Aflatoxin M1 causes cytotoxicity and intestinal epithelial cell integrity damage in differentiated human Caco-2 cells

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Abstract

Aflatoxin M1 (AFM1) is a mycotoxin that is commonly found as a milk contaminant, and its presence in milk has been linked to cytotoxicity. The present study aimed to evaluate the acute cytotoxic effects of AFM1 on intestinal CaCo2 cells. Initially, we checked the morphology and viability of CaCo2 cells after treatment with different concentrations of AFM1 (5ng/L, 50ng/L, 250ng/L, 500ng/L, 1000ng/L, and 2000ng/L) for different time intervals (6hrs, 12hrs, and 24hrs). It was found that AFM1 didn't show any effect on cell morphology and viability. Further, DCFDA assay showed increased ROS production after 6hrs treatments. qPCR analysis showed an increased expression of epithelial specific cytoskeleton markers Cytokeratin, Villin, Vimentin, and JAM1, and a decreased expression of tight junction proteins, Claudin, Occludin, and ZO1. Similarly, we found an increased expression of Cyp1a1 transcript with an increasing AFM1 concentration and incubation time. This gene expression analysis showed AFM1 can causes disruption of gap junctions between intestinal cells, which was further confirmed by a transwell experiment. In conclusion, consumption of AFM1-contaminated milk doesn't show any effect on cells morphology and viability but decreases the expression of intestinal barrier transcripts that may leads to the disruption of intestinal barrier function and leaky gut.

Introduction

For better health, maintaining food quality and safety are important challenges. Thus, there is a need for continuous exploration of food contaminants and to study their cytotoxic effects. One such ubiquitous contaminant is mycotoxin. Mycotoxins are low molecular weight, naturally occurring secondary metabolites produced by certain genera of fungi such as Aspergillus, Fusarium, Penicillium and many more (Mary et al., 2015; Kensler et al., 2011). Several studies suggested that the mycotoxins cause various detrimental health effects, such as cancer, hepatic disease, nephropathy, etc. and can also give rise to mutations in humans and animals (Claeys et al., 2020; Ahmed Adam et al., 2017; da Rocha et al., 2014; Sun et al., 2011; Abbès et al., 2010; Stoev et al., 2010; Theumer et al., 2010; Wild et al., 2010).

Aflatoxins, a type of mycotoxin are mainly produced by certain fungal genera like Aspergillus and Penicillium. It is one of the commonest mycotoxins detected in different foodstuffs, feed and fodder (Zhang et al., 2015; Caloni et al., 2006). The animals may consume feed and fodder contaminated with fungal growth and there is a high chance that the animals might get exposed to aflatoxins (Streit et al., 2012; Monbaliu et al., 2010; Gonzalez et al., 2008; Zinedine et al., 2007; Sangare-Tigori et al., 2006). To date, more than 20 types of aflatoxins are known, but the common ones are Aflatoxin B1 (AFB1), Aflatoxin B2, Aflatoxin M1, Aflatoxin M2, Aflatoxin G1 and Aflatoxin G2 (Moss, 2002) and amongst them, AFB1 is the most toxic aflatoxin which causes hepatocarcinogenesis in humans and animals. The liver is the key organ where AFB1 is metabolized into its hydroxylated metabolite, aflatoxin M1 (AFM1), by the Cytochrome P450-associated enzymes; consequently, AFM1 drains into the milk and dairy products (Battacone et al., 2003). For addressing these issues, current research work was designed to assess the cytotoxicity of the most frequently detected mycotoxin, AFM1.

Studies have reported AFM1 as a potent carcinogen in humans, but its carcinogenicity is approximately 10% of that of AFB1. The toxic effects of AFM1 include DNA damage, gene mutation and cell transformation in mammalian cells, insects, dairy animals etc (Prandini et al., 2009; Creepy, 2002; Govaris et al., 2002).

Milk is considered a complete food and is among the most consumed food by humans around the world. Due to its high prevalence in milk, various food regulatory authorities across the world have predetermined a maximum residual limit (MRL) of aflatoxins in milk. The MRL of AFM1 in milk, established by EU and CODEX, is 0.05 µg/kg and 0.5 µg/kg, respectively (Prandini et al., 2009). As AFM1 is stable at higher temperatures, it might be present in pasteurized market milk and dairy products.

India is one of the largest producers and consumers of milk in the world. Food Safety and Standards Authority of India (FSSAI) has reported AFM1 as the predominant milk contamination. Due to the high occurrence of AFM1 in milk, it is necessary to study its cytotoxic effect on human health.

Following the accidental ingestion of food contaminated with aflatoxins especially AFM1, it passes through the gastrointestinal tract (GIT). As the intestinal epithelial cells act as a primary barrier against contaminants they prevent the entry of toxins into the body circulation. A recent In-vitro digestion study suggests that AFM1 digestion is possible at intestinal phase (Kumar et al., 2022). The intestinal epithelium has different types of proteins, resembling a barrier-like structure which helps in maintaining cobblestone morphology of epithelial cells as well as cell-to-cell interaction and performs the function of a barrier. Considering the importance of these protein markers, they may be evaluated further in order to determine the cytotoxicity of different food toxicants.

To understand the cytotoxic effects of food contaminants on intestinal cells, there is a need for an intestinal model system. Among all the available models, cell lines are widely used to study TJ barrier function. As recommended by FDA, Caco-2 cells lines, (Alassane-Kpembi et al., 2015; 2013; Sambuy et al., 2005; Delie and Rubas, 1997), are ideal for studying Tight Junction (TJ) proteins functions and evaluating the effects of drugs and toxins on intestinal barrier function.

Studies have been done on the effect of AFM1 on intestinal cells at higher dose and higher exposure time but none of the study till date reported the acute toxicity effect of AFM1 on intestinal cells at recommended AFM1 MRL. In the present study, the human colon derived cell line, Caco-2 cells, were used as an in vitro model for studying toxicity effects of AFM1. Hence, the current study was designed in order to assess the acute cytotoxicity effect of AFM1 in differentiated CaCo2 cell line model system and to understand the effects of AFM1 on CaCo2 cells' morphology, ROS production viability and intestinal specific transcript marker.

Materials and methods

2.1. Chemicals

AFM1, Dulbecco's modified Eagle's medium (DMEM), Nonessential amino acid (NEAA), 3-(4, 5- dimethyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), and dichlorfluorescein diacetate (DCFH-DA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibiotics and fetal bovine serum (FBS) solution were purchased from Gibco (Thermo-Fisher scientific).

2.2 Caco-2 cell culture

The human adenocarcinoma cell line, Caco-2 cell line having passages no. 25–30 was obtained from National Centre for Cell Science (Pune), India. The Caco-2 cells were cultured in DMEM with higher glucose concentration along with 10% FBS (Gibco), 2 mM glutamine, non-essential amino acids, and antibiotics (100 U/ml of penicillin, 10mg/ml of streptomycin, and 5 mg/ml of amphotericin).

2.3 AFM1 stock solution preparation

The AFM1 toxin was procured from Sigma, and the stock solutions of AFM1 (5µg/mL) were prepared according to the manufacturer's instructions and were stored at − 20°C. AFM1 toxins were dissolved in the DMEM media without FBS, which was added prior to culturing the cells.

2.4 Evaluation of cell viability by MTT assay

The effects of AFM1 on Caco-2 cells' proliferation and viability were evaluated by a dye, tetrazolium salt 3- (4, 5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT). Firstly, CaCo2 cells were seeded in 96 well cell culture plates (25,000 cells/well) and incubated for 48hrs for cells adherence. Once the cells reach 70% confluency, they were treated with different concentrations of AFM1 (5ng/L, 50ng/L, 250ng/L, 500ng/L, 1000ng/L and 2000ng/L) for 6hours, 12hours, 24hours. After completing the incubation time, the culture medium was discarded, and the cell monolayer was washed twice with PBS. Cells were then incubated with 180 µl DMEM and MTT (20µL per well at a concentration of 0.5 mg/ml) for 4hrs at 37°C in a CO2 incubator. After incubating the cells, the supernatant was discarded, and then formazan dissolved in 100 µl of DMSO was added. Plates were gently mixed for 5 min in a shaker, and precautions were taken so that no air bubbles remained present in the plate. The absorbance was measured at 570 nm with a reference wavelength of 630 nm using a 96-well ELISA plate reader. The cells' viability was calculated as viability (%) = (absorbance of treated cells/ (absorbance of control) X 100.

2.5 Cells staining

Caco-2 cells were seeded in 96 well plates at a density of 25,000 cells/well and were allowed to grow for 48 hrs in a cell culture medium. Then the cells were exposed to AFM1 (5ng/L, 50ng/L, 250ng/L, 500ng/L, 1000ng/L and 2000ng/L) for 24 h, and cell morphology was examined using Hematoxylin & Eosin stain (HE stain). After the incubation period, growth media was discarded and washed with PBS two times.

Cells were fixed in cold methanol (100%) and then washed with distilled water. For staining, 1%-hematoxylin was applied and incubated for 15 min at room temperature, then washed with distilled water. After that, eosin was applied and incubated at room temperature for 5 min followed by washing with distilled water. The culture plate was kept for air drying, and the stained cells were observed under a phase-contrast inverted microscope at 20X magnification.

2.6 Intracellular ROS measurement

Intracellular ROS was measured using 2, 7-dichlorodihydrofluorescein diacetate (H2-DCFDA). Cells were seeded in 96-well black cells culture plate with a cell density of 25,000 cells/well (Ilboudo et al., 2014). CaCo2 cells were allowed to grow for 48hrs in the DMEM with 10% FBS and without supplementation of phenol red. Then cells were washed three times with PBS. A solution having AFM1 toxin was prepared in media containing H2-DCFDA 5μM, and afterwards, cells were treated with different concentrations of AFM1 (5ng/L, 50ng/L, 250ng/L, 500ng/L, 1000ng/L and 2000ng/L) for a particular incubation time of 6 h, 12 h and 24 h. After the incubation period, the medium was removed, and the cells were washed twice with PBS. The fluorescence intensity was measured with a Tecan® microplate reader at the excitation and emission wavelength of 485nm and 530nm, respectively.

2.7 Gene expression study of AFM1 treated CaCo2 cell

Caco-2 cells (1.5 lakh cells/well) were cultured on a 24 well cell plate, and cells were treated as mentioned above in the viability experiment. According to the manufacturer's instructions, total cellular RNA was extracted using Tri reagent (Sigma-Aldrich, USA). After extraction, the RNA sample was treated with RNase-free DNase I (Takara Bio, Japan) for removing residual DNA contamination. The RNA purity was assessed with NanoDropTM 2000/2000c Spectrophotometers (CAT#ND-2000) and agarose gel electrophoresis. The RNA samples, having a purity range of less than 2.0, were used for further gene expression study. RNA samples were used as a template for cDNA preparation; that is, for the synthesis of cDNA. RevertAid First Strand cDNA Synthesis Kit (CAT#K1622) method was followed for cDNA synthesis. The prepared cDNA was stored at -20ºC till use. Before using real-time PCR, the cDNA was diluted to 1:10 with nuclease-free water. A total of 12 μL of the reaction mixture was prepared for each reaction, and an individual sample was used in duplicate. The qPCR reaction mixture contained 6μL of LightCycler® 480 SYBR Green master mix 2X (Roche Diagnostics, Mannheim, Germany), 0.5μL of 5μM forward primer and reverse primer and 5μL of diluted cDNA. Similarly, for non-template control instead of cDNA, Nuclease free water was used. The β-Actin gene was used as a housekeeping gene. A light thermocycler real-time PCR (Applied Biosystem 7500 Fast) was used for amplification and quantification of genes under the following cyclic conditions: - Pre-incubation at 95°C for 5 min, followed by 40 consecutive thermal cycles, with each cycle containing 3 discrete temperature steps, which includes denaturation at 95°C for 20 sec, annealing for 15sec and final extension at 72°C for 15sec. After amplification, Ct values were obtained and analyzed using the Livak ΔΔCt method (Livak and Schmittgen, 2001).

2.8 Heat Map

To understand the collective role of the AFM1 treated Intestinal epithelial cells specific transcript markers (Claudin, Occludin, Villin, Vimentin, Cytokeratin, JAM1, ZO-1, and Cyp1A1) involved in the disruption of gap junctions between intestinal cells. A hierarchical clustering approach was used by the online webserver heatmapper (http://www.heatmapper.ca/expression/). The quantified mRNA expressions of AFM1 treated Intestinal epithelial cells at different time intervals were utilized as per the given instructions on the heatmapper webserver. Afterwards, heatmap cluster of the transcripts was visualized by the Heatmapper.

2.9 AFM1 trans-epithelial transport experiment

Caco-2 cells were seeded in a sterile polyester membrane insert with a pore size of 0.4 mm (Himedia, Cat#TCP084-2x6no) with a $1X10^5$ cells/well density. This experiment was performed post 21 days culture of Caco-2 cells to allow the formation of a differentiated confluent monolayer. Firstly, the cells were seeded on the lid (Apical side) insert, and cells were cultured in the media (DMEM with phenol red) containing 10% FBS and on the basal side media (without phenol red) or PBS was added. Before AFM1 treatment, cells' permeability or integrity was checked, and it was confirmed that the cells' permeability was less than 5%. After that, cells were exposed to AFM1 with different concentrations of 5ng/L, 50ng/L, 500ng/L and 2000ng/L for 24hrs. At last, the effect of AFM1 on the permeability of Caco2 cells was checked by phenol red assay. The phenol red assay method was performed as described previously (Rani et al., 2017). Both apical and basolateral chambers were washed with PBS three times, and 300 µL of DMEM containing phenol red and 500 µL of PBS were added in the upper and basal chamber, respectively. The cell culture plate was kept in an incubator for 60 minutes, and after incubation, 100 µL of solution from apical and basal chambers were transferred into a 96 well plate. After that, it was tested for phenol red absorbance at 560 nm and accordingly, the percentage of diffusion was calculated.

2.10 Statistical analysis

Values are representative of the Means ± SEM (standard error of the mean) of three independent experiments performed at least in triplicate. The gene expression data were analyzed using a one-way analysis of variance (ANOVA) with a posthoc Tukey's test. Statistical significance was considered at a P value < 0.05.

Results

3.1 CaCo2 Cells Morphology by AFM1 treatment

For morphology study, CaCo2 cells were treated with AFM1 at different concentration for 24hrs (Fig. 1). Initially, the AFM1 treated Caco2 cells were observed directly under the microscope, and no changes in the

cellular morphology were found, even at higher AFM1 concentrations. Later, the cell morphology after AFM1 treatment was observed after staining with Hematoxylin and Eosin stain (Fig. 2). Similarly, no change was found in the cellular morphology of AFM1 treated CaCo2 cells compared to the untreated cells. Hence, the cells morphology result revealed that AFM1 did not affect the cellular morphology of Caco2 cells.

3.2 Effect of AFM1 on CaCo2 cell viability by MTT assay

MTT assay was used to determine the viability of AFM1-treated Caco2 cells. The MTT assay results showed that AFM1 had no effect on the viability of CaCo2 cells after 6 and 12 hrs of treatment. However, after 24 hrs of treatment, the viability of Caco2 cells was reduced by 10% at 2000ng/L AFM1 dose (Fig. 1S).

3.3 Effect of AFM1 on Reactive Oxygen Species (ROS) production

The DCFDA assay was used to examine the ROS production of AFM1-treated caco2 cells. The ROS production was increased with an increased dose of AFM1 after 6hrs of treatment (Fig. 3) and decreased ROS production at 12hrs and 24hrs exposure time.

3.4 Effect of AFM1 on intestinal specific Caco2 cells transcript markers

Initially, epithelial-specific transcript markers such as cytokeratin, villin, and fibroblast specific marker vimentin were checked. These transcript markers are component of intermediate filaments, which maintains the structural integrity and shape of the epithelial cells. Expression of cytokeratin was checked after 6hrs, 12hrs and 24hrs of treatment. We observed a slight elevation in the expression of the cytokeratin gene at higher concentrations of AFM1 after 6hrs of its treatment. However, the expression of cytokeratin was significantly higher after 12hrs and 24hrs of treatment with 2000ng/L AFM1 in comparison to the control group (Fig. 4a-4c). Likewise, the expression of another epithelial-specific transcript marker gene 'Villin' was checked. Villin is a tissue-specific actin-binding protein associated with the actin core bundle of the intestinal brush border cells. Villin is mainly produced in epithelial cells that develop brush borders, responsible for nutrient uptake. The Villin expression was significantly higher in 1000ng/L and 2000ng/L of AFM1 treatment groups than in other groups after 6hrs and 24hrs of treatment (Fig. 4d-4f).

Another fibroblast specific transcript marker, 'Vimentin' expression, was checked after 6hrs, 12hrs and 24hrs of treatment. Vimentin is an Intermediate filament (IF) structural protein and a marker of mesenchymal-derived cells. It provides mechanical support to the cell and its cytoplasmic constituents. There was an escalation in the Vimentin gene expression with an increased concentration of AFM1 treatment after 24hrs (Fig. 4g-4i). Overall, the expression of epithelial-specific transcript markers was

confirmed by morphological analysis, indicating that AFM1 had no effect on the morphology of Caco2 cells.

Expression of intestinal tight junction specific transcript markers which are involved in the intestinal cells' function and maintaining intestinal cells' structural integrity (e.g., Occludin, Claudin, Junctional Adhesion Molecules (JAM) and Zonula Occludens (ZO1)) were also checked. These molecules play a role in cell-to-cell interaction and provide a barrier function. A hike was observed in the occludin gene expression in Caco2 cells along with the increased concentrations of AFM1 treatment after 6hr; however, the expression of occluding gene was significantly higher than the control after 12 hrs treatments with 2000ng/L AFM1. Interestingly, an increase in the Occludin gene expression was significantly decreased compared to control after 24hrs treatment with 1000 ng/L and 2000 ng/L AFM1 (Fig. 5a-5c).

Likewise, the expression of one more intestinal transcript marker, “Claudin”, was also checked. There was a decline in the Claudin gene expression in Caco2 cells after 6hrs treatment as the concentration of the AFM1 increased. The decrease became very significant after 12 hrs of treatment with 2000ng/L of AFM1. Later, the claudin expression was reduced significantly after 24 hrs of treatment with all the selected AFM1 concentrations (5-2000ng/L) (Fig. 5d-5f).

The expression of another transcript marker, JAM, was also checked and was found to have a higher expression in CaCo2 cells treated by AFM1. After 6hrs and 12hrs treatments, the JAM gene expression was significantly higher at a concentration of 2000ng/L AFM1 than in the control or untreated sample. After 24hrs of treatment at the dosage of 500ng/L, 1000ng/L and 2000ng/L concentration of AFM1, the JAM gene expression was also significantly higher (> 2-fold) as compared to the control sample (Fig. 5g-5i).

The gene expression of an intra-cytoplasmic tight junction protein such as ZO1 showed a significant increase in the expression in Caco2 cells after 6hrs treatments with 2000ng/L of AFM1 compared to control. Similarly, an increase in the expression of the ZO1 transcript was observed after 12hrs treatment with 1000ng/L and 2000ng/L of AFM1 than the control and other treatments of AFM1. However, after 24hrs of the treatment, the gene expression was decreased in the treatment group of 50ng/L to 2000ng/L AFM1. The gene expression was significantly lower in the treatment group, having an AFM1 dose of 500ng/L to 2000ng/L compared to the control sample (Fig. 6a-6c).

Along with these transcript markers, we also checked the gene expression of a xenobiotic metabolism enzyme such as Cyp1A1. After 6hrs treatments of AFM1, the Cyp1A1 gene expression was found to be higher as the AFM1 concentration was increased. At the AFM1 dose of 1000ng/L and 2000ng/L, the Cyp1A1 gene expression was significantly higher than control at 6hrs, 12hrs and 24hrs treatments (Fig. 6d-6f). Overall, heat map illustrated an increased expression of epithelial specific cytoskeleton markers Cytokeratin, Villin, Vimentin, and JAM1, and a decreased expression of tight junction proteins, Claudin, Occludin, and ZO1. Similarly, we found an increased expression of Cyp1a1 transcript with an increasing AFM1 concentration and incubation time (Fig. 7).

3.6 Effect of AFM1 on Cell membrane permeability

To validate the tight junction gene expression results, intestinal permeability experiment was conducted using trans-well experiment. Caco2 cells were treated with AFM1 for a particular incubation time (24hrs.). The intestinal permeability was assessed by phenol red assay. After performing phenol red assay, we found that the intestinal permeability was significantly increased (approx. 30%) at 2000ng/L AFM1 concentration in comparison to the untreated cells. Similarly, a slight increase in intestinal permeability was also found at 500 ng/L AFM1 concentrations (Fig. 8).

Discussion

The gastrointestinal tract (GIT) serves as a primary tissue barrier to foreign materials like food products and contaminants such as mycotoxin, toxins, xenobiotics, and so on. As a result, if the food is contaminated with toxic elements, the intestinal tract, particularly the duodenum and jejunum, will be the most vulnerable to toxicity, and will be exposed to the toxicants at higher amounts than other tissues of the body (Smith et al., 2016). Claudin, Occludin, JAM, and ZO1 are intercellular and intracellular tight junction (TJ) proteins found in the GIT, particularly the intestine. These intercellular proteins help to selectively transport large molecules, ions, solutes, and water (Qasim et al., 2014; Suzuki, 2013). Zonula occludens (ZO)-1, ZO-2, and ZO-3 are cytoplasmic scaffolding proteins that help to stabilise intercellular connections (González-Mariscal et al., 2000; Martin-Padura et al., 1998; Mitic and Anderson et al., 1998; Furuse et al., 1993). Toxins may thus cause defects in the structure and function of the intestinal epithelial barrier, particularly the disruption of TJ protein integrity, resulting in intestinal dysfunction or leaky gut.

In-vitro methods are commonly used to assess a toxicant's cytotoxicity. The in-vivo approach has several disadvantages, including the use of live animals for testing, the high cost, the time required, the need to scarify the animals, and so on. In-vitro methods are the best options for understanding cytotoxicity and minimizing these issues. In our study, the Caco-2 cell line was used as an in vitro model to assess the cytotoxicity, cell morphology, cellular absorption, and functional characteristics of intestinal cells after exposure to AFM1 on the intestine.

After exposing Caco2 cells to AFM1, a cellular morphology study was performed first. The primary goal of this experiment was to better understand the morphological changes caused by AFM1 in Caco-2 cells. There has been no research published that shows the effect of AFM1 on the cellular morphology of intestinal cells. Caco2 cells exposed to different AFM1 doses (5ng/L, 50ng/L, 250ng/L, 500ng/L, 1000ng/L, and 2000ng/L) for 6hrs, 12hrs, and 24hrs showed no change in morphology. CaCo2 cells are susceptible to morphological changes, but the current study found no change in CaCo2 cell shape. Caloni (2012) supported this observation by claiming that the cellular morphology of cells treated with AFM1 remains unchanged. The reason for these results could be a shorter AFM1 exposure time, as well as the concentration of toxins used in the experiment being insufficient to cause any change in cellular morphology. Similarly, cellular morphology was observed in treated renal epithelial cells using different

mycotoxins such as ochratoxin A (OTA) or citrinin (CIT), and it was discovered that when both mycotoxins were added in combination and incubated for 48 hours, there was a change in cellular morphology (Schulz et al., 2018).

The current study found that exposing cells to a higher concentration of AFM1 (2000ng/L) for 24 hours reduced cell viability slightly. The recommended mechanisms for AFM1-mediated cytotoxicity on cell viability are oxidative DNA damage (Gao et al., 2017; Gao et al., 2016; Zhang et al., 2015; Tavares et al., 2013; Wild and Turner, 2002) and disruption of cell cycle regulatory protein expression (Bao et al., 2019). Notably, Bao et al. (2019) proposed that AFM1 affects cell proliferation by modulating specific proteins such as CDK1, SOS1/Akt, and AMPK signaling molecules, resulting in cell cycle arrest and decreased cell viability. Previous research has shown that among all mycotoxins, AFM1 is the least toxic in terms of affecting cell viability (Tatay et al., 2014; Wang et al., 2014; Tavares et al., 2013; Behm et al., 2012), which is consistent with our findings. Finally, the viability results of our study showed that at the concentration level of AFM1 used in this study, it only caused minor cell damage, which was insufficient to affect CaCo2 cell proliferation.

We examined the effect of AFM1 on intestinal epithelial-specific transcript markers to determine whether AFM1 causes any damage to the morphology of intestinal cells. Cytokeratins are intermediate filament protein family members that are expressed as a network radiating from the nucleus to the plasma membrane. Cytokeratins are intracytoplasmic cytoskeleton proteins that are commonly used to identify (Giotakis et al., 2021; Chaudhary et al., 2018; Coch and Leube, 2016). Cytokeratins play an important role in protecting epithelial cells from mechanical and non-mechanical stressors (Karantza, 2011). There have been a few studies that show the effect of AFM1 on epithelial-specific gene expression. To understand AFB1 induced cytokeratin expression on epithelial cells a normal rat liver-derived cell line BL8L showed that AFB1 had a similar effect on cytokeratin expression as AFM1 (Green et al. 1990). Reisinger et al. (2019) also used mycotoxins to treat intestinal cells, including deoxynivalenol, nivalenol, fumonisin B1, and enniatin B. Cytokeratins were found to be expressed as a network radiating from the nucleus to the cytoplasm. This result suggested that mycotoxin may cause increased cytokeratin expression, which is consistent with the findings of our expression study. AFM1 was identified as a stressor in the current study. As a result, during stressful conditions, cells must maintain their stability, and our findings revealed increased expression of the cytokeratin gene.

The expression of an epithelial-specific gene, such as Villin, was also examined in the AFM1 treated CaCo2 cells. Villin is an actin-binding protein found in the brush border of the intestinal epithelium. Villin is primarily produced by epithelial cells, which form a brush border to facilitate nutrient uptake. In our study, we found that villin has an increased expression pattern, implying that it aids in the assembly of the actin core bundle of microvilli and maintains the structural integrity of the cells. Another study suggested that villin may play a role in cancer, and it discovered that its expression in intestinal cells is consistently maintained in their corresponding carcinomas, even when the organised brush-border structure is lost. The presence or absence of villin aids in determining the type and location of the primary tumors. Other mycotoxins, such as deoxynivalenol, were studied to see how they affected the intestine of

chickens. The chicken was fed 4000 g/kg of deoxynivalenol (DON) in this experiment, and its intestinal integrity was examined. The study found that the expression of VIL1 was significantly lower in the exposed sample compared to the control sample (Santos et al., 2021). Because they used a higher concentration of mycotoxins to test the toxicity in chicken, their findings were the polar opposite of ours. Arango et al. (2012) previously proposed that villin expression appears to be lost during the formation of compact tumor cells. Using this knowledge and the current study results, which showed an increase in villin expression, we concluded that AFM1 may be less likely to cause intestinal carcinoma.

In the AFM1-treated Caco2 cell line, we also looked at the gene expression of another intermediate filament protein called Vimentin. Vimentin is a structural protein that provides mechanical support to the cell and its cytoplasmic constituents. It is also a marker of the epithelial-mesenchyme transition. Vimentin expression was found to be higher in the current study. A similar observation was made when lung cells were treated with aflatoxin G1, which increases vimentin gene expression via the TNF-/NF-B pathway (Yi et al., 2017). In AFM1-treated cells, a similar mechanism of increased TNF-/NF-B pathway expression was observed (Gao et al., 2020). Another study suggested that AFM1 treatment may increase vimentin expression by activating the MAP kinase signaling pathway (Gao et al., 2020). As a result, the increased expression of Vimentin in Caco2 cells exposed to AFM1 in the current study could be attributed to the TNF-/NF-B or MAP kinase signaling pathways. Other mycotoxins, such as ochratoxin A (OTA) or citrinin (CIT), also increased Vimentin expression in human proximal tubule-derived epithelial cells (HK-2) (Schulz et al., 2018). Taken together, epithelial-specific gene expression data supported our cell morphology study findings that AFM1 treatment had no effect on the cell.

The current study's second goal was to determine the effect of AFM1 on the gene expression of intestinal-specific genes such as claudin, occludin, JAM, and ZO1, which aid in the maintenance of the intestinal barrier function. AFM1 has been linked to an increase in the expression of the MAPK singling pathway (Gao et al., 2020), and other studies have linked MAPK (p38/ERK) protein to the suppression of intestinal specific barrier proteins such as claudin (Carrozzino et al., 2009). We hypothesized that the decreased expression of the claudin gene in AFM1-treated cells after 24 hours was due to increased expression of the MAPK protein. A similar result in AFM1-treated caco2 cells for claudin protein level confirmed our qPCR-based gene expression study (Gao et al., 2017). An in-vivo study with AFB1 and AFM1 exposure backed up our findings (Gao et al., 2021).

We also looked at the expression of another intestinal barrier protein, occludin, and found that it had a similar pattern to claudin. Wu et al. (2013) demonstrated the involvement of the p38 MAPK signaling pathway in the reduction of TJ proteins like occludin and ZO-1, as well as the functional comptonization of the intestinal barrier in human gastric epithelial cells. Furthermore, Gao et al. (2017) examined the effect of AFM1 on occludin-silenced cells and discovered that the TEER value was significantly lower ($p < 0.05$) than in control and NC-silenced cells. This report suggests that occludin is important for TJ integrity and contributes to epithelial permeability maintenance. Aside from AFM1, other mycotoxins that affect CaCo2 cells include Aflatoxin B1 (AFB1), fumonisin B1 (FB1), ochratoxin A (OTA), and T-2 toxin (T2).

These toxins reduce the expression levels of tight junction protein components such as claudin-3, claudin-4, and occludin (Romero et al., 2016).

Junctional adhesion molecules-1 (JAM1), which regulates junctional integrity, morphology, permeability, and polarity, was also studied in this study. JAM-A has been shown to play a variety of roles in intestinal homeostasis by regulating epithelial permeability, inflammation, and proliferation (Kang et al., 2007; Mandell et al., 2005). After AFM1 treatment, we saw an increased JAM-A expression, which is involved in maintaining intestinal homeostasis and preserving epithelial morphology. This could explain the increased expression of the JAM1 gene, which is required for maintaining epithelial shape after AFM1 treatment.

The ZO-1 is an intracellular epithelial marker that connects tight junction proteins to the actin cytoskeleton, which is in charge of maintaining the structure and function of the intestinal barrier. Numerous intestinal-specific diseases are caused by defects in intestinal barrier integrity. AFM1 and OTA cause increased intestinal permeability due to decreased expression of tight junction proteins claudin-3, claudin-4, occludin, and ZO-1 (Wu et al., 2013). The activation of the p38 MAPK pathway may be responsible for the decreased expression of tight junction proteins such as occludin and ZO-1 in intestinal epithelial cells (Gao et al., 2018).

Finally, our intestinal junction gene expression study indicates that AFM1 exposure compromises intestinal barrier integrity. The disruption of the intestinal barrier allows for greater penetration of luminal substances that are normally excluded and may promote intestinal disorders. Many studies have found that the expression of claudin and occludin is reduced in Crohn's disease (CD). As a result, more research is needed to understand the mechanisms underlying this AFM1-induced decrease in TJ permeability.

Finally, our intestinal junction gene expression study indicates that AFM1 exposure compromises intestinal barrier integrity. The disruption of the intestinal barrier allows for greater penetration of luminal substances that are normally excluded and may promote intestinal disorders. Many studies have found that the expression of claudin and occludin is reduced in Crohn's disease (CD). As a result, more research is needed to understand the mechanisms underlying this AFM1-induced decrease in TJ permeability. The possible molecular mechanism underlying Cyp1a1 expression is the activation of AHR agonist molecules such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (Mary et al., 2012; Chen et al., 2009; Rumora et al., 2007), which induces the stimulation of AHR expression (Tan et al., 2004; Yim et al., 2004). According to one study, both the CYP1A and CYP3A isoforms oxidise AFB1 and convert it to a less potent carcinogen (Ayed-Boussema et al., 2012). Because AFM1 is a metabolic product of AFB1, we expect that increased expression of CYP1A in CaCo2 cells in this study may be involved in the biotransformation of AFM1 into a different metabolite, which needs to be investigated further.

The current study suggests that AFM1 reduces the expression of tight junction proteins, implying that reducing cell-to-cell interaction leads to repression of the intestinal barrier's function. A transwell assay was used to confirm this, and it was discovered that the permeability of the intestinal cells increased by up to 30% after 24 hours of treatment with 2000ng/L AFM1. This finding supported previous research

(Gao et al., 2018; Gao et al., 2017; Caloni et al., 2012), which found a dose-dependent decrease in TEER and an increase in Caco-2 cell paracellular permeability after 48 hours of AFM1 and/or OTA treatment. However, previous findings suggested an increase in paracellular transport after 48 hours of treatment, despite the fact that the toxicant used was of a higher concentration, whereas our findings suggested an increase in paracellular transport after 24 hours of treatment. As a result, the current study's transwell experiment results support the gene expression observation.

Finally, we investigated ROS production following AFM1 treatment. After 6 hours of treatment, we noticed an increase in ROS production. However, the level of ROS decreased after 12 and 24 hours of incubation. It is well understood that free radicals directly cause injury to the cell membrane by oxidizing unsaturated fatty acids within the cell membrane's phospholipids, altering membrane resistance or permeability (Ilboudo et al., 2014). According to Mary et al. (2012), AFB1 may also be involved in the activation of phospholipase A2, an enzyme involved in the production of arachidonic acid, which then produces hydroperoxide free radicals. In a similar experiment, Zhang et al. (2015) found that increasing the concentrations of AFB1 and AFM1 caused a significant ($p < 0.05$) increase in cellular ROS accumulation in a dose-dependent manner compared to the control. ROS levels were measured after 24 hours, 48 hours, and 72 hours in this study. However, in our study, we measured ROS levels after 6 hours, 12 hours, and 24 hours of incubation, and our findings contradict the previous study. In the experiment, we discovered high ROS levels after 6 hours, but ROS levels began to decline as the time increased up to 24 hours. The cause could be an initial oxidative burst, but the cells may have developed a defense mechanism to neutralize the ROS level later on. Finally, the ROS production study conducted in the experiment revealed that AFM1 increased ROS production in CaCo2 cells in a shorter incubation time.

Conclusion

The present study revealed that consumption of AFM1 contaminated milk doesn’t show any effect on cells morphology, viability. Similarly, we found that AFM1 at 2000ng/L significantly increased the free radical generation after 6 hours of treatment. In addition to that, gene expression study showed an increased expression of epithelial specific cytoskeleton markers such as cytokeratin, villin and vimentin, Cyp1A1 and a junctional adhesion marker, JAM1, and a decreased expression of tight junction proteins, Claudin, Occludin, and ZO1 with the increasing AFM1 concentration and incubation time. The gene expression results showed that AFM1 causes disruption of gap junctions between the intestinal cells, which was further confirmed by a tranwell experiment. On the basis of these observations we concluded that consumption of AFM1 contaminated milk doesn’t show any effect on cells morphology and viability but decreases the gene expression of intestinal barrier transcripts that leads to the disruption of intestinal barrier function and thereby causes leaky gut.

Declarations

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Author Contributions

Dr. Suneel Kumar Onteru, Dr. Dheer Singh, and Dr. Rajni Kumar Paul conceived the idea and organized the present study. Lal Krishan Kumar, Surya Kant Verma, Rajeev Chandel and Meet Thumar conducted experiments. Lal Krishan Kumar and Surya Kant Verma performed data analysis and wrote the manuscript. Suneel Kumar Onteru corrected, Rajni Kumar Paul Surya Kant Verma and Meet Thumar edited the manuscript.

Declarations/Conflict of interest

The authors declare no competing interests.

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Figures

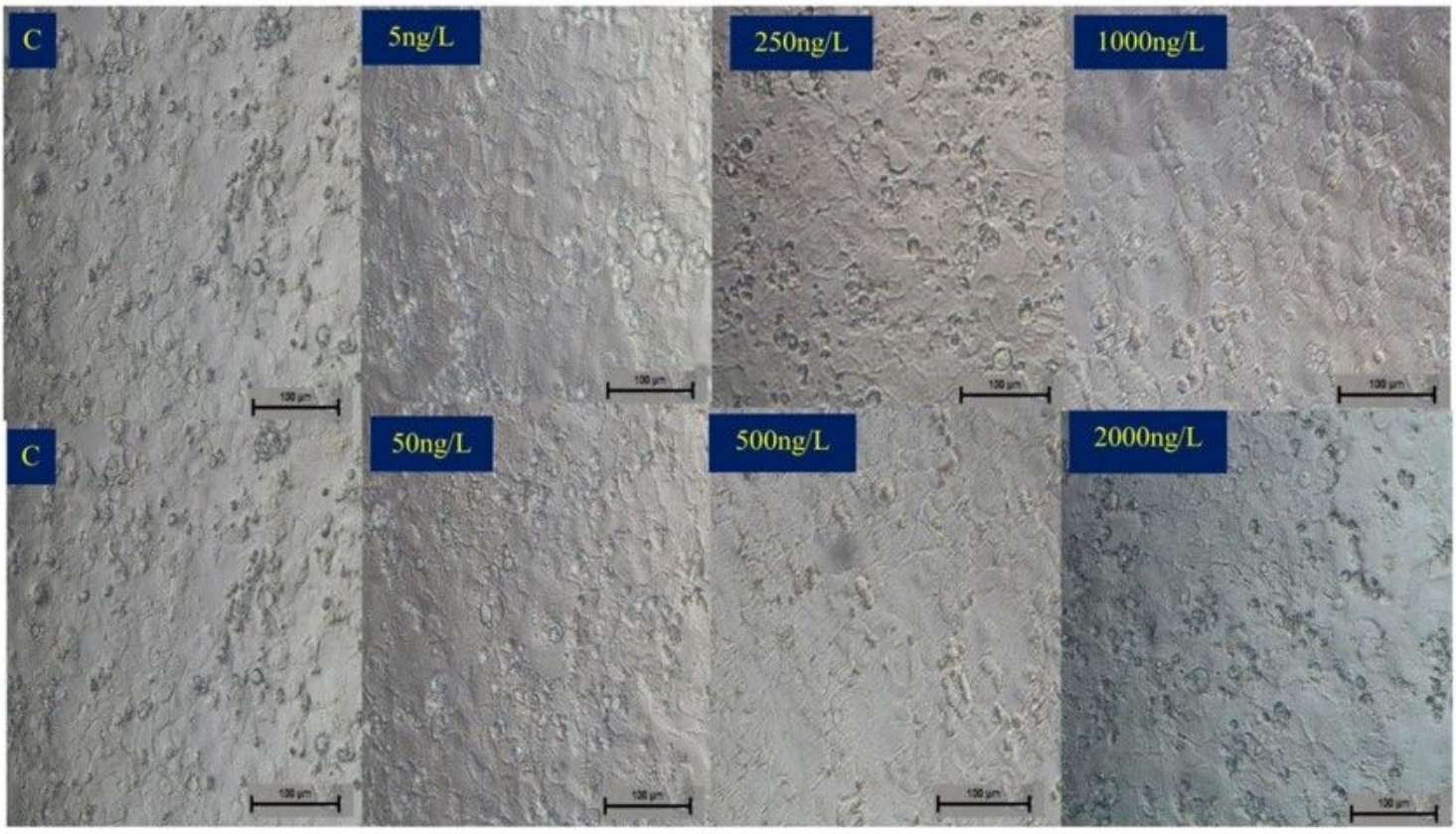

Figure 1

AFM1 induced morphological changes in Caco-2 cells. Caco-2 cells were seeded in 96-well plates and allowed to grow for 48hrs. Thereafter, cells were exposed to AFM1 for 24hrs and morphological changes were observed by using Nikkon inverted microscope. Selected Images are representative of three independent experiments. Images were observed with a magnification of 20X.

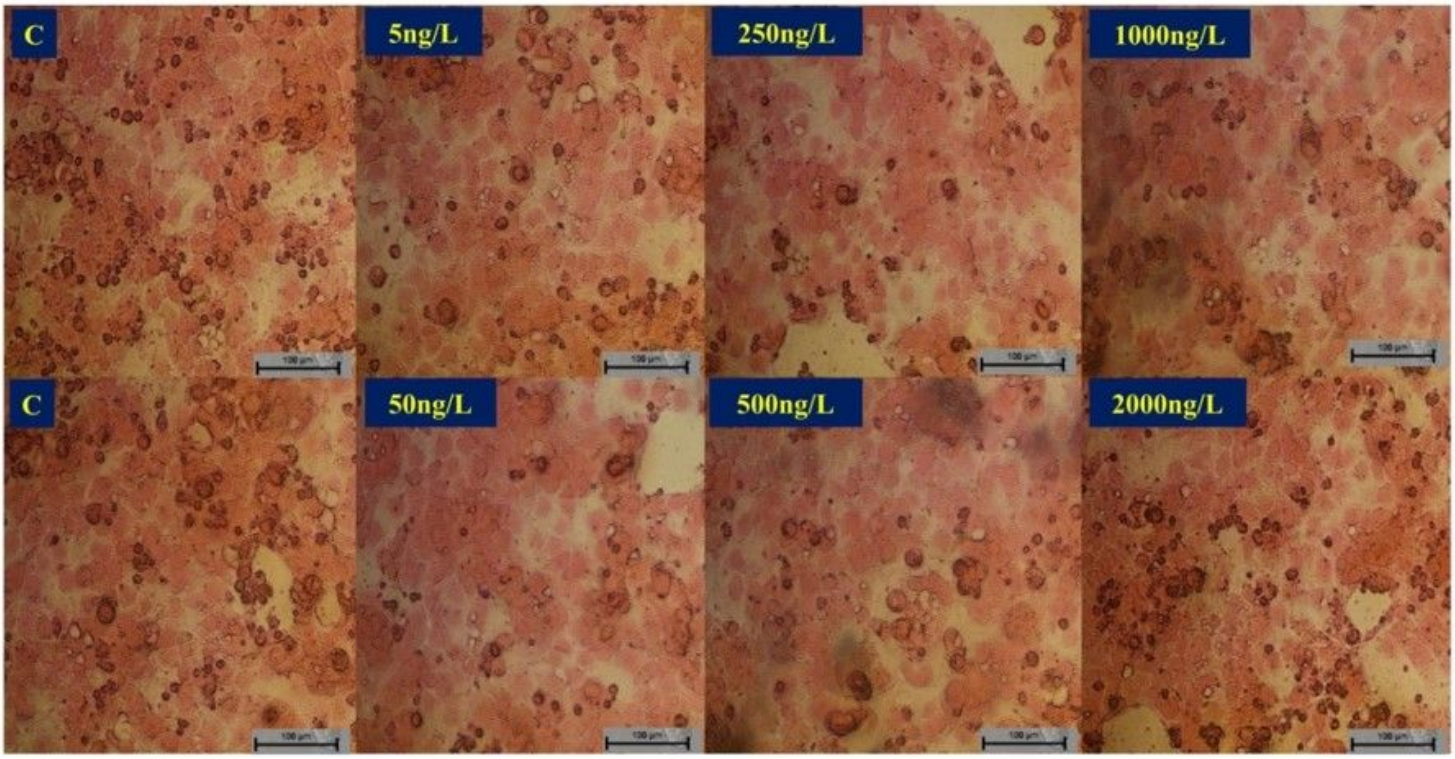

Figure 2

AFM1 induced morphological changes in Caco-2 cells by HE stain. Caco-2 cells were seeded in 96-well plates and allowed to grow for 48hrs. Thereafter, cells were exposed to AFM1 for 24hrs followed by cells were stained with Hematoxylin & Eosin stain and morphological changes are observed by using Nikkon inverted microscope. Selected Images are representative of three independent experiments. Images were observed with a magnification of 20X.

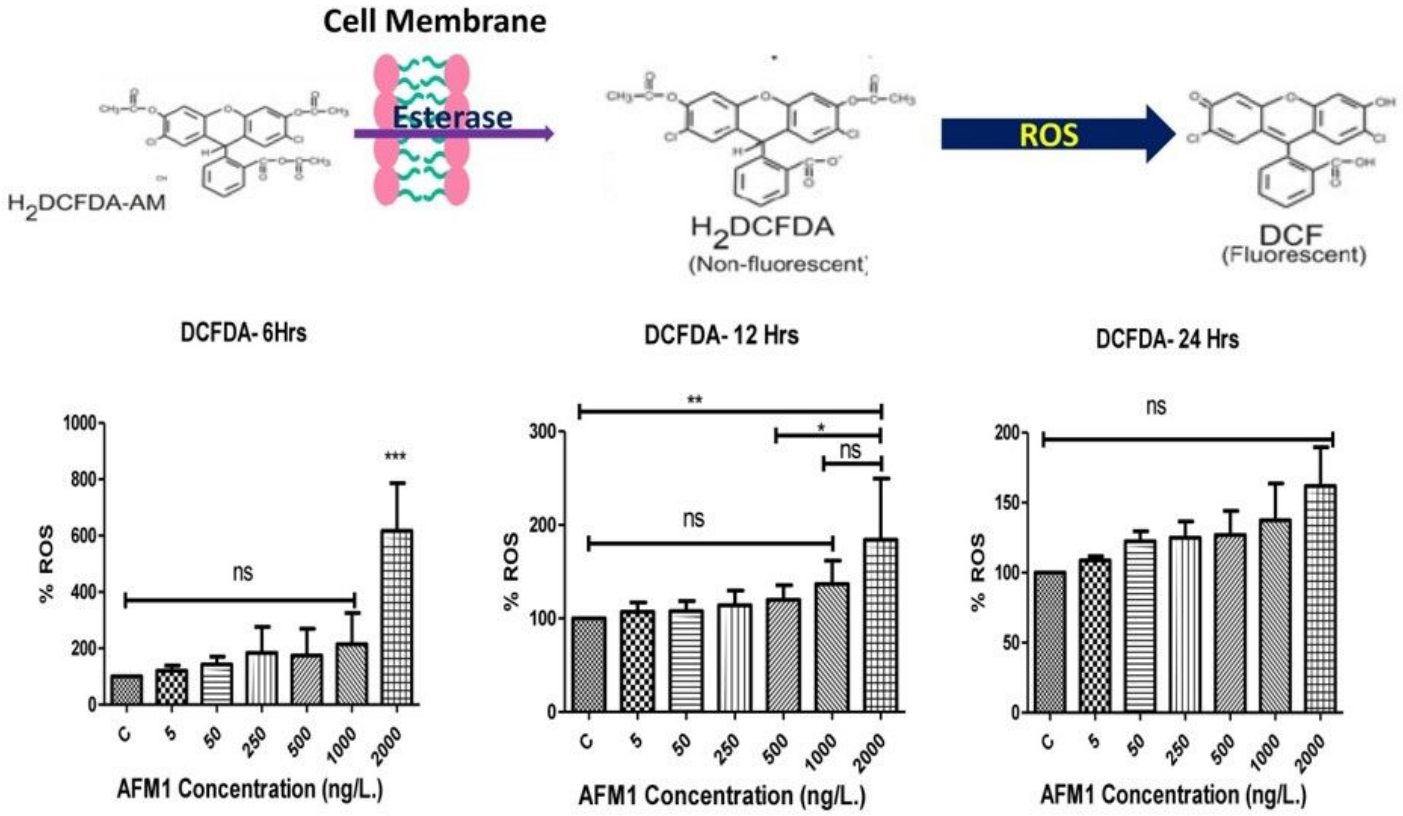

Figure 3

DCFDA dye working principle and ROS generation in Caco2 cells treated with AFM1 at different doses and time periods, 6hrs, 12hrs and 24hrs treatment with AFM1. The DCFDA dye was used for ROS study. Results are expressed as mean ± SEM (n= 3). *p < 0.05 - p < 0.005 indicate significant difference between the control cells and AFM1 treated cells at different concentrations.

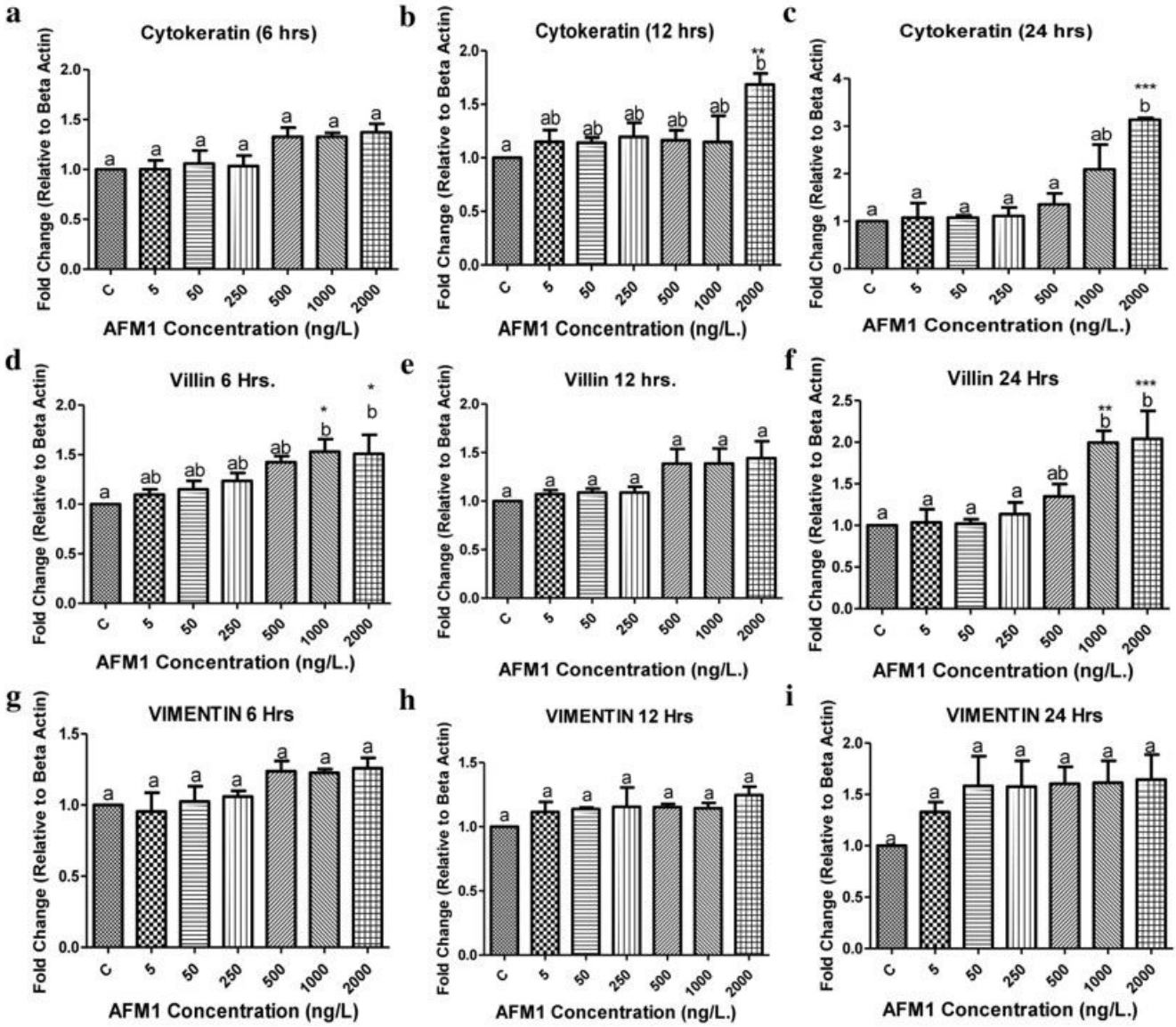

Figure 4

Cytokeratin, Villin and Vimentin gene expression in Caco2 cells treated with AFM1 at different doses and time, 6Hrs 12Hrs and 24Hrs treatment with AFM1. (a-i) Real time PCR analysis of Cytokeratin gene expression in the Caco2 cells are expressed as mean ± SEM (n= 3). *p < 0.05 - p < 0.005 indicate significant difference between control cells and AFM1 treated cells at different concentrations. **(d-f)** Real time PCR analysis of Villin gene expression in the Caco2 cells are expressed as mean ± SEM (n= 3). *p < 0.05 - p < 0.005 indicate significant difference between control cells and AFM1 treated cells at different concentrations. **(g-i)** Real time PCR analysis of Vimentin gene expression in the Caco2 cells are expressed as mean ± SEM (n= 3). *p < 0.05 - p < 0.005 indicate significant difference between control cells and AFM1 treated cells at different concentrations.

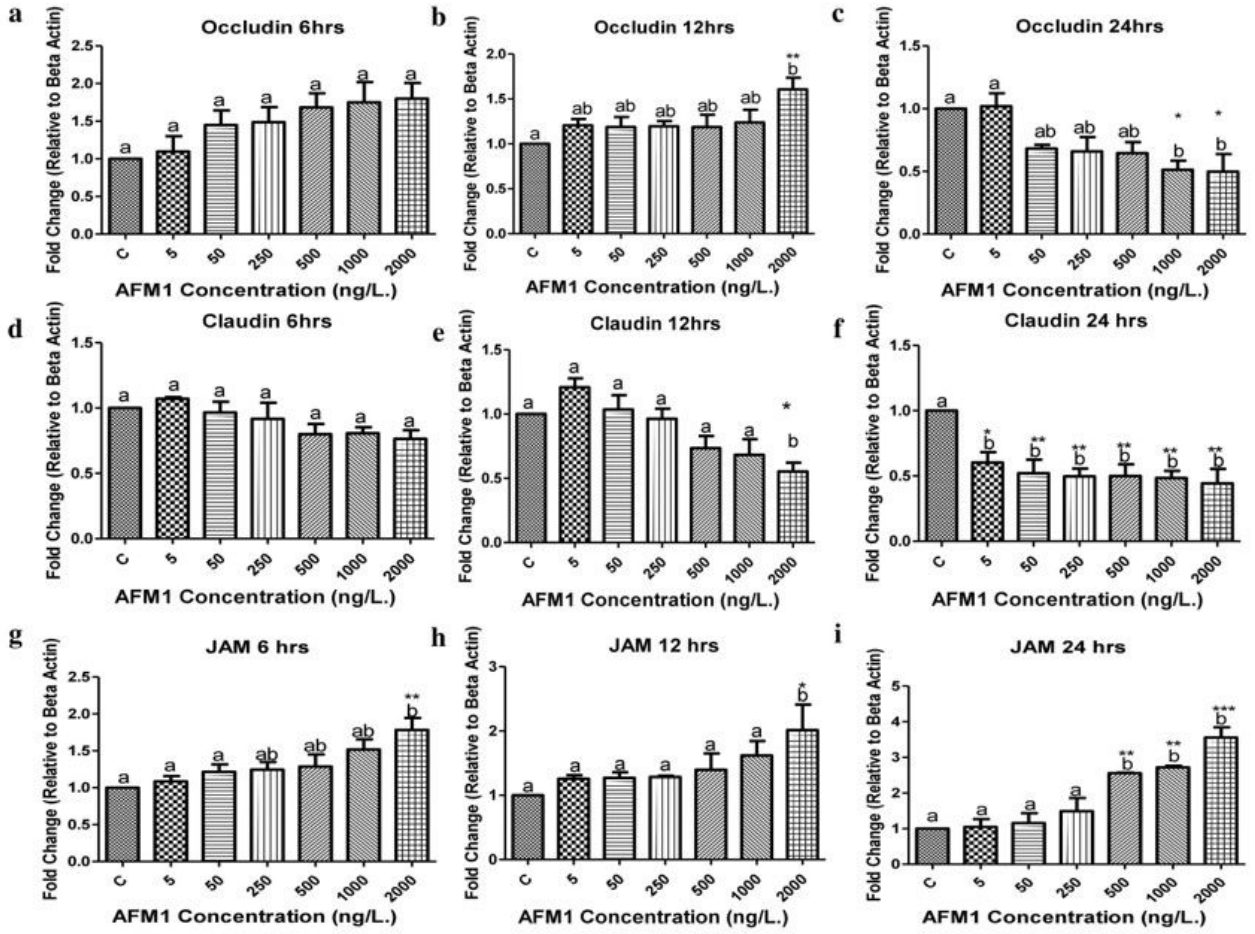

Figure 5

Occludin, Claudin and JAM gene expression in Caco2 cells treated with AFM1 at different doses and time, 6hrs 12hrs and 24hrs treatment with AFM1. (a-c) Real time PCR analysis of Occludin gene expression in the Caco2 cells are expressed as mean ± SEM (n= 5). *p<0.05 - p<0.005, significantly different from control cells to AFM1 treated differentiated and undifferentiated cells with the same toxin in different concentration. **(d-f)** Real time PCR analysis of Claudin gene expression in the Caco2 cells are expressed as mean ± SEM (n= 5). *p< 0.05-p<0.005, significantly different from control cells to AFM1 treated differentiated and undifferentiated cells with the same toxin in different concentration. **(g-i)** Real time PCR analysis of JAM gene expression in the Caco2 cells are expressed as mean ± SEM (n= 3). *p<0.05-p<0.005, significantly different from control cells to AFM1 treated differentiated and undifferentiated cells with the same toxin in different concentration.

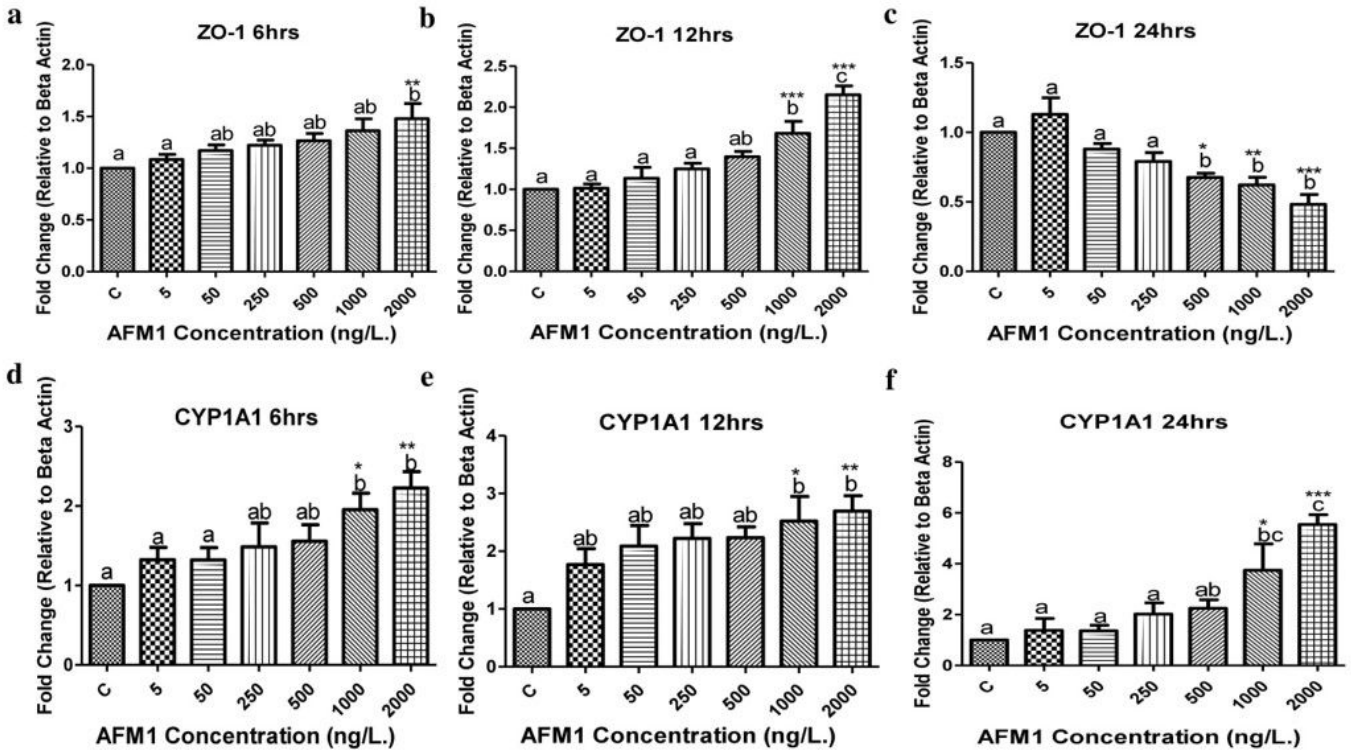

Figure 6

ZO-1 and Cyp1A1 gene expression in Caco2 cells treated with AFM1 at different doses and time, 6hrs 12hrs and 24hrs treatment with AFM1. (a-c) Real time PCR analysis of ZO-1 gene expression in the Caco2 cells are expressed as mean ± SEM (n= 5). *p<0.05-p<0.005, significantly different from control cells to AFM1 treated differentiated and undifferentiated cells with the same toxin in different concentration. **(d-f)** Real time PCR analysis of ZO-1 gene expression in the Caco2 cells are expressed as mean ± SEM (n= 5). *p<0.05-p<0.005, significantly different from control cells to AFM1 treated differentiated and undifferentiated cells with the same toxin in different concentration.

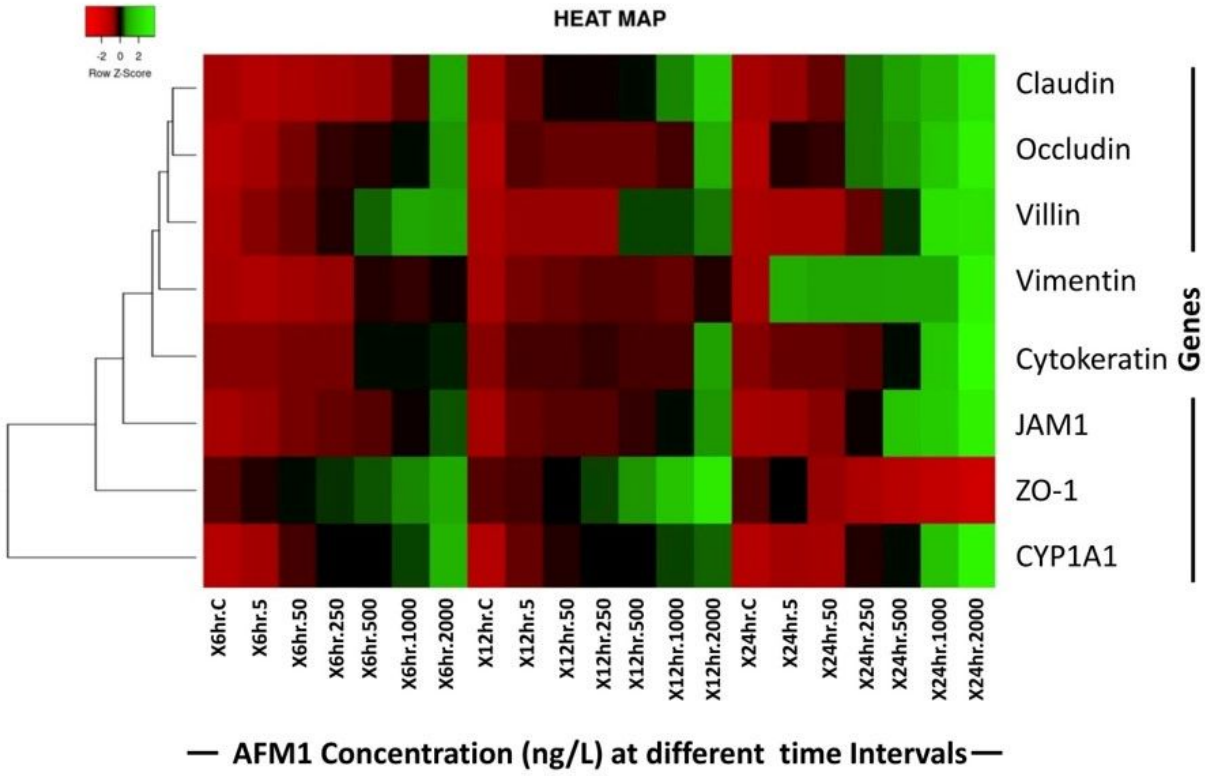

Figure 7

Heat map of the AFM1 treated Intestinal epithelial cells specific transcript markers. Hierarchical clustering of the mRNA expression pattern of the Claudin, Occludin, Villin, Vimentin, Cytokeratin, JAM1, ZO-1, and CYP1A1 genes at different doses as Control, 5, 50, 250, 500, 1000 and 2000ng/L and at different time intervals 6hrs, 12hrs and 24 hrs. Colors showed Bright green, relatively high expression; Black, relatively average expression; and Brown, relatively low expression.

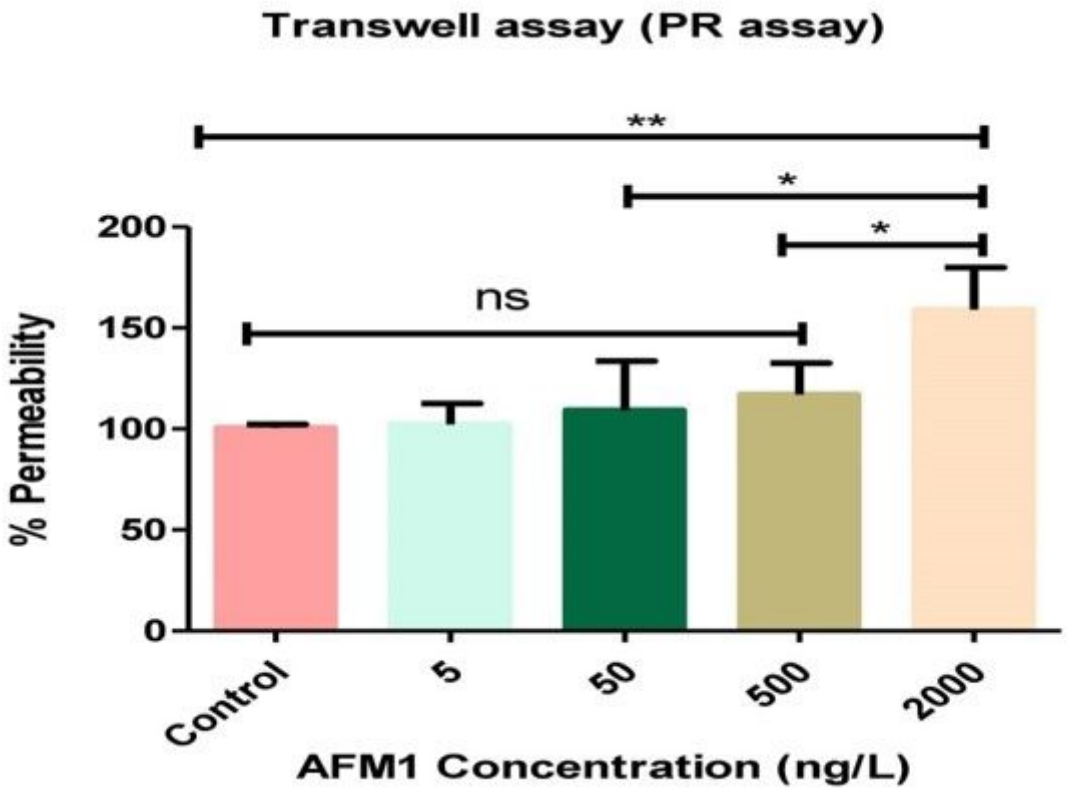

Figure 8

Trans-well based permeability study of Caco2 treated with AFM1 at different dose for 24hrs incubation time. Permeability was observed by Phenol red assay Results are expressed as mean ± SEM (n= 3). *p < 0.005, significantly different from control cells to AFM1 treated differentiated and undifferentiated cells with the same toxin in different concentration.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 4.SupplementaryMaterials.docx