب طبيعي مسبب للسرطان ولنقص المناعة. هذه الدراسة تقيّم الآثار السامة للأفلاتوكسين على الخلايا البشرية اللمفاوية. تمت هذه الدراسة بين تشرين ثاني 2008 وحزيران 2012. **الطريقة**: عزلت خلايا دموية لمفاوية من سبعة متطوعين أصحاء. رصد التنفس الخلوي بواسطة المؤشر الفسفوري لكشف الأكسجين. وكشف نشاط انزيم الكاسبيس داخل الخلية باستخدام المادة الفسفورية المشتقة من كومارين القادرة على قياس نشاط الإنزيم. تم قياس موت الخلية عن طريق قياس التعرض لمادة الفوسفاتيديل سيرين و زيادة نفاذية غشاء الخلية لبروبديم ايوداید باستخدام تقنية التدفق الخلوي. **النتائج**: الأفلاتوكسين (2.5 µM و 25 µM) ثبط الاستهلاك الخلوي للأكسجين. ظهرت فعالية الكاسبيس داخل الخلايا بعد ساعتين من إضافة الأفلاتوكسين. عدد الخلايا الميتة الايجابية لمادة الانيكزن–V ارتفع بزيادة جرعة الأفلاتوكسين وبزيادة زمن تعرض الخلايا لهذا السم. عند إضافة 50 µM من الأفلاتوكسين أصبحت نسبة معتبرة من الخلايا نخرية بعد 24 ساعه (انيكزن (V^+PI^+). **الخلاصة**: الأفلاتوكسين مادة سامة للخلايا البشرية اللمفاوية وسميته تتم عبر الموت الخلوي المبرمج والنخر.

مفتاح الكلمات: الأفلاتوكسين؛ مؤشر كشف الأكسجين؛ التنفس الخلوي؛ الميتوكوندريا؛ كاسبيس؛ الدم؛ الخلايا اللمفاوية.

Advances in Knowledge

- *Aflatoxin B_1 (AFB_1) is a potent immunosuppressant.*
- *AFB_1 induces apoptosis and necrosis in human lymphocytes.*
- *AFB_1 inhibits lymphocyte respiration (mitochondrial oxygen consumption).*

Application to Patient Care

- *The cytotoxicities of aflatoxin in humans include immunosuppression, mediated by lymphocyte apoptosis and necrosis.*
- *Public awareness of the potential immunotoxicity of aflatoxins is needed.*
- *Effective health regulations are required to minimise the exposure to aflatoxins, especially in immunocompromised hosts.*

Aflatoxin B_1 (AFB_1) is a mycotoxin commonly found in food contaminated by organisms, such as *Aspergillus flavus*.[1,2] Exposure to AFB_1 is linked to several human diseases, including hepatocellular carcinoma,[3,4] mutagenesis,[5] immunosuppression,[6–8] impaired infant growth,[9–12] and adverse birth outcomes.[12,13] Synergistic interactions with hepatitis B and human immunodeficiency viruses have also been reported.[14,15]

The toxin is activated by hepatic cytochrome P450 3A4, and its active form (AFB_1-exo-8,9-

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epoxide) rapidly reacts with cellular deoxyribonucleic acid (DNA) and proteins.[16,17] Most of the information regarding its immunotoxicity has been derived from animal studies.[18–20] The exposure of young children to dietary aflatoxin and its toxic effects on human lymphocytes are also well established,[14,21–24] including the inhibition of lymphocyte respiration.[25]

The term apoptosis describes the caspase- (or apoptosome)-mediated cytotoxic processes that cause mitochondrial dysfunction, membrane damage and DNA fragmentation.[26] The apoptotic pathways that are independent of apoptosomes are also known.[27] This study explores the mechanisms (biomarkers) of the toxic effects of AFB_1 in human lymphocytes.

Methods

This study was carried out from November 2008 to June 2012. The PBMC were isolated from the whole blood of 7 healthy adult volunteers as previously described.[28] The Institutional Review Board for the protection of human subjects of the United Arab Emirates University approved the collection of blood from the healthy volunteers. Informed consent was obtained from the participating volunteers.

The reagents and solutions used were as follows. The Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin ([Pd phosphor] at molecular weight [M_r]~1300) was purchased from Porphyrin Products (Frontier Scientific, Inc., Logan, Utah, USA). The dactinomycin was purchased from Merck & Co., Inc. (Whitehouse Station, New Jersey, USA). The benzyloxy-carbonyl-val-ala-DL-asp-fluoromethylketone (zVAD-fmk) was a Calbiochem® product (EMD Millipore, Billerica, Massachusetts, USA). The *N*-acetyl-asp-glu-val-asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was purchased from Alexis Biochemicals (Enzo Life Sciences, Inc., Farmingdale, New York, USA). The AFB_1 (produced by *Aspergillus flavus*), glucose oxidase, D(+)-glucose and remaining reagents were from Sigma-Aldrich Co. (St. Louis, Missouri, USA).

The Pd phosphor (at 2.5 mg/mL and 2.0 mM), glucose oxidase (at 10 mg/mL) and sodium cyanide (at 1.0 M) solutions were prepared in distilled water (dH_2O) and stored at -20 °C. The phosphate buffered saline (PBS) solution was made daily. The AFB_1, dactinomycin, zVAD-fmk (at 2.14 mM) and Ac-DEVD-AMC (at 7.4 mM) solutions were prepared and stored as previously described.[29–31]

Oxygen detection was based on the principle described previously,[32] and the phosphorescence oxygen (O_2) analyser used as reported elsewhere.[33–36] For the fluorescence-activated cell sorting (FACS) analysis, an aliquot of 10^6 of PBMC for each subject were cultured with 0, 5, 10, 50 or 165 µM of AFB_1. The cells were harvested after 2, 16, and 24 hours, and analysed as previously described.[37] The AFB_1 was prepared and measured as described.[38]

The reaction mixtures contained 1.5 x 10^6 cells in PBS, 10 mM of glucose and 68 µM of Ac-DEVD-AMC, with and without zVAD-fmk (at 20 µM). The mixtures were incubated at 37 °C in glass vials (in the dark with continuous agitation) for two hours, with and without AFB_1 or dactinomycin. The suspensions were then diluted with 1.0 mL of ice-cold PBS-glucose, sonicated for 60 secs and passed through 23-G needles. The supernatants were collected by centrifugation (12,300 x g for 10 mins) and separated on high-performance liquid chromatography (HPLC) as described.[37] The released 7-amino-4-methylcoumarin (AMC) moiety (peak retention time of ~8.7 mins) was detected by fluorescence. A control reaction mixture containing PBS-glucose, 68 µM of Ac-DEVD-AMC and 5 µl of dimethyl sulfoxide (DMSO) (the vehicle for zVAD-fmk) without added cells was monitored periodically at 37 °C for the spontaneous release of AMC moieties; in these control reactions, the AMC peak areas at 0.5, 1, 2 and 3 hours were negligible.

The HPLC analysis of the released AMC moieties was done on a Waters Corporation (Milford, Massachusetts, USA) reversed-phase HPLC system (excitation wavelength of 380 nm and emission wavelength of 460 nm). Solvent A was acetonitrile (CH_3CN) and water (H_2O) at a ratio of 1:3 and solvent B was dH_2O. The column, a 4.6 x 250 mm Ultrasphere® ion pair column (Beckman Coulter, Inc., Brea, California, USA), was operated at 25 °C at 1.0 mL/min (0.5 mL/min of each pump). The run time was 15 mins and the injection volume was 20 µL.[37]

Results

The cellular respiration results were as follows. The

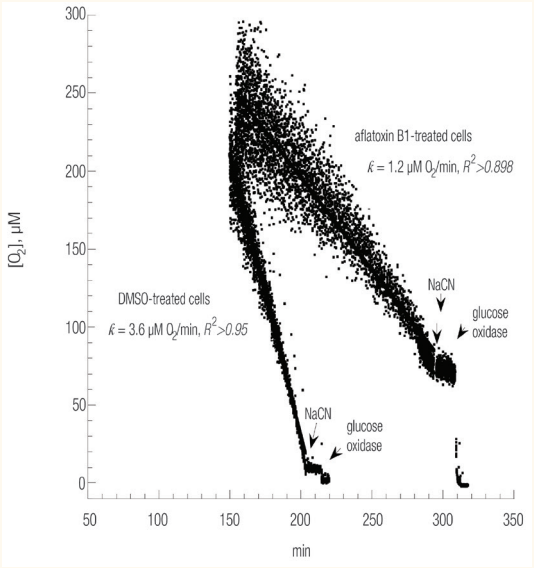

Figure 1: The AFB_1 (25 μM in DMSO) inhibited the PBMC respiration. The PBMC (2.5 x 10^7 cells/mL in PBS-glucose, 3 μM of Pd phosphor and 0.5% of fat-free albumin) were incubated at 37 °C with 12 μL/mL of DMSO or 25 μM of AFB_1 (in DMSO). Min zero corresponded to the addition of AFB_1. At t = 154 mins, 1.0 mL of each mixture was simultaneously placed in the phosphorescence O_2 analysers for O_2 measurements at 37 °C. The rates of respiration (k) were calculated from the best fit curves. The additions of 5.0 mM of NaCN and 50 μg/mL of glucose oxidase are shown.

O_2 = oxygen; DMSO = dimethyl sulfoxide; k = zero-order rate constant for cellular mitochondrial O_2 consumption; R^2 = regression coefficient; NaCN = sodium cyanide; AFB_1 = aflatoxin B_1; PBMC = human peripheral blood mononuclear cells; PBS = phosphate buffered saline; Pd phosphor = Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin.

representative O_2 consumption runs are shown in Figure 1 and 2. In Figure 1, the PBMC (2.5 x 10^7 cells/mL) were incubated with 12 μL/mL of DMSO or 25 μM of AFB_1. At t = 154 mins, 1.0 mL of each mixture was simultaneously placed in the instruments for the O_2 measurements. The respiration rate, zero-order rate (constant for cellular mitochondrial O_2 consumption (k) in μM O_2 min^{-1}) for untreated cells was 3.6 and 1.2 for AFB_1-treated cells (67% inhibition). In Figure 2, the PBMC (10^7 cells/mL) were incubated with 1.2 μL/mL of DMSO or 2.5 μM of AFB_1. At t = 95 mins, 1.0 mL of each mixture was simultaneously placed in the instruments for the O_2 measurements. The k values were 1.6 and 1.3 μM of O_2/min, respectively (19% inhibition).

In four separate experiments, the values of k for untreated lymphocytes were 2.2 ± 1.1 μM O_2 min^{-1} per 10^7 cells. The corresponding values for lymphocytes treated with AFB_1 (cells exposed to 2.5–75 μM of AFB_1 for 60–154 mins) were 1.3 ± 0.6 μM O_2 min^{-1} per 10^7 cells; the inhibition was dose-dependent and ranged from 19–67%.

The caspase activity was monitored after incubation for two hours at 37 °C with 100 μM of AFB_1 (which was added as a powder), with and without 20 μM of zVAD-fmk, using the caspase-3 substrate analogue Ac-DEVD-AMC. Caspase-3 cleaved Ac-DEVD-AMC, releasing the fluorogenic

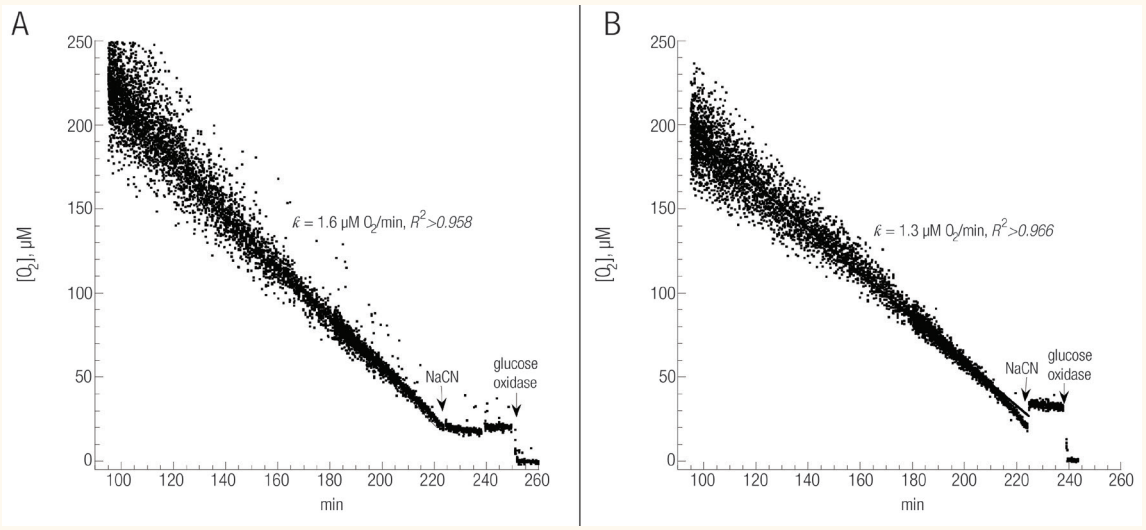

Figure 2 A & B: The AFB_1 (2.5 μM in DMSO) inhibited the PBMC respiration. The PBMC (10^7 cells/mL in PBS-glucose, 3 μM of Pd phosphor and 0.5% of fat-free albumin) were incubated at 37 °C with 1.2 μL/mL of DMSO (**A**) or 2.5 μM of AFB_1 (**B**). Min zero corresponded to the addition of the AFB_1. At t = 95 mins, 1.0 mL of each mixture was simultaneously placed in the phosphorescence O_2 analysers for O_2 measurements at 37 °C. The rates of respiration (k) were calculated from the best-fit curves. The additions of 5.0 mM of NaCN and 50 μg/mL of glucose oxidase are shown.

O_2 = oxygen; k = zero-order rate constant for cellular mitochondrial O_2 consumption; R^2 = regression coefficient; NaCN = sodium cyanide; AFB_1 = aflatoxin B_1; DMSO = dimethyl sulfoxide; PBMC = the human peripheral blood mononuclear cells; PBS = phosphate buffered saline; Pd phosphor = Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin.

moiety AMC. After cell disruption, the AMC was separated on HPLC and detected by fluorescence. For untreated cells [Figure 3A], the AMC peak area (in arbitrary units) was 273,367 and abolished by zVAD-fmk. For cells treated with AFB_1 alone [Figure 3B], the AMC peak area was 32,347,746 (118-fold higher). For cells treated with AFB_1 and zVAD-fmk [Figure 3B], the AMC peak area was 3,522,589 (89% inhibition). Similar results were obtained in two additional experiments. For comparison, the cells were also treated with 20 μM of dactinomycin, which is well known to activate caspases. The AMC peak area in the presence of dactinomycin alone was 54,679,510; in the presence of dactinomycin and zVAD-fmk, the peak area was 4,561,062 (92% inhibition) [Figure 3C].

The caspase activation was monitored as a function of the time of incubation with AFB_1. Three conditions were tested: untreated cells, cells treated with 100 μM of AFB_1 alone and cells treated with 100 μM of AFB_1 and 20 μM of zVAD-fmk. The Ac-DEVD-AMC cleavage was monitored at 15, 30, 60 and 120 mins after treatment. The AMC moiety appeared only after two hours of incubation with AFB_1; zVAD-fmk blocked ~94% of the AMC peak area. This profile of caspase activation was similar to that described for dactinomycin and doxorubicin in human immortalised T-lymphocytes.[28,29]

The induction of the lymphocyte apoptosis by AFB_1 was monitored by flow cytometry, using the cell membrane's permeability to propidium iodide (PI) and the phosphatidylserine exposure. There were 10 independent experiments performed, and the results of these are summarised in Figure 4. The PBMC were isolated from the blood of 7 healthy volunteers and exposed to 0, 5, 10 or 50 μM of AFB_1. The FACS analysis was performed at 2, 16 and 24 hours. With the total number of mononuclear cells isolated at 2.5 x 10^6, the number of Annexin V-positive cells increased with incubation time; this increase was statistically significant (P <0.05) after 16 hours of incubation for each concentration. Although the difference in the effect of 5 μM and 10 μM was not significant (P = 0.082), the increase in the number of apoptotic cells after treatment with 50 μM was significant [Figure 4 A and B]. It is noteworthy that 50 μM of AFB_1 also produced a significant number of necrotic cells (annexin V-positive and PI-positive), which became more evident at 24 hours [Figure 4C].

Thus, the results of this study show that AFB_1 inhibits cyanide-sensitive cellular respiration. The toxin also induces apoptosis and necrosis.

Discussion

The phosphorescence O_2 analyzer, caspase assay and flow cytometry were used here to confirm the toxic effects of AFB_1 on human lymphocytes.[25] The results show that AFB_1 impairs human lymphocyte

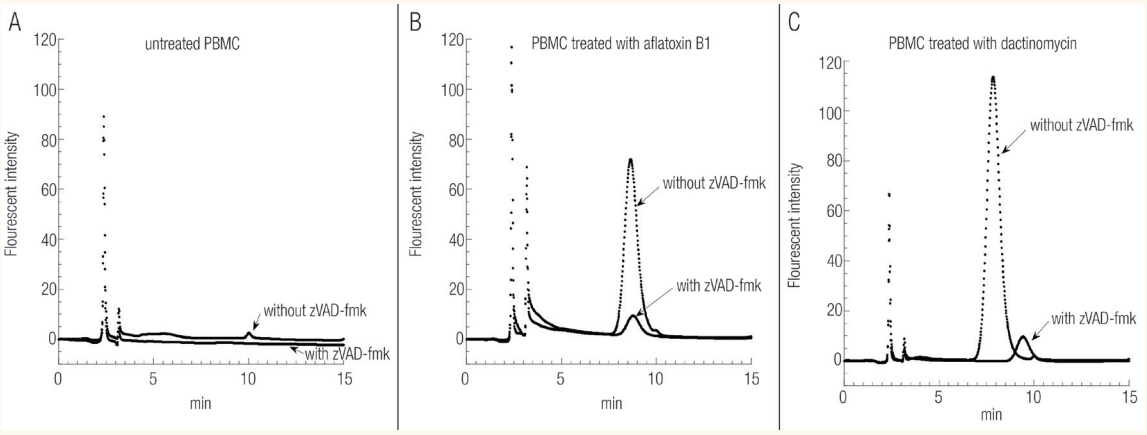

Figure 3 A–C: The intracellular caspase activation by AFB_1 and dactinomycin. Each mixture (final volume of 0.5 mL) contained 1.5 x 10^6 cells in PBS-glucose and 68 μM of Ac-DEVD-AMC, with and without 20 μM of zVAD-fmk. The suspensions were incubated at 37 °C for two hours without addition (**A**), with the addition of 100 μM of AFB_1 (**B**) or with the addition of 20 μM of dactinomycin (**C**). At the end of the incubation period, the cells were disrupted and their supernatants were separated via HPLC. The AMC moiety was monitored by fluorescence. The retention time for the Ac-DEVD-AMC was ~2.4 mins and ~8.7 mins for the AMC.

PBMC = the human peripheral blood mononuclear cells; zVAD-fmk = benzyloxy-carbonyl-val-ala-DL-asp-fluoromethylketone; AMC = 7-amino-4-methylcoumarin; AFB_1 = aflatoxin B_1; PBS = phosphate buffered saline; Ac-DEVD-AMC = N-acetyl- asp-glu-val-asp-7-amino-4-methylcoumarin; HPLC = high-performance liquid chromatography.

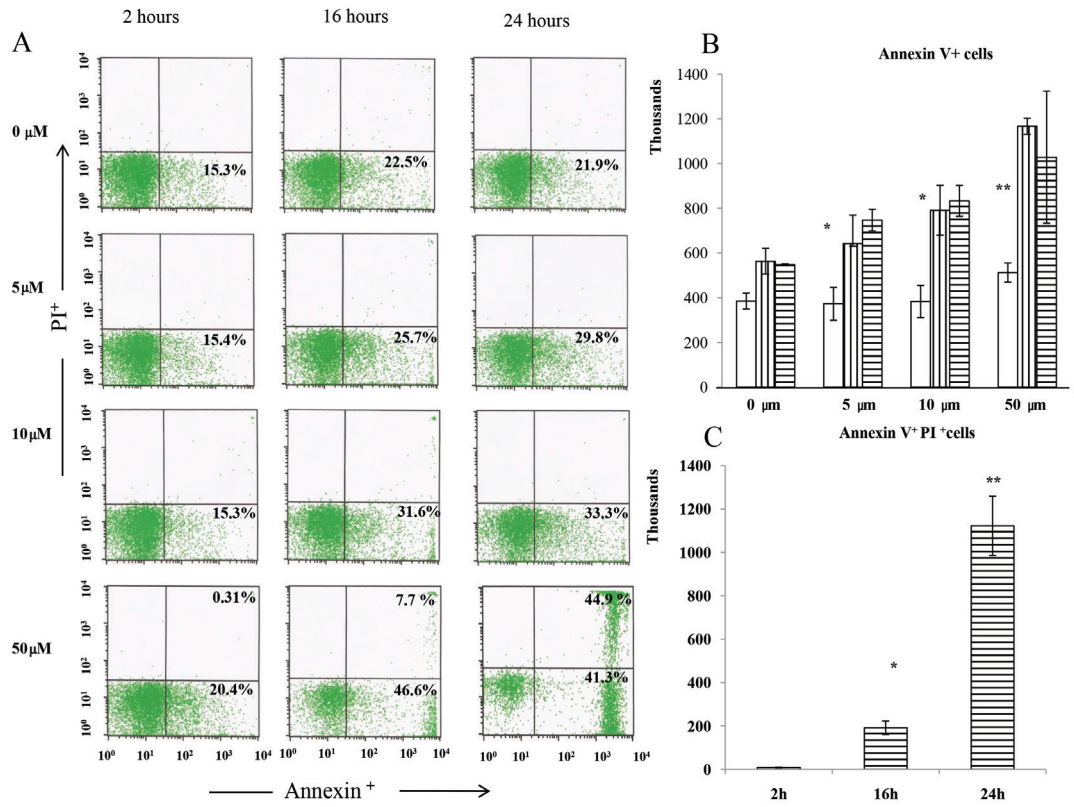

Figure 4 A–C: The flow cytometry analysis of the apoptosis. **A:** The percentages of the Annexin V-positive cells; data are representative of samples from 7 healthy individuals. **B:** The quantification of the FACS analysis of the apoptotic (Annexin V-positive) cells from 7 healthy donors stained with PI and Annexin V at 24 hours (horizontal lines), 16 hours (vertical lines) and two hours (no lines). **C:** The quantification of the FACS analysis of the necrotic (Annexin V-positive and PI-positive) cells after treatment with 50 µM of AFB_1.

PI = propidium iodide; FACS = fluorescence-activated cell sorting; AFB_1 = aflatoxin B_1.

**P <0.05; **P <0.005.*

mitochondrial function [Figure 1 and 2] and activates intracellular caspases [Figure 3]. The AFB_1 also produces lymphocyte apoptosis and necrosis [Figure 4]. The caspases become active in the cells within two hours of the AFB_1 addition [Figure 3]. This period is similar to that needed to inhibit cellular respiration [Figure 1 and 2].

In one study, 32 µM of AFB_1 had a minimum effect on human lymphocyte proliferation following phytohaemagglutinin-P stimulation.[39] However, an earlier study on human lymphocytes showed less lymphocyte proliferation in the presence of 16 µM of AFB_1.[40] The AFB_1 also inhibited concanavalin A-promoted lymphocyte proliferation (50% inhibition at 60 nM).[20] Moreover, AFB_1 was shown to induce apoptosis in human lymphocytes.[41,42]

The concentrations of AFB_1 used here were relatively high, especially since the average human exposure in Eastern China is only ~0.5 mmol/day.[3] It is important, however, to emphasise that the stability of AFB_1 in solutions is poor and the bulk of the toxin is deactivated by the rapid reaction with H_2O.[43] Thus, the data presented here mainly point to the potential immunotoxicity of AFB_1.

The caspase activity reaction described was previously calibrated using recombinant human caspase-3.[28] The AMC was expressed as peak areas (in arbitrary units) per number of cells. The reactions were conducted in the presence and absence of the pan-caspase inhibitor zVAD-fmk. It is of note that the substrate Ac-DEVD-AMC can be cleaved by several caspases, including caspase-3 (turnover number [k_{cat}]/ Michaelis constant [K_m] = 218,000 s-1); caspase-7 (k_{cat}/K_m = 37,000 s-1); caspase-1/interleukin-1 converting enzyme (k_{cat}/K_m = 30,000 s-1); caspase-6 (k_{cat}/K_m = 2,000 s-1), and caspase-4 (k_{cat}/K_m = 1,800 s-1).[44]

Conclusion

Human lymphocytes exposed *in vitro* to AFB_1 exhibit impairments in cellular respiration, caspase activation and necrosis. The results underscore the immunosuppressive activity of aflatoxins in humans exposed to this natural fungal toxicant.

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