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# Toxic Mechanisms Induced by Fumonisin $B_1$ Mycotoxin on Human Intestinal Cell Line

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**Abstract** The gastrointestinal tract is the main target of exposure to mycotoxin fumonisin $B_1$ ($FB_1$), common natural contaminant in food. Previous studies reported that proliferating cells are more sensitive than confluent cells to the toxic effect of $FB_1$. This study aims to investigate, by dose- and time-dependent experiments on human colon proliferating intestinal cell line (HT-29), the modifications induced by $FB_1$ at concentrations ranging from 0.25 to 69 μM. The choice of highest $FB_1$ concentration considered the low toxicity previously reported on intestinal cell lines, whereas the lowest one corresponded to the lower $FB_s$ levels permitted by European Commission Regulation. Different functional parameters were tested such as cell proliferation, oxidative status, immunomodulatory effect and changes in membrane microviscosity. In addition $FB_1$-FITC localization in this cell line was assessed by using confocal laser scanning microscopy. Lipid peroxidation induction was the main and early (12 h) effect induced by $FB_1$ at concentrations ranging from 0.5 to 69 μM, followed by inhibition of cell proliferation (up to 8.6 μM), the immunomodulatory effect (up to 17.2 μM), by assessing IL-8 secretion, and increase in membrane microviscosity (up to 34.5 μM). The toxic effects observed in different functional parameters were not dose-dependent and could be the consequence of the $FB_1$ intracytoplasmatic localization as confirmed by confocal microscopy results. The different timescales and concentrations active of different functional parameters could suggest different cellular targets of $FB_1$.

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Fumonisin $B_1$ ($FB_1$) is a mycotoxin produced mainly by isolates of *Fusarium verticillioides* and *F. proliferatum*, the most common fungi growing on maize. Human exposure estimates for fumonisins (FBs) ranged from 0.017 to 0.089 μg/kg body weight/day in Canada and the United States, to 14 μg/kg body weight/day in South Africa (IPCS 2000). The International Agency for Research on Cancer (IARC) classified $FB_1$ and $FB_2$ in group 2B (possible carcinogenic to humans) because there is no direct established causal association between $FB_1$ exposure and cancer in humans (WHO and IARC 1993). Several in vivo studies performed in laboratory animals reported the development of cancer in liver and kidney (IPCS 2000). However, controversies regarding the toxic and carcinogenic properties of the FBs were reported by Gelderblom and Marasas (2012) suggesting that further studies on modulating role of dietary constituents should be performed. Contradictory results on $FB_1$ genotoxicity by using different tests have been reported. Recently, Theumer et al. (2010) reported a genotoxic effect induced by $FB_1$ on spleen mononuclear cells using Comet and micronuclei tests.

The kinetics and metabolism of $FB_1$ in human are of interest because, although $FB_1$ is poorly absorbed by the intestinal system when dosed orally, it is highly toxic. This

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condition was qualified by Shier (2000) as the "fumonisin paradox." To date there have been no reports about the kinetics and metabolism of $FB_1$ in humans. $FB_1$ was rapidly eliminated from plasma with a half-life of 40 min in vervet monkeys when dosed by intravenous injection (Shepard et al. 1994). Low levels of residues were recovered in skeletal muscle (1 %), liver (0.4 %), brain (0.2 %), and kidney (0.1 %), whereas analysis of faeces, intestinal contents, and urine samples showed >90 % of $FB_1$ and its hydrolysis products (Shepard et al. 1994). Although major organs did not retain large amounts of the toxin or its residues, the liver, which plays the major role in eliminating $FB_1$ from the circulation, retained the highest level. This release transfers $FB_1$ from plasma into the bile, and large amount of toxin (10.5 % of $FB_1$ dose) was still contained within the gall bladder after 24 h and was still biologically active (Shephard et al. 1995).

Fumonisin is excreted mainly in the faeces either unchanged or depleted of one ester-linked tricarballylic acid. Only traces of aminopentol, the fully hydrolyzed $FB_1$, are found in faeces. Van der Westhuizen et al. (2011) reported urinary $FB_1$ excretion of <0.075 % in humans. Although no hydrolyzed product has been found in urine or bile, it is assumed that hydrolysis occurs in the gut and is probably performed by microorganisms (Bouhet and Oswald 2007). Moreover, after ingestion of mycotoxin-contaminated food or feed, intestinal cells could be exposed to a high concentration of $FB_1$ (Bouhet and Oswald 2007). In fact, 24 h after administration of radiolabeled $FB_1$, intestinal epithelial cells of nonhuman primates contained 25 % of the dose (Shephard et al. 1995). The toxicity of $FB_1$ is due to the inhibition of ceramide synthase through its free amino group because of its chemical similarity to sphingoid bases (such as sphingosine and sphinganine). Ceramide synthase is a key enzyme in de novo sphingolipid biosynthesis and in the sphingolipid salvage pathway. Its inhibition by $FB_1$ induces the increased intracellular sphinganine and sphingosine ratios (Soriano et al. 2005). Free sphingoid bases and sphingolipid degradation products are mediators of growth, differentiation, and cell death (Riley et al. 2001). Thus, $FB_1$ could indirectly modify the biological properties of plasma membrane by inducing changes in its phospholipid composition (as consequence of ceramide synthase inhibition) (Theumer et al. 2008) and could have a direct effect on the cellular membrane structure (due to its amphipathic sphingoid-like structure) with consequent modification of the cell membrane (Yin et al. 1996).

Several other mechanisms, consequent to the inhibition of ceramide biosynthesis, have been invoked and have been correlated with oxidative stress and apoptosis induction (Kouadio et al. 2005). A recent study using transmission and scanning electron microscopy showed immuno-localization of $FB_1$ within mitochondria, nucleus, and nucleolus of oesophageal cancer cells and considered these organelles to be specific targets (Myburg et al. 2009). Similarly, on astrocytes and neuroblastoma cells, mitochondrial complex I inhibition, depolarization of the mitochondrial membrane, and deregulation of calcium signaling have been observed, thus confirming mitochondria as targets of $FB_1$ (Domijian and Abramov 2011). All of these results suggest that $FB_1$ exerts its biological effect by binding to cellular macromolecules or membrane components within the affected organelles as hypothesized by Myburg et al. (2009).

Because intestinal epithelium could be the primary target of $FB_1$, HT-29 cells could be a useful model for studying the influence of $FB_1$ toxicity. Previous studies have reported that proliferating cells are more sensitive than confluent cells to the toxic effect of $FB_1$ (Bouhet et al. 2004; Riley et al. 2001; Schmelz et al. 1998; Yoo et al. 1996) because there is a continuous dynamic turnover of sphingolipids in growing cells as well as an ongoing demand for their de novo synthesis. The intestinal epithelium not only represents a physical barrier but also participate actively in inflammatory response. In fact, intestinal epithelial cells, in response to inflammatory stimuli, release chemokines and other proinflammatory molecules that provoke an acute inflammatory response. The immunotoxicity of $FB_1$ has been less documented. The available data indicate that $FB_1$ affects innate and humoral immunity, and cell response (Mahmoodi et al. 2012). These immunomodulatory effects of $FB_1$ may result from changes in the expression of cell surface molecules, which are important in immune cell communication (Bouhet et al. 2006) and/or may be due to changes in various cytokines and chemokines in the immune system (Mahmoodi et al. 2012).

Considering the main targets of $FB_1$, such as cellular membrane and oxidative status, the dose- and time-dependent effect induced by $FB_1$ on cell proliferation, lipid peroxidation status, cytokine profile, and membrane microviscosity, was assessed on a human intestinal cell line. In addition, $FB_1$ localization in the cell line was assessed using confocal laser scanning microscopy. In order to evaluate the toxic mechanisms induced by $FB_1$ on its main targets, in this study $FB_1$ concentrations, that did not correspond to the natural levels of human exposure through food as has also been performed in previous in vitro experiments, were tested (Bouhet et al. 2004; Caloni et al. 2002; Schmelz et al. 1998).

## Materials and Methods

### Reagents

McCoy's 5A medium, Dulbecco's phosphate-buffered saline (PBS), trypsin-ethylene diamine tetraacetic acid (EDTA) solution, L-glutamine 200 mM, antibiotic and

antimycotic solution, thiazolyl blue tetrazolium bromide (MTT), trypan blue solution, and 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). Foetal bovine serum (FBS) was purchased from Gibco (Milan, Italy). $FB_1$ standard was purchased from Orsell (Modena, Italy). Fluorescein-labeled $FB_1$ was purchased from Aokin (Berlin, Germany). Lipid Peroxidation [malondialdehyde (MDA)] Assay Kit (Biovision) was purchased from Vinci-Biochem (Florence, Italy). OptEIA set human interleukin (IL)-8 was purchased from BD Bioscience Pharmigen (Milan, Italy).

### Human Intestinal Cell Line

The human colon cell line HT-29 (ECACC; Sigma Aldrich) was grown in 25 $cm^2$ flasks at a starting density of 250,000 cells/mL in McCoy's 5A medium with 3 g/L glucose supplemented with 10 % of FBS, 1 % of L-glutamine, and 1 % of antibiotic and antimycotic solution. Cells were harvested twice a week up to 70–80 % confluence using trypsin-EDTA solution. Cell density and viability were determined by dilution in trypan blue with Bürker's chamber. The cells used for experimental protocols showed a mean viability of 90 %.

### Evaluation of Toxicity Induced by $FB_1$ on Intestinal Model

#### *$FB_1$ Preparation*

$FB_1$ (5 mg) was dissolved in PBS at a concentration of 1,380 μM (stock solution) and stored at −20 °C. The stock solution was diluted 1:10 in culture medium, and serial dilutions (1:2) were performed starting from a concentration of 69 μM. The selection of the greatest $FB_1$ concentration considered the toxicity found in previous in vitro studies on intestinal cell lines (Bouhet et al. 2004; Caloni et al. 2002; Schmelz et al. 1998).

#### *Assessment of Cell Viability and Proliferation*

The effect of $FB_1$ on cell viability and proliferation of HT-29 cells was assessed using Trypan blue dye exclusion and a colorimetric MTT assay, respectively. Absorbance was measured at 580 nm in a spectrophotometric plate reader [enzyme-linked immunosorbent assay (ELISA) Reader Multiskan MS Plus MK II; Labsystem, Helsinki, Finland] as previously reported by our unit (Minervini et al. 2005). Briefly, HT-29 cells were seeded at a cell density of 500,000 cells/mL in a 96-well plate, exposed to $FB_1$ concentrations (from 1.1 to 69 μM), and incubated from 24 to 72 h. The selection of these incubation times was based on previous studies performed on this cell line (Schmelz et al. 1998). Control samples consisted in 100 μL of cell suspension and 100 μL of complete medium supplemented with 10 % of PBS. Each mycotoxin concentration and control were tested in six wells of the plate. For each incubation time and $FB_1$ concentration, three independent experiments were performed.

#### *Assessment of Lipid Peroxidation*

Lipid peroxidation induced by $FB_1$ was assessed by using a Lipid Peroxidation Assay Kit (Biovision), a tool for the sensitive detection of MDA, which is an end product of lipid peroxidation. The MDA in the sample reacted with thiobarbituric acid (TBA) to generate the MDA-TBA adduct, which can be easily quantified colorimetrically with sensitivity as low as 0.1 nM/well. MDA determination was performed according to the manufacturer's instructions.

Time- (6–24 h) and dose-dependent (0.25–69 μM) experiments were performed on HT-29 intestinal cell line: 900 μL/well of HT-29 cells ($1 \times 10^6$ cells/mL) were seeded in a 24-well plate and supplemented with 100 μL of different concentrations of $FB_1$ toxin. For each incubation time and $FB_1$ concentration, three independent experiments were performed. The incubation times range was selected according to previous experiments using a specific inductor (hydrogen peroxide 9 mM). Negative control samples were also prepared by adding to 900 μL of HT-29 cells 100 μL of complete medium with 10 % of PBS. Cells were trypsinized and centrifuged, and the pellets (10 mg) were homogenized in 300 μL of distilled water with 9 % of Triton X-100 for 15 min. Cell samples were centrifuged (13,000×*g* for 10 min) to remove insoluble material, and 200 μL of the supernatant from each homogenized sample were transferred into a microcentrifuge tube. At the same time, the different dilutions (0.4–20 nM) of MDA standard were prepared to obtain the standard curve. Then 600 μL of TBA solution were added, and each standard concentration and cell samples was incubated for 60 min at 95 °C. The samples were cooled to room temperature in an ice bath for 10 min, and 200 μL from each sample were transferred into a 96-well microplate for colorimetric analysis (532 nm) using a Reader Multiskan MS Plus MK II ELISA reader (Labsystems, Helsinki, Finland).

#### *Assessment of Immunomodulatory Effect Induced by $FB_1$*

To evaluate the impact of $FB_1$ on the immune system at the intestinal level, we evaluated the effect of the mycotoxin on IL-8 production by HT-29 cells with and without LPS stimulation as inflammatory cytokine induction. Preliminary experiments were performed to verify the

intestinal cell line's sensibility to LPS. HT-29 cells ($1 \times 10^6$ cells/mL) were exposed for 24 h to 1 and 10 μg/mL LPS, and cell culture supernatants were collected for the quantification of IL-8. Subsequently, dose- (1.1–69 μM) and time-dependent (24–48 h) experiments were performed: 900 μL/well of HT-29 cells were seeded in a 24-well plate, supplemented with 100 μL of different concentrations of $FB_1$ toxin, and incubated for 24 and 48 h alone or in combination with LPS at a final concentration of 1 μg/mL. For each incubation time and $FB_1$ concentration, three independent experiments were performed. The selection of incubation times with $FB_1$ was performed considering both previous studies on different cell lines (Mahmoodi et al. 2012) that the cytotoxicity found on this cell line in this study after longer incubation times.

The plates were centrifuged and the cell culture supernatants collected and tested with ELISA kits (BD OptEIA set human IL-8) for the quantification of IL-8. Cytokine production was measured according to the manufacturer's instructions, and the cytokine concentration of the samples was calculated by a standard curve obtained by linear regression analysis of purified IL-8 concentration versus the optical density in a double logarithmic plot.

### *Fluorescence Anisotropy to Assess Membrane Microviscosity After $FB_1$ Exposure*

Assessment of plasma membrane microviscosity was performed by fluorescence anisotropy. The cationic derivative of diphenylhexatriene, TMA-DPH probe, incorporated very rapidly (1 min) into the plasma membrane of whole living cells and remained restrictively localized therein for at least 30 min (Kuhry et al. 1985). Fluorescence polarization is detectable with a monochromatic light source that passes through a vertical polarizing filter and excites fluorescent molecules in the sample tube. Only those molecules that are oriented properly in the vertically polarized plane absorb light, become excited, and subsequently emit light, which is measured in both the horizontal and vertical planes. This assay was applied to intestinal cells to evaluate the changes in membrane fluidity after $FB_1$ exposure. This mycotoxin does not interfere with the spectroscopic properties of TMA-DPH as reported by Theumer et al. (2008). In this study, HT-29 cells at a density of $1.25 \times 10^5$ cells/mL were incubated in 1 mL of complete medium in the absence or presence of different concentrations of $FB_1$ (1.1–69 μM) for 6–24 h. Control samples were prepared with 10 % PBS in medium culture. The medium was removed by centrifugation (2,500 rpm for 4 min), and the cells were diluted in PBS and labeled with TMA-DPH ($2.5 \times 10^{-6}$ M) for 1 min. For each incubation time and concentration, three independent experiments were performed. The selection of these incubation times useful to evaluate membrane microviscosity was previously verified using specific inductor (Tween-20 600 μg/mL) and also considered results reported in previous studies (Ferrante et al. 2002).

Fluorescence was measured with a Perkin Elmer LC-240 fluorescence detector equipped with a xenon lamp and automated polarizer. Excitation and emission wavelengths were set to the optimal intensities for TMA-DPH excitation (340 and 425 nm, respectively) (Haugland 1996). Membrane microviscosity was measured by determining the ratio between emission intensities parallel and perpendicular to the excitation plane (Shinitzky and Barenholz 1978). Ten anisotropy values for each sample were measured continuously in a 1.4 mL fluorescence quartz cuvette and corrected for background light-scattering and autofluorescence. A higher anisotropy value, $r$, corresponds to lower lipid mobility. Steady-state fluorescence anisotropy ($r$) was calculated as follows (Eq. (1)):

$$r = \frac{Ivv - IvhG}{Ivv + 2IvhG} \qquad G = \frac{Ihv}{Ihh} \tag{1}$$

where $I_{VV}$, $I_{HH}$, $I_{VH}$, and $I_{HV}$ are the values from different fluorescence intensity measurements taken with polarizers either both vertical (VV) or both horizontal (HH) or, elsewhere, the excitation polarizer is vertical and the emission polarizer horizontal (VH) and vice versa (HV). $G$ is a correction factor for differences in detection system sensitivity for vertically and horizontally polarized light. For each concentration of $FB_1$ and incubation time, three independent experiments were performed.

### *Localization of $FB_1$ in Intestinal Cell Line by Using Confocal Laser Scanning Microscopy*

HT-29 cells were seeded on glass coverslips at a cellular density of 50,000 cells/mL. After reaching 70–80 % of confluence, a time-course exposure (5 min to 24 h) of intestinal cell line was performed using 10 μL of fluorescein (FITC)-labelled $FB_1$ ($1.22 \times 10^{-7}$ M). Cell monolayers were rinsed twice with cold PBS and fixed with 4 % *p*-formaldehyde in PBS for 20 min at 4 °C. Cells were observed at 600× magnification in oil immersion with a laser-scanning confocal microscope (C1/TE2000-U Nikon) equipped with an Argon Ions 488 laser and a 495–519 (B2-A) nm excitation/emission filter. Parameters related to fluorescence intensity (60 × objective, zoom = 0.30 mm pinhole size) were maintained at constant values for all measurements.

### Statistical Analysis

Statistical analysis was performed using SigmaPlot software v.11 (SigmaPlot for Windows; Systat San Jose, California, USA). Multiple-comparison Dunn's method was

used to evaluate the significant difference between cells samples treated with and without (control samples) $FB_1$ concentrations. Values of $p < 0.05$ were considered statistically different.

## Results

### Assessment of Cell Viability and Proliferation

The highest $FB_1$ concentration significantly ($p < 0.05$) affected cellular viability (77 ± 3.3 % vs. 88 ± 3 % in control samples) of HT-29 after 72 h of exposure. Other $FB_1$ concentrations and decreased incubation times did not affect the cellular viability (data not shown). The effect induced by $FB_1$ on cell proliferation did not exceed 30 % of inhibition. As seen in Fig. 1, after 72 h exposure the HT-29 cell line proved to be sensitive to $FB_1$ concentrations: in fact, significant ($p < 0.05$ vs. control) inhibition of cell proliferation was observed from 8.6 to 69 μM of $FB_1$. Shorter exposure time (24 and 48 h) to $FB_1$ did not significantly modify cell proliferation in HT-29 cells (data not shown).

### Assessment of Lipid Peroxidation

A significant ($p < 0.05$) increase of MDA versus controls was observed only after 12 h of exposure to $FB_1$ concentrations ranging from 0.5 to 69 μM (Fig. 2), whereas after 6 and 24 h, MDA levels were lower than the detection limit for the method (0.1 nM/mg) (data not shown).

Assessment of immunomodulatory effect induced by $FB_1$. As shown in Fig. 3, after 48 ours of incubation HT-29 cells synthesized I-8 levels of 1.6 ± 0.33 and 2.9 ± 0.6ng/mL for unstimulated and LPS-stimulated control samples, respectively. Exposure to $FB_1$ without LPS stimulation did not influence the immune response of HT-29 cells (IL-8 values ranged from 1.56 ± 0.25 to 1.82 ± 0.5 ng/mL). After LPS stimulation, $FB_1$ induced a significant decrease in IL-8 amount versus LPS-stimulated controls ($p < 0.5$) at concentrations ranging from 17.2 to 34.5 μM, thus showing an immunosuppressive effect on the intestinal cell line. After 24 h of incubation with $FB_1$, no modifications of IL-8 secretion were observed with or without LPS stimulation (data not shown).

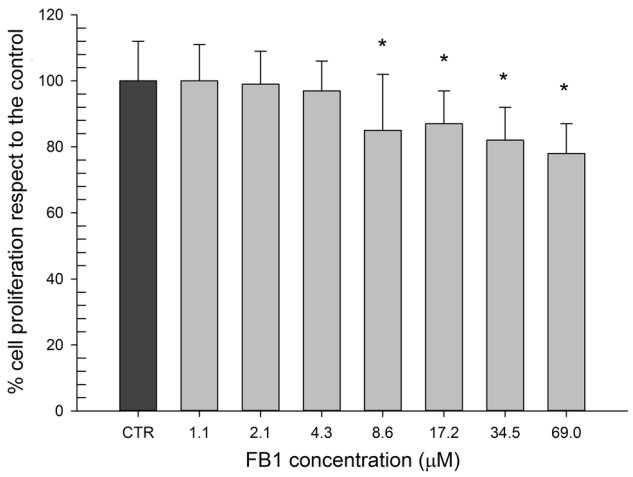


**Fig. 1** Inhibition of HT-29 cell proliferation induced after 72 h of $FB_1$ exposure using MTT test. Data, assessed as percentage of control samples, are expressed as mean ± SD; ($n = 24$). *$p < 0.05$ with respect to controls as assessed by Dunn's methods

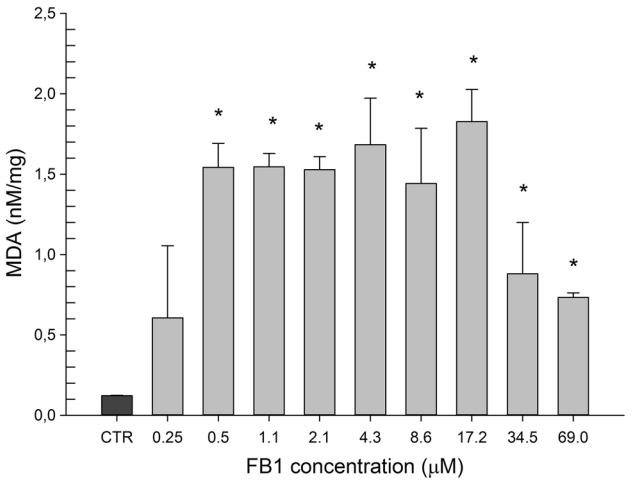


**Fig. 2** Lipid peroxidation induced by $FB_1$ after 12 h of exposure of HT-29 intestinal cell line assessed as quantification of MDA levels (nM/mg). Data are expressed as mean ± SD ($n = 24$). *$p < 0.05$ with respect to control samples assessed by Dunn's method

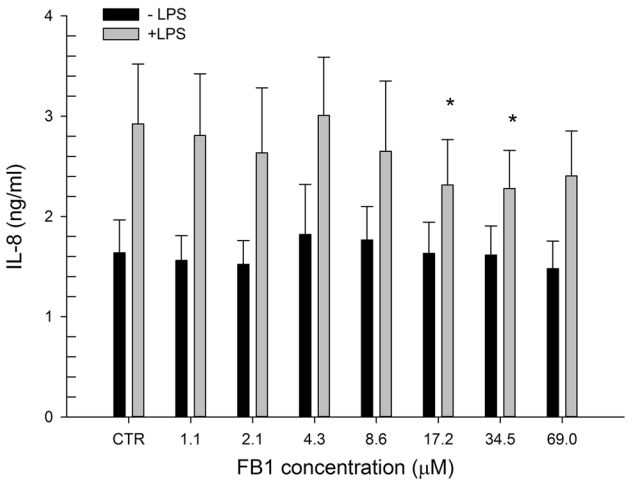


**Fig. 3** IL-8 secretion (ng/ml) by HT-29 cell line stimulated with and without LPS after 48 h of $FB_1$ exposure using immunoenzymatic bioassay. Data are expressed as mean ± SD ($n = 24$). *$p < 0.05$ with respect to control samples assessed by Dunn's method

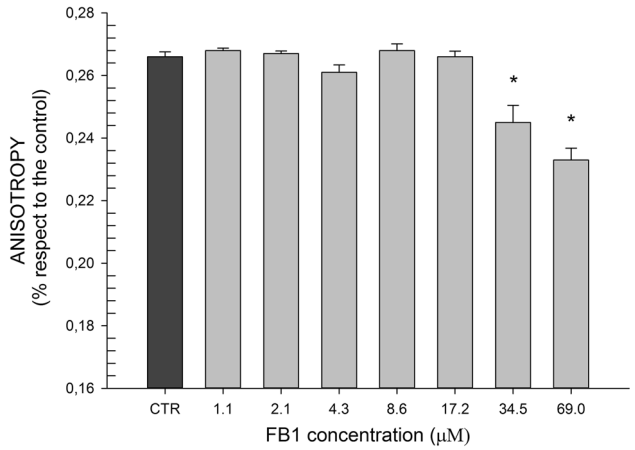


**Fig. 4** Modifications of membrane microviscosity induced by $FB_1$ after 24 h exposure of HT-29 intestinal cell line as assessed by fluorescence anisotropy of TMA-DPH probe. Data are expressed as mean ± SD ($n = 24$). *$p < 0.05$ with respect to control samples assessed by Dunn's method

### Assessment of Membrane Microviscosity After $FB_1$ Exposure by Fluorescence Anisotropy

The effect induced by $FB_1$ on membrane microviscosity of HT29 cells was observed only after long-term exposure (24 h); 34.5 and 69 μM levels of $FB_1$ significantly ($p < 0.05$) increased membrane microviscosity, which was recorded as a decrease in fluorescence polarization of 13 and 8 %, respectively (Fig. 4). No effect on membrane microviscosity induced by $FB_1$ was observed at shorter incubation times (data not shown).

### Localization of $FB_1$ Into Intestinal Cell Line Using Confocal Fluorescence Microscopy

After 30 min of incubation, fluorescence of $FB_1$-FITC was detected in HT-29 cells. As shown in Fig. 5, in treated samples a marked staining in light green was observed within the cytoplasm and membrane compartments with a major accumulation along the plasma membrane. This fluorescence was weakly present up to 24 h, whereas no staining was detected in negative controls (Fig. 5).

## Discussion

$FB_1$ is a common mycotoxin contaminant of corn, and humans are continuously exposed to low doses (WHO and IARC 1993). The large amount of $FB_1$ detected in faeces, associated with its low bioavailability, showed that the gut is a primary target because it is exposed to high concentrations of $FB_1$. Previous in vitro studies on human (Caco2 and HT-29) and porcine (IPEC-1) intestinal cell lines showed a different $FB_1$ toxicity on cellular proliferation, apoptosis induction, oxidative status, and barrier integrity (Bouhet et al. 2004; Caloni et al. 2002; Kouadio et al. 2005; Schmelz et al. 1998). The results of the present study aimed to assess toxicity induced by $FB_1$ on different functional parameters of human intestinal cell line (HT-29) using dose- and time-dependent experiments.

The inhibition of cell proliferation found in the intestinal cell line did not exceed 30 %, and there was high variability between experiments, probably due to the variable accumulation in cells of specific end-products and intermediates of the sphingolipid pathway consequent to the balance between anabolic and catabolic processes (IPCS 2000). Theoretically, at any specific time, the balance between the intracellular concentration of sphingolipids, which protect cells from apoptosis (decreased ceramide, increased sphinganine-1-P), and the concentration of effectors that induce apoptosis (increased ceramide, free sphingoid bases, fatty acids, or specific glycosphingolipids) determines a cell response (Riley et al. 2001). In this study, the results obtained on the inhibition of cellular proliferation of HT-29 cells were partially in line with data reported

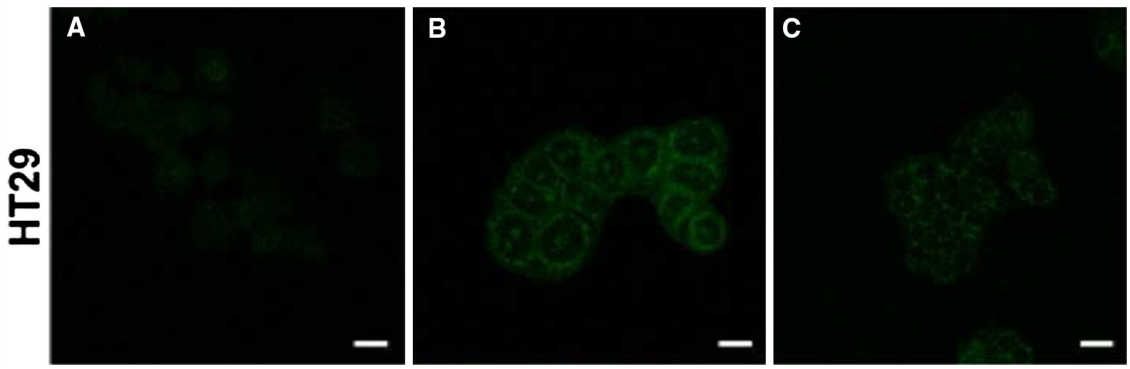


**Fig. 5** Localisation of $FB_1$-FITC in HT-29 intestinal cell line as assessed by confocal microscopy observation. **a** HT-29 control. **b** After 30 min of exposure. **c** After 24 h of exposure. *Scale bar* represents 20 μm **a** and **b** or 30 μm **c**

by Schmelz et al. (1998). These differences were probably related to the different experimental protocols and culture conditions used. Similar results were reported by Bouhet et al. (2004) on porcine intestinal cell line, whereas high sensitivity of a Caco2 intestinal cell line was found by Kouadio et al. (2005) with an $IC_{50}$ value of 20 μM. The effect of $FB_1$ on the inhibition of proliferation of other cell lines, as determined using MTT test, was different and was probably related to cell type (Ferrante et al. 2002; Galvano et al. 2002a, b; Liu et al. 2002; Meli et al. 2000; Minervini et al. 2004).

$FB_1$ influenced intracellular redox status with early induction of lipid peroxidation observed on HT-29 cells at lower tested $FB_1$ concentrations. These results confirmed the hypothesis, as reported by other investigators (Abado-Becognee et al. 1998; Ferrante et al. 2002; Kouadio et al. 2005; Meca et al. 2010; Mobio et al. 2003), that lipid peroxidation is one of most sensitive mechanisms whereby $FB_1$ affects the toxicity of different cell lines after 24 h of exposure. In addition, in this study MDA was a "sensitive" biomarker of oxidative imbalance with respect to GSH content as reported by other investigators in different cell lines where a greater increase in MDA production, rather than GSH depletion, was found in glioblastoma cells after $FB_1$ in vitro exposure (Stockmann-Juvala et al. 2004a, b). Studies exploring $FB_1$ toxicity provided discordant results [no effect or increased reactive oxygen species (ROS) production] on human and rat neural cells (Stockmann-Juvala et al. 2004a, b; Galvano et al. 2002a, b).

Concerning the modulation of inflammatory processes on intestinal cell line, $FB_1$ induced a decrease in IL-8 synthesis only after exposure to LPS stimulation of HT-29 cells, which is in agreement with results obtained on porcine intestinal epithelial cell line IPEC-1 and on human gastric and colon cell lines, although these were observed after longer incubation times and lower $FB_1$ concentrations (Bouhet et al. 2006; Mahmoodi et al. 2012). IL-8 is a potent chemoattractant to the site of infection or injury resulting in microbicidal activity involving both the oxidative and non-oxidative pathways in host defense systems. IL-8 also induces degranulation, respiratory burst, and leukotriene B4 release by polymorphonuclear leukocytes (PMNs). IL-8 activity is also directed toward intestinal epithelial cells because this interleukin enhances cell proliferation and controls the repair processes during injury to the intestinal mucosa or during cytotoxic stress (Mahmoodi et al. 2012). A decrease in IL-8 response could be responsible for a decrease in the number of PMNs recruited to the lamina propria and/or a functional decrease in these PMNs, thus leading to impaired elimination of bacteria from the intestinal tract (Bouhet et al. 2006). In fact, in $FB_1$-treated pigs increased intestinal colonization by pathogenic *Escherichia coli* was reported by Bouhet et al. (2006).

Fluorescence polarization, which was previously used to assess $FB_1$-dependent modifications to membrane microviscosity (Ferrante et al. 2002; Theumer et al. 2008), was used on intestinal cell line. A significant increase in microviscosity was induced on HT-29 cells after 24 h of $FB_1$ exposure at the highest concentrations. Previous studies (Ferrante et al. 2002; Yin et al. 1998) indicated that $FB_1$ can disturb membrane structure and affect oxygen-transport properties of membranes. In fact, modifications in membrane fluidity are known to be linked to alterations in the physiological process of cell membrane-mediated transport, in the activities of membrane bound enzymes, and in receptor-binding phagocytosis, endocytosis, cytotoxicity, and cell growth (Ferrante et al. 2002). In previous studies (Ferrante et al. 2002; Yin et al. 1998), a contemporaneous occurrence of disrupted membrane structure and lipid peroxidation was found after 24 h, and this was explained as a consequence of increase in oxygen diffusion-concentration products as well as the enhancement effects on membrane permeability. In this study, the different timescales and $FB_1$ concentrations that affected lipid peroxidation and anisotropy in HT-29 cells could suggest different cellular targets of $FB_1$. The early induction of lipid peroxidation observed on HT-29 cells could be due to the involvement of mitochondria as reported by Domijian and Abramov (2011). In fact, in neuronal cells $FB_1$ impaired mitochondrial functions and increased cROS levels without influence on cell viability. In addition, Myburg et al. (2009) showed the immunolocalization of $FB_1$ in mitochondria, nucleus, and nucleolus of oesophageal cancer cells. Our results on membrane and intracytoplasmatic $FB_1$-FITC localization in HT-29 cells are in line with results reported by Myburg et al. (2009), although different staining procedures were used, and FITC labeling may determine slight physicochemical modifications of $FB_1$.

On HT-29 cells, early intracellular localization of $FB_1$-FITC, lipid peroxidation, and lack of effect on cell viability were found; on the contrary, a late increase of microviscosity (observed after 24 h at the highest $FB_1$ concentrations) could be explained as an indirect effect on modifications in the phospholipid composition consequent to the enzyme inhibitor-type interactions (Theumer et al. 2008). This supposition was shown by Schmelz et al. (1998) on HT29 cell line as an increase in sphinganine with its half-life inside cells being much more longer than the half-life of $FB_1$ as found by Riley et al. (1998), who reported on rat kidney cell line (LLC-$PK_1$). In fact, $FB_1$ not only blocks biosynthesis of complex sphingolipids, but it also causes accumulation of sphingoid bases, which in turn can alter plasma membrane composition and function (Ferrante et al. 2002). Consequently, because lipid-protein interactions play a crucial role in maintaining the structure

and function of integral membrane proteins and receptors, modifications of cellular membrane could influence the cell surface molecules important in immune cell communication (Bouhet et al. 2006) as shown by the inhibition of IL-8 secretion by HT-29 cells after $FB_1$ exposure.

In conclusion, lipid peroxidation—followed by modifications to membrane microviscosity and inflammatory response—was the main and most sensitive effect of $FB_1$ on human intestinal cell line. The lowest $FB_1$ concentration that affected lipid peroxidation [$\leq$0.5 μM (corresponding to 400 ppb)] was 10 times greater than the mean $FB_1$ food levels reported by SCOOP (2003) in Europe (31.5–74.2 ppb) and corresponded to conditions not reproducible in nature. Further studies will be performed to assess lipid peroxidation induced by $FB_1$ in the intestinal system.

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