NUTRITION AND FOOD SCIENCE JOURNAL Open Access

SCIENTIFIC LITERATURE

RESEARCH ARTICLE

Toxic Effects of Fumonisin B1 Mycotoxin on Caco-2 Human Intestinal Cell Line

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ARTICLE INFO

Article history:
Received: 05 January 2017
Accepted: 04 September 2017
Published: 15 September 2017

Keywords:
Microviscosity;
Fumonisin B1;
Intestinal cell line;
MTT test;
Oxidative status

Citation this article: Antonella G, Lucantonio D, Fiorenza M. Toxic Effects of Fumonisin B1 Mycotoxin on Caco-2 Human Intestinal Cell Line. Nutri Food Sci J. 2017; 1(1):111.

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ABSTRACT

The gastrointestinal tract is the main site of exposure to Fumonisin B1 (FB_1), common mycotoxin contaminant of corn and its derivative products. This study, therefore, aims to investigate the effects of such exposure analysing the time- and dose-dependent modifications of some functional parameters induced by FB_1 on the human colon intestinal line (Caco-2). Cell proliferation, oxidative status and changes in membrane microviscosity were assessed using MTT test, malondialdehyde levels related with lipid peroxidation and fluorescence anisotropy, respectively. Short-term exposure of Caco-2 cells to FB_1 induced inhibition of cell proliferation with temporary changes in membrane microviscosity and increased oxidative stress. The observed early FB_1 toxicity was probably due to interactions between the mycotoxin and the cellular membrane, presumably related to amphipathic sphingoid-like structure of mycotoxin.

Abbreviations: FB_1: Fumonisin B_1; MDA: Malondialdehyde; TMA-DPH, N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatriene-1-yl) Phenylammonium p-toluenesulfonate.

Introduction

Fumonisin B1 (FB_1) is a mycotoxin produced mainly by isolates of Fusarium *verticillioides* and *F. proliferatum* and classified by IARC as "possible carcinogenic to humans" [1]. The WHO reported that FB_1 was commonly found in corn and its derivative products all over the world: the contamination was found in 77% of samples from Africa, 85% of samples from Latin America, 63% of samples from North America, 53% of samples from Europe and 52% of samples from Asia [2]. Due to the high occurrence of FB_1 in corn, although at levels permitted by worldwide regulations, humans are constantly exposed to low levels of FB_1, with a higher risk for those countries where corn is the prevalent food; hence, the gastro-intestinal tract could represent the first target of exposure to FB_1 that, although not absorbed by the intestine when administered orally, is found to be hazardous to human health, a condition qualified by Shier [3] as the "fumonisin paradox". For its chemical similarity to sphingoid bases, such as sphingosine and sphinganine, the FB_1 toxicity is related to the inhibition of ceramide synthase, a key enzyme in *de novo* sphingolipid biosynthesis and in the sphingolipid salvage pathway.

The inhibition of ceramide synthase by FB_1 induces the increased intracellular sphinganine and sphingosine ratio, normally used as biomarker for the assessment of mycotoxin exposure [4]. Consequent to inhibition of ceramide biosynthesis, oxidative stress and apoptosis induction have been reported as toxicity mechanisms on intestinal cell line [5]. Since, FB_1 could modify the biological properties of plasma membrane directly, interfering with membrane configuration, due to its amphipathic sphingoid-like structure [6], and indirectly inducing changes on phospholipid composition [7], the aim of the present study was to investigate these specific effects on human intestine using cell line (Caco-2) as model. Dose- and time-dependent effects induced by exposure to FB_1 on cell viability, proliferation, oxidative status and membrane microviscosity were assessed.

Materials and methods

1 Reagents

Dulbecco's Modified Eagle's medium, Dulbecco's Phosphate Buffered Saline (PBS), Trypsin-EDTA Solution, L-glutamine 200 mM, Antibiotic and Antimycotic Solution, Thiazolyl Blue Tetrazolium Bromide (MTT), Trypan Blue Solution, N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatriene-1-yl) phenylammonium p-toluenesulfonate (TMA-DPH) were purchased from Sigma Aldrich (Milan, Italy). Non Essential Amino acid Solution was purchased from Euroclone (Milan, Italy). Foetal Bovine Serum (FBS) was purchased from Gibco (Milan, Italy). Fumonisin B_1 standard was purchased from Orsell (Modena, Italy). Lipid Peroxidation (MDA) Assay Kit (BIOVISION) was purchased from Vinci-Biochem (Florence, Italy).

2 Human intestinal cell line

The human colon carcinoma cell line Caco-2 (ECACC, Sigma Aldrich) was grown in 25 cm^2 flasks at a starting density of 250,000 cells/ml in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose supplemented with 10% of FBS, 1% of L-Glutamine, 1% Antibiotic and Antimycotic solution, 1% non essential amino acid solution. Density and cell viability were determined using Trypan blue dye and a Bürker counting chamber. The cells used for experimental protocols showed a mean viability of 90%.

3 Evaluation of toxicity induced by FB_1 on intestinal cells

3.1 Preparation of Fumonisin B_1 solution

Fumonisin B_1 solution was prepared following the protocol described in Minervini et al [8]. The choice of highest FB_1 concentration (69 µM) was based on the FB_1 toxicity evaluation found in previous *in vitro* studies [3,9,10].

3.2 Assessment of cell viability and proliferation

To test the influence of FB_1 on cell viability and the proliferation of Caco-2 intestinal cell line, Trypan blue dye exclusion and a colorimetric MTT assay were used, respectively. Caco-2 human colon cell line was seeded at a cell density of 100,000 cells/ml in 96-well plate, exposed at FB_1 concentrations (from 0.5 to 69 µM) and incubated from 24 to 72 hours. MTT test and viability assay followed the protocols described by Minervini et al [8].

3. 3 Assessment of lipid peroxidation.

Time (from 6 to 24 hours) and dose-dependent effect induced by FB_1 on the lipid peroxidation was assessed using Lipid Peroxidation Assay Kit (Biovision), as reported by Minervini et al [8].

3.4 Fluorescence anisotropy to assess membrane microviscosity after FB_1 exposure

Considering previous references on FB_1-dependent modifications to membrane fluidity [7,11] assessed by fluorescence anisotropy, we applied this method in order to assess the microviscosity of intestinal cell line after FB_1 exposure.

Time (from 6 to 24 hours) and dose-dependent experiments, using cationic derivative of diphenylhexatriene, TMA-DPH probe, and calculation of anisotropy values, were performed following the protocol described by Minervini et al [8].

4 Statistical analysis

Statistical analysis was performed using SigmaPlot software v.11 (Systat Software, Inc. SigmaPlot for Windows). Multiple Comparisons Dunn's Method was used to evaluate the significant difference between FB_1 concentrations and control samples. Values of $p<0.05$ were considered statistically different.

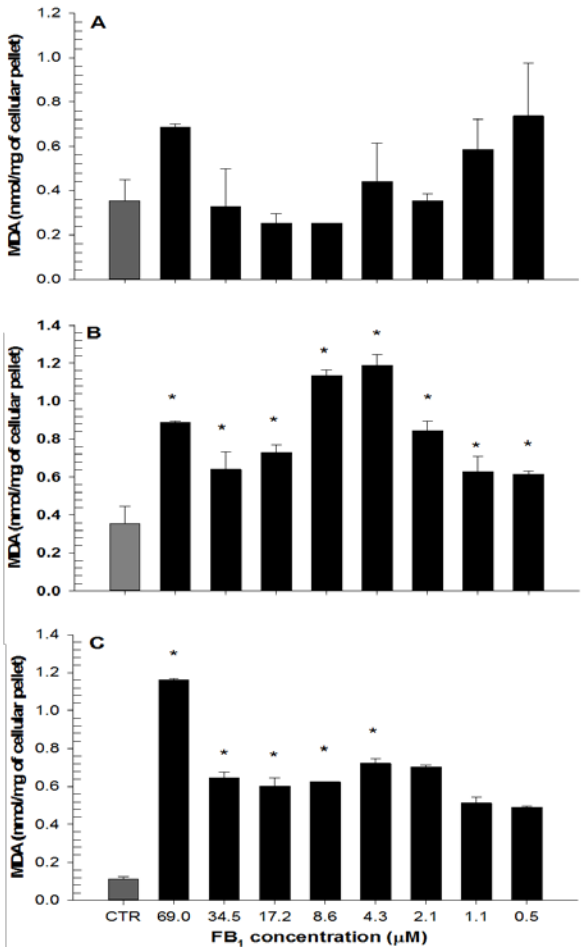

Figure 1: Lipid peroxidation assessed as malondialdehyde (MDA) production induced by dose- and time-dependent exposure (A, B and C after 6, 12 and 24 hours, respectively) of human intestinal cell line Caco-2 to fumonisin B_1. Data are expressed as mean ± standard deviation of three independent experiments. *p<0.05 with respect to control sample assessed by Dunn's method.

Results and Discussion

1 Assessment of cell viability and proliferation

Time- and dose-dependent experiments were carried out in order to assess the effect of exposure to FB_1 (24, 48 and 72 hours) on cell viability and proliferation of intestinal Caco-2 cell line. Exposure to FB_1 did not affect cell viability (79% ± 6 after exposure to 69 μM of FB_1 vs. 92% ± 9.9 in control sample) after each time incubation tested in the protocol, in agreement with data reported by Caloni et al [9]. Effect of FB_1 on cell proliferation was not marked and did not exceed 30% of inhibition. A significant ($p<0.05$ vs. control) but dose-independent reduction in cell proliferation was observed after 24 hours exposure at FB_1 concentrations ranging from 4.3 to 69 μM. No influence of FB_1 on cell proliferation was observed after 48 and 72 hours of exposure. Our results are not in agreement with the inhibition of cell proliferation found on Caco-2 cells by Kouadio et al [5] and the discrepancy is probably related to different culture conditions.

2 Assessment of lipid peroxidation

Fumonisin B_1 induced time-related redox imbalance on intestinal cell line. An early induction of lipid peroxidation was observed on Caco-2 cell line after 6 hours of incubation with FB_1. As reported in Figure 1A, an increase in MDA production, ranging from 0.3 to 0.7 nmol/mg of cellular pellet, was observed. The rise was not related to FB_1-concentration. A significant ($p<0.05$) production of MDA, ranging from 0.6 to 1.2 nmol/mg of cellular pellet, was observed after 12 hours of exposure to FB_1 at each concentration tested (Figure 1B). Similar significant ($p<0.05$) MDA production was observed after 24 hours of exposure to FB_1 with an evident dependence in the range from 2.1 to 69 μM of FB_1 (Figure 1C). These results were in agreement with the strong oxidative stress (assessed as MDA production after 24 hours) reported by Kouadio et al [5], on Caco-2 intestinal and by Ferrante et al [11] on macrophage cell lines, although at different FB_1 concentrations.

3 Assessment of membrane microviscosity after FB_1 exposure by fluorescence anisotropy

Fumonisin B_1 induced a significant ($p<0.05$) early increase in Caco-2 cell membrane microviscosity (recorded as 10% reduction in fluorescence polarization) after 6 hours of exposure at all tested concentrations (from 0.5 to 69 μM). After increased exposure times (12 and 24 hours), FB_1 did not induce any change in membrane microviscosity. Similar results were found by Ferrante et al [11] on macrophage cell line, but with higher FB_1 levels (up to 10 μM) and longer exposure time (24 hours).

In our previous study [8] performed on HT-29 human intestinal cell line, different time-dependent toxic effects of FB_1 was found in respect with the present data on Caco-2 cells. In fact, HT-29 intestinal cell line exposed to FB_1 showed late inhibition of cell proliferation, a rise of the oxidative status together with an increase of membrane fluidity. These effects are probably related

to FB_1 intracellular absorption, as also demonstrated by our microscopic confocal observations [8] and consequent inhibition of the sphingolipid metabolism, as reported by Schmeltz et al [12]. By contrast, the earlier toxic effect induced by FB_1 on Caco-2 cells, reflected by the increase in microviscosity and induction of lipid peroxidation, could be consequent to the interaction between FB_1 and the cell membrane. As reported by several Authors [6,7], the interaction with FB_1 leads to modifications in the lipid composition and functionality of the cell membrane. Yin et al [13] indicated that the interaction between of the FB_1 and lipid bilayers has multiple effects, such as modification of the membrane structure and influence on the cell membrane's oxygen transport properties. The results reported in this study confirmed the existence of different toxic mechanisms cell/organ type-related induced by FB_1 [7,14].

Conclusion

The toxic mechanisms induced by FB_1 were different in relation to cell/organ type. On Caco-2 human intestinal cell line, in our conditions, lipid peroxidation and modification of membrane microviscosity were the early toxic mechanisms induced by FB_1 probably related to the direct interaction between mycotoxin and cellular membrane. Such toxic effects were observed at FB_1 concentrations (up to 0.5 μM corresponding to 0.4 ppm) much higher than the mean FB_1 food levels reported by SCOOP [15] in Europe (from 31.5 to 74.2 ppb).

Acknowledgement

This work was supported in part by EC KBBE-2007-222690-2 MYCORED.

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