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Cytotoxicity and Genotoxicity of Fumonisin B1 on Cultured Human Lymphocytes

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ABSTRACT

The present investigation was planned with the aim to assess cytotoxic, genotoxic and oxidative effects of fumonisin B1 (FB1) on human lymphocytes *in vitro*. The cytotoxicity and genotoxicity were assessed by methyl tetrazolium (MTT) and Comet assays, respectively, while superoxide dismutase (SOD) and malondialdehyde (MAD) levels in culture supernatant were considered as oxidative stress markers. The results revealed that FB1 has growth inhibitory effects on cultured lymphocytes at all concentrations tested (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 μg/ml), and in a concentration-dependent manner. FB1 was also genotoxic as suggested by single cell electrophoresis of cultured cells treated with two concentrations of FB1 (150 and 500 μg/ml), and a significant increased damage to DNA was observed in these cells. The markers of oxidative stress were also deviated in cultured cells treated with FB1; SOD level was significantly decreased while MAD level was significantly increased in treated cells compared to untreated cells. In conclusion, the present results suggest that FB1 was cytotoxic (increased growth inhibition) and genotoxic (increased DNA damage), and induced oxidative stress in cultured cells that were treated with FB1 even at low concentrations.

Keywords: Fumonisin B1, Lymphocytes, Growth inhibition, Comet assay, Oxidative stress.

1. INTRODUCTION

Mycotoxins are low molecular weight toxic secondary metabolites produced by certain genera of fungi; for instance, *Aspergillus*, *Penicillum* and *Fusarium*, which can enter grains in the field, and their growing in the grains continue during storage under good conditions of temperature and humidity [1]. They can also be found in animal products [2]. Mycotoxins cause a fundamental consequential economic loss, and furthermore, human and animals are also at risk for their adverse effects when they consume mycotoxin-contaminated food. These include infertility, vaginal or rectal prolapse, abnormal offspring, lung edema and liver cancer [3]. Globally, there is no region that can escape the adverse effects of mycotoxins, as about 25% of the world crops contain fungal toxins [1]. The main mycotoxins that found frequently in food are aflatoxins, ochratoxins, fumonisins, deoxynivalenol, patulin, and the ergot alkaloids, which are produced by various fungi , and one of them is *Fusarium* [4,5].

The genus *Fusarium* causes diverse types of diseases in human; for instance, keratitis, onychomycosis, sinusitis, disseminated infection in immunosuppressed patients, and food poisoning after ingestion of *Fusarium* toxins [5]. The most predominant and toxic fumonisins is fumonisin B1 (FB1), which is produced by *Fusarium verticillioides* and other species of *Fusarium*. Structurally, FB1 is similar to sphingosine, which is an important component of phospholipids that are found in cell membranes and have a role in cell signal transduction pathways, and growth, differentiation and death of cell [6]. The FB1 mechanism of action results from its competition with sphingosine and sphinganine in sphingolipid metabolism, and end-up in blocking sphingolipids synthesis; causing raised sphingoid bases and reducing sphingolipids [7].

Different adverse effects in animals and human have been associated with FB1. Experimentally, it has been reported that rats exposed to FB1 were at a greater risk of carcinogenesis. In human, a close relationship between FB1 levels in maize and high incidence of esophageal cancer has been suggested [6].

This investigation was planned with the aim to assess cytotoxic, genotoxic and oxidative effects of FB1 on human lymphocytes *in vitro*.

2. MATERIALS AND METHODS

2.1 Preparation of FB1 concentrations

Standard FB1 (5 mg) was dissolved in 5 ml of acetonitrile:distilled water (50:50, v/v) and used as stock to prepare the following concentrations: 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/ml.

2.2 Isolation and Counting of Lymphocytes

Ten milliliters of venous blood was collected in a tube containing heparin (10 IU/ml) from a 35 year old healthy man. Lymphocytes were isolated from whole blood by a density-gradient centrifugation using histopaque as a separating medium [8]. The collected cells were suspended in RPMI-1640 medium, and the cell concentration was adjusted to 1×10^6 cell/ml, after assessing their viability by a trypan-blue-exclusion test. The viability was greater than 95%.

2.3 Establishment of Cell Culture

To assess the cytotoxic, genotoxic and oxidative effects of FB1, two types of cultures were set-up. In the first, the isolated cells were cultured without adding FB1 (control culture), while in the second, the isolated lymphocytes were co-cultured with the different concentrations of FB1 (50-500µg/ml). In both cases, the cells were stimulated with phytohemagglutinin (PHA). The cultures were established in wells of 96-well plate, and incubated for 48 hours at 37ºC (5% CO_2 and 80% relative humidity). All assessments were based on three cultures (triplicate) of each treatment [9].

2.4 Cytotoxicity assay using Methyl Tetrazolium (MTT)

This test was performed by adding MTT dye to each well containing cultured lymphocytes after the incubation period, and incubated for 30 minutes at 37ºC. After that, the optical density (OD) of each well was measured using an ELISA reader at a wavelength of 630 nm. The growth inhibition (GI) was given as a percentage, as a previously described [10], by using the following equation:

$$\text{Growth Inhibition (\%)} = \frac{\text{OD of control} - \text{OD of Sample}}{\text{OD of control}} \times 100$$

2.5 Genotoxicity assay (Comet assay)

The DNA damage of cultured lymphocytes was assessed for two concentrations of FB1 (150 and 500 µg/ml) using a single cell electrophoresis (Comet assay) technique [11]. An aliquote (20 µl) of cultured cells was added to 250 µl of low melting agarose (LM agarose) at 37°C and well-mixed. Then, 100 µl of this mixture was applied to a Comet slide, which was placed in a dark place for 10 minutes at 4°C. Then, the slide was immersed in lysis solution for 30-60 minutes, followed by immersion in alkaline solution for 20 minutes at room temperature or in a dark placed for one hour at 4°C. To carry out the Comet assay electrophoresis, alkaline electrophoresis solution was added to the tank tray, and then the slide was placed in the tray. The electrophoresis was run at 25 volts for 30 minutes. After electrophoresis, the slide was immersed twice in deionized distilled water for 5 minutes, and once in 70% ethanol for 5 minutes. The slide was left to dry at 37°C for 10-15 minutes and then the Comet slide was stained with 100 µl of SYBR for 30 minutes in a dark place at room temperature. Finally, excess SYBR solution was removed, and the slide was rinsed with distilled water. Then, the slide was left to dry completely at 37°C, and examined by a fluorescent microscope at 40X magnification. Fifty randomly selected cells were examined per sample, and Comet scoring was determined. Four scores were considered, and they were based on the degree of DNA damage (no damage, and low, medium and high damage) [12].

2.6 Determination of Oxidative Stress

The oxidative stress was determined by assessment of superoxide dismutase (SOD) and malondialdehyde (MDA) in the supernatant of cultured lymphocytes. Such assessments were carried out using commercially available kits (Cohesion Bioscience, UK), and instructions of manufacturer were followed.

2.7 Statistical Analysis

Data were given as means ± standard deviation (SE), and significant differences between means were assessed by one-way analysis of variance (ANOVA), followed by Duncan test. The level of significance was $p < 0.05$. These analyses were carried out using the statistical package SPSS version 22.

3. RESULTS AND DISCUSSION

3.1 Growth Inhibition

The FB1 was observed to have growth inhibitory effects on cultured lymphocytes at all concentrations tested (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/ml). The GI rate showed a concentration-dependent manner, and the highest inhibition was observed at the concentration 500 µg/ml (Figure 1). Such growth inhibitory effects may be due to the accumulation of sphingoid bases, which may interfere with DNA synthesis[13], and cause signaling changes by cAMP [9], and disruption of cell cycle [14].

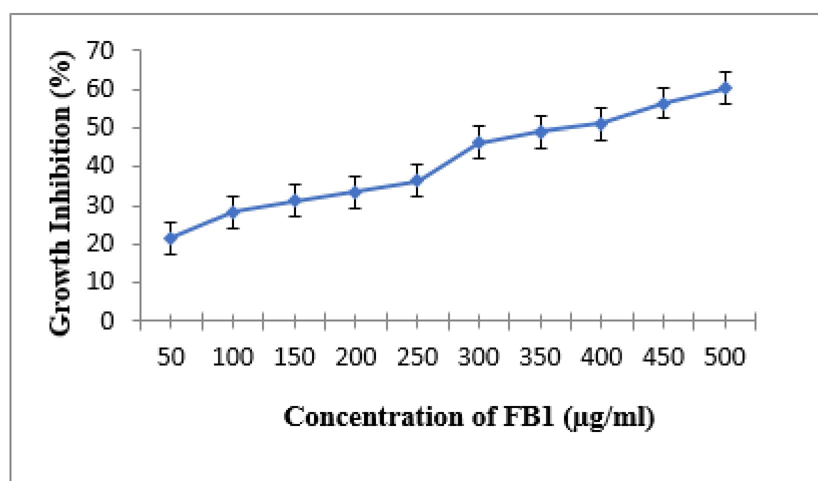

Figure 1: Growth inhibition effects of different concentrations of FB1 on human lymphocytes after 48 hour incubation period using MTT assay

In agreement with such findings, *in vitro* studies have confirmed that FB1 has toxic effects on human cell lines. In this context, it has been demonstrated that FB1 arrested human liver cell line HL-7702 at G0/G1 phase in a dose-dependent manner, and the cell cycle was negatively affected by FB1 treatment. The authors explored the underlying mechanism of such effects and demonstrated that they were associated with alterations in the molecular expression of *cyclin E* and *P21* genes [14]. In a further study, DNA damage was reported in human fibroblasts treated with FB1 [15]. In addition, FB1 was reported to have the ability to induce apoptosis in human proximal tubule-derived cells (IHKE cells) [16], and caused oxidative stress in cells human intestinal cell line Caco-2 [17].

3.2 Comet Assay

The genotoxic effects of FB1 was further confirmed by the results of single cell electrophoresis of human lymphocytes treated with two concentrations of FB1 (150 and 500 µg/ml), and a significant increased damage to DNA was observed in these cells (Table 1 and Figure 2). Such findings highlighted the genotoxic effects of FB1.

Table 1: Assessment of DNA damage (Comet assay) in cutured human lymphocytes treated with FB1.

Concentration (µg/ml)	Percentage of Cells (Mean ± SD)			
	No damage	Low damage	Medium damage	High damage
Control (zero)	45.3 ± 2.5^{A}	40.2 ± 3.2^{A}	7.4 ± 2.3^{C}	7.1 ± 2.4^{B}
150	40.8 ± 4.4^{B}	28.8 ± 3.4^{B}	15.6 ± 2.3^{B}	14.9 ± 2.7^{A}
500	39.2 ± 5.0^{B}	25.1 ± 1.9^{B}	18.4 ± 2.2^{A}	17.3 ± 3.7^{A}

Similar superscript letters represent no significant difference ($p > 0.05$) between means in columns, while different superscript letters represent a significant difference ($p < 0.05$) between means in columns

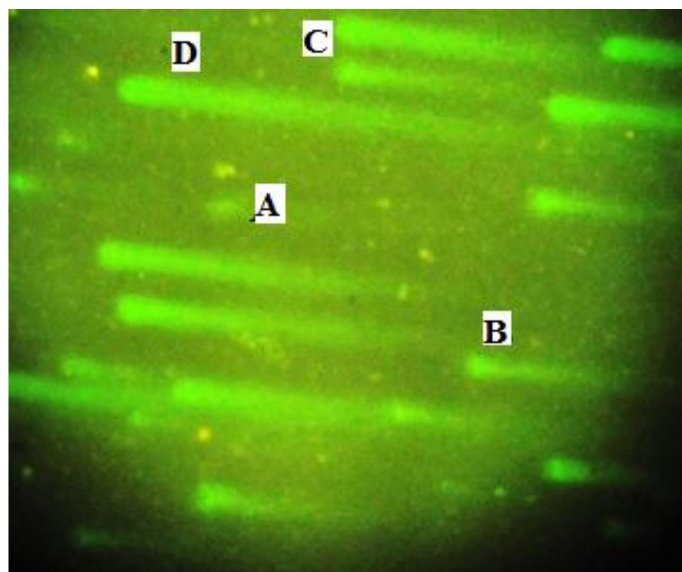

Figure 2: Single cell electrophoresis of cultured human lymphocytes after being treated with FB1 at concentration 500 µg/ml (A: No damage; B: Low damage; C: Medium damage; D: High damage).

Comet assay is a simple, cost-effective procedure, and a sensitive method for detecting breaks in single or double strand DNA of apoptotic cells. It also gives a background level of DNA damage in normal and abnormal cells [12]. A previous study found that FB1 caused a fragmentation of DNA, and many apoptotic bodies were observed, but incubating these cells with vitamin E (25 μM) prior to FB1 (18 μM) reduced these effects [22]. A further study demonstrated that a single dose exposure to FB1 caused apoptosis and increased lipid peroxidation in exposed cells even after a very short period of exposure and DNA lesions was occurred after a longer exposure to the lowest concentration of B1 [23].

3.3 Oxidative Stress

Table 2 shows the effect of FB1 on SOD and MAD in the supernatant of cultured lymphocytes after 48 hours incubation with FB1 at two concentrations (150 and 500 μg/ml). The results revealed that SOD level was significantly decreased while MAD level was significantly increased in treated cells compared to untreated cells.

Table 2: Level of SOD and MDA in the supernatant of human lymphocytes cultured with FB1 for 48 hours.

Concentration μg/ml	Supernatant Level (Mean ± SD)	
	SOD (μg/mg)	MDA (nmol/ml)
Control (zero)	16.19 ± 1.06^{A}	6.35 ± 0.64^{B}
150	11.29 ± 0.62^{B}	10.39 ± 0.57^{A}
500	7.79 ± 0.78^{C}	13.18 ±1.16^{A}

Similar superscript letters represent no significant difference ($p > 0.05$) between means in columns, while different superscript letters represent a significant difference ($p < 0.05$) between means in columns.

SOD is an essential antioxidant enzyme that is involved in partitioning superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2). It represents an important defense mechanism against the superoxide radical toxicity [18]. MDA is an organic molecule and one of the final products of lipid metabolisms that cause toxic stress in the cells. It is also mutagenic by reacting with deoxyguanosine and deoxyadenosine in DNA to form adducts. The level of MAD is usually known to parallel oxidative stress status [17, 18]. A previous study shares the findings of present study, in which the authors made a comparison between three mycotoxins (FB1, DON and ZEN) and demonstrated their capability to induce MDA production (lipid peroxidation), but in the following sequence: FB1 > DON > ZEN. Such effect was related to their common target, which is the mitochondria as observed by MTT test [21].

4. CONCLUSION

The present results suggest that FB1 was cytotoxic (increased GI) and genotoxic (increased DNA damage), and induced oxidative stress in cultured cells that were treated with FB1 even at low concentrations.

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