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Low level of Ochratoxin A enhances Aflatoxin B_1 induced cytotoxicity and lipid peroxydation in both human intestinal (Caco-2) and hepatoma (HepG2) cells lines

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Abstract: Aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) are contaminants which co-occurred in the same food such as cereals. The few studies performed on their interactive effect had revealed additive or antagonistic cytotoxic effect according to cells endpoints and concentrations of both mycotoxins. The aim of the present study was to investigate in a possible influence of very low level of ochratoxin A in aflatoxin B_1 toxic action regarding cellular endpoints such as malonedialdehyde (MDA) production and cells viability as evaluated by lysosome and mitochondria integrities and cell lactate dehydrogenase (LDH) leakage. OTA (20nM) and AFB_1 were tested in combination in both human intestinal (Caco-2) and hepatoma (HepG2) cells lines. As results, OTA alone tested at 20nM was not cytotoxic and did not induce MDA production in both Caco-2 and HepG2 cells line. Interestingly, combined to AFB_1 (10µM), OTA enhanced markedly AFB_1 cytotoxic effect. OTA significantly increased cell lysosomes damage induced by AFB_1 from 24% to 38% (+14%) and from 28% to 43% (+15%) respectively in Caco-2 and HepG2 cells line ($p<0.05$). Similarly, OTA enhanced inhibition of mitochondria succinate dehydrogenase activity induced by AFB_1 until to +15% and +6% respectively in Caco-2 and HepG2 cells line ($p<0.05$). On cell necrosis marker, the mixture of OTA and AFB_1induced more LDH leakage when compared to AFB_1 alone with increase of +14% and +12% respectively in Caco-2 and HepG2 cells line ($p<0.05$). Finally, on MDA production, AFB_1 + OTA induced more intensively MDA production when compared to AFB_1 alone with +49% and +110% of increasing in both Caco-2 and HepG2 cells line ($p<0.01$). Taken together, our results suggested that combined AFB_1 and OTA induced all the toxicities observed with the mycotoxins separately but more intensively suggesting synergistic or potentiating effect. Moreover, AFB_1 or its association with OTA had been found very potent in human hepatic cells HepG2 in necrosis induction but especially in lipids oxidative damage confirming oxidative stress as one of keys pathways in toxic action of AFB_1.

Keywords: Aflatoxin B_1, Ochratoxin A, Low Level, Interactive Effect, Influence

1. Introduction

Mycotoxins are structurally diverse groups composed mainly of small molecular weight compounds. These compounds are produced mainly by the mycelial structure of filamentous fungi, or more specifically, the molds. Mycotoxins are secondary metabolites synthesized during the end of the exponential phase of growth of mold and are found in a wide range of countries, feeds and foods [1, 2]. The most significant mycotoxins in terms of public health and agronomic perspective include the aflatoxins, ochratoxin A (OTA), trichothecenes, fumonisins, zearalenone and patuline [3] and they were largely studied concerning their toxic aspects. The toxic effect of mycotoxin ingestion in both humans and animals depends on a number of factors including intake levels, the toxicity of the compound, duration of exposure (acute or chronic), the body weight of the individual, the presence of other mycotoxins (synergistic effects), mechanisms of action, metabolism, and defense mechanisms [4, 1, 5, 6, 7]. Aflatoxins B_1 (AFB_1) and ochratoxin A are mycotoxins abundantly produced in tropical areas such as Côte d'Ivoire.

AFB_1 is acutely toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds. The liver is the main target organ for aflatoxins and chronic exposure to low levels in foodstuffs causes liver fibrosis and primary liver cancer [8] but, AFB_1 may also cause tumors in other organs, such as colon and kidney [9]. It is bioactivated in liver by cytochrome P450 and its epoxide metabolite attacks DNA forming adducts [10] that might evolve to secondary injuries such as apurinic sites (AP) or imidazole AFB_1 formamidopyrimidine opened rings (AFB_1-FAPY) [11]. The metabolite AFB_1-FAPY induces G-T transversion, and is a good indicator of AFB_1 exposure in urine [12]. The evaluation of epidemiological and laboratory results carried out in 1993 by the International Agency for Research on Cancer (IARC) found that there is sufficient evidence in humans for the carcinogenicity of naturally occurring mixtures of aflatoxins, which are therefore classified as Group 1 carcinogens [13]. Moreover, the *in vitro* toxic action of AFB_1 remained few studied [14]. However, it had been reported that AFB_1 reduced cell viability in a dose-dependent manner, caused DNA damage by DNA strand breaks or oxidative damage, activation of intracellular ROS and apoptosis in a concentration dependent manner with activation of gene p53 but significantly decreasing of the antiapoptotic factor bcl-2 expression [14 - 16].

Ochratoxin A has been shown to be nephrotoxic, immunosuppressive, carcinogenic and teratogenic in all experimental animals tested so far [17] and recent studies are relating it to neurodegenerative diseases such as Parkinson and Alzheimer [18]. Owing to the similarity of morphological and functional kidney lesions in ochratoxin A-induced porcine nephropathy and endemic nephropathy, this mycotoxin has been proposed as the causative agent of endemic nephropathy [19], although the evidence for this is not substantial. Mycotoxin OTA mechanisms of action are not clearly determined but apparently, OTA may disrupt phenylalanine metabolism, reduce gluconeogenesis, and induce apoptosis via protein/DNA synthesis inhibition. In addition, the OTA capability to generate reactive oxygen species (ROS) may explain the lipid, protein and DNA damage [3].

Human population is probably exposed to multiple mycotoxins because human diet is generally varied and because the same food might be contaminated by several mycotoxins [20, 21]. For example, studies focused on mycotoxins presence in foods from Côte d'Ivoire reported co-occurrence of aflatoxin B_1, ochratoxin A, fumonisin and zearalenone in rice, maize, peanuts [21]. Since, multi-exposure could lead to additive, synergistic or antagonistic effects [5, 7, 15, 16], several combinations of mycotxins such as binary or tertiary combination were tested on cells line in the aim to observe their simultaneous effect on cellular damages namely cytotoxicty, genotoxicity, or cells metabolism [5, 7, 15, 16] but very few had concerned the mixture of AFB_1 and OTA [15, 16].

In a study on the mixture of AFB_1 and OTA, it had been reported an additive cytotoxic effect and an increase in DNA fragmentation and p53 level and downregulated bcl-2 expression as compared to mycotoxins taken separately in Vero cells [15]. These findings were partially supported by those more recently reported [16] which revealed that the co-exposure to OTA (tested at low or non genotoxic concentration) significantly decreased DNA damage induced by AFB_1, not only in breaks and apurinic sites but also in FPG-sensitive sites suggesting antagonistic effect so the low level of OTA may influence AFB_1 toxic action [16]. However, in the same study [16], when AFB_1 was associated with OTA tested at high concentration or cytotoxic concentration that leaded to additive effect as previously reported [15]. Thus, the combined effect resulting from OTA and AFB_1 interaction is not always additive effect as reported previously [15] that may depend to the level of OTA or AFB_1 or the type of cell line used. Concerning the general cell toxicity, the findings on interaction between very low level of OTA and AFB_1 remain not elucidated. Indeed, in tropical areas such as West Africa, at contrast to AFB_1 which was found abundantly in foods, OTA appeared less alarming with absence or low levels [22]. For these reasons, it is interesting to clarify the interactive effect between OTA and AFB_1 regarding not only their combined effect in divers cells lines but also their natural occurrence in foods.

The aim of this study is to evaluate whether very low level or non cytotoxic level of OTA may influence or modulate AFB_1 - induced cytotoxic effect following several cells endpoints namely mitochondria and lysosomes integrities, necrosis and lipid peroxydation in both intestinal and hepatic cells lines, Caco-2 and HepG2 respectively.

2. Materials and Methods

2.1. Chemicals

Mycotoxins OTA and AFB_1 were obtained from Sigma Chemical Company (St Louis, MO, USA) and were dissolved in ethanol/water (90:10). Dulbecco's Modified Eagle Medium (DMEM), foetal calf serum (FCS) and neutral red (NR) solution were provided from Sigma-Aldrich (Saint Quentin Falavier, France). All other chemicals used were of analytical grade.

2.2. Cell Culture and Treatment

Caco-2 cells, a human colon cancer cell line, were obtained from Dr. Jing Yu, Tufts School of Medicine (Medford, MA, USA). The cells were grown as monolayer culture in a high glucose concentration (4.5 g/l) DMEM medium supplemented with 10% foetal calf serum (FCS), 8mM L-glutamine, 1% of mixture penicillin (100 IU/ml) and streptomycin (100g/ml) incubated at 37 °C in an atmosphere of 5% CO2—95% air mixture. For cell counting and subculture, the cells were dispersed with a solution of 0.05% trypsin and 0.02% EDTA.

The HepG2 cell line was kindly provided by Dr S. Knasmüller (Institute of Cancer Research, Medical University of Vienna, Austria). The cells were stored in liquid nitrogen and cultivated in Dulbecco's minimal essential medium, supplemented with 15% fetal bovine serum, both from Gibco (Paisely, Scotland) and 50 µg/ml penicillin–50 IU/ml streptomycin in 75 cm^2 tissue culture flasks (Greiner, Kremsmuenster, Austria). Cells were incubated at 37 °C in an atmosphere of 5% CO2—95% air mixture and for their counting and subculture, the cells were dispersed with a solution of 0.05% trypsin and 0.02% EDTA.

2.3. Cytotoxicity Assay by Neutral Red (NR) Test

The NR test was performed to assess cytotoxicity, as previously described by [7]. Viable cells actively transport this dye across their cell membrane; therefore, after subsequent lyses absorbance can be used as a measure of cell viability. The solution stock of NR (3.3 g/l) was diluted to 1/100 in the cell culture medium and the extract solution consisted of 50% (v/v) ethanol in Milli-Q water with 1% (v/v) acetic acid. Caco-2 or HepG2 cells (1 x 10^4 cells/ml/well) were preincubated in 96-well multidishes for 24 h at 5% CO_2 – 95% air at 37°C following the incubation in presence of toxins OTA (20 nM), AFB_1 (10 µM) and their mixture. After72 h of incubation in presence of each mycotoxin alone and their mixture or the vehicle, 150 ml of freshly prepared NR solution pre-warmed to 37°C was added to each well and all plates returned to the incubator at 37°C for 4 h. The cells were washed two times and 150 ml of the extract solution were added in each well and plates were shaken for 15 min. The absorbance at 540 nm was determined using a Microplate Reader DYNATECH MR 4000.

2.4. Cytotoxicity Assay by MTT Test

MTT test was used to assess cell viability based on the capacity for viable cells to metabolise a tetrazolium colourless salt to a blue formazan in mitochondria [7]. Caco-2 or HepG2 cells (1 x 10^4 cells/ml/well) were preincubated in 96-well multidishes for 24 h at 5% CO_2 – 95% air at 37°C prior incubation in presence of toxins OTA (20 nM), AFB_1 (10 µM) and their mixture. After 72 h of incubation, 100 µl of 0.5% solution of thiazolyl blue tetrazolium bromide (MTT) were added to each well and 2 h later the medium was eliminated. Subsequently, 100 µl of dimethyl sulfoxide (DMSO) were added to the wells to extract the formazan formed in the viable cells. After 5 min of continuous stirring, the absorbance was determined at 540 nm using a Microplate Reader DYNATECH MR 4000. The absorbance is proportional to the number of viable cells.

2.5. Cytotoxicity Assay by Lactate Dehydrogenase Measure

The LDH assay was performed to assess cytotoxicity, as previously described [23]. Caco-2 or HepG2 cells (1 x 10^5 cells/ml/well) were preincubated in 24-well multidishes for 24 h at 5% CO_2 – 95% air at 37°C. Cell viability was assessed by LDH leakage through the membrane into the medium. After 48 h of incubation in presence of mixture of toxins (OTA, 20 nM) and (AFB_1, 10 µM) or the vehicle, cells supernatant were used for the presence of LDH by LDH assay kit (Biomerieux, Lyon, France). In this test, three wells were used for each mycotoxin (OTA and AFB_1) and their mixture. The amount of LDH measured is related to the protein content of cellular homogenates, determined using the colorimetric method of Bradford. Leakage was expressed as percentage difference from controls.

2.6. Extraction and Determination of Malonedialdehyde (MDA)–Thiobarbituric acid (TBA) Adduct

Cells Caco-2 or HepG2 (1 x 10^5 cells/ml) were cultured in 24-well multidishes (Polylabo, France) for 24 h at 37°C as described above, and then cultures were incubated in the presence of each mycotoxin alone and their mixture [ZEA (20 nM) + AFB_1 (10 µM)] for 24 h at 37°C. After this incubation, cells were trypsinised, centrifuged and resuspended in SET buffer (0.1M NaCl, 20mM EDTA, 50mM Tris–HCl, pH 8.0). As previously described [24], extraction and determination of the malonedialdehyde (MDA)–thiobarbituric acid (TBA) adduct by HPLC and fluorimetric detection after extraction in n-butanol (50 ml injected for analysis) was performed. The amount of MDA measured is related to the protein content of cellular homogenates, determined using the colorimetric method of Bradford.

2.7. Statistical Analysis

The data generated by this study were statistically processed using SPSS 11.19 statistical software. The analysis of variance (ANOVA) was used to treat all data. Whenever a significant difference ($p<0.05$) was revealed, the ANOVA test is complemented by the Tukey post ANOVA test to identify the variable (s) with very significant differences compared to the values of the control group.

3. Results

3.1. Cells Viability

3.1.1Cell Viability as Measured by Mitochondria Succinate Dehydrogenase Activity

"Fig.1" summarized viability of both HepG2 and Caco-2 cells as measured by MTT test after incubation of mycotoxin AFB_1 (10µM) or OTA alone (20nM) or their mixture. AFB_1 (10µM) decreased cells viability about 28 - 29% in both Caco-2 and HepG2 cells line while OTA did not produce any effect. However, the mixture AFB_1 + OTA reduced cell viability at 43% and 35% in Caco-2 and HepG2 cells respectively indicating an enhancement of AFB_1 effect by OTA.

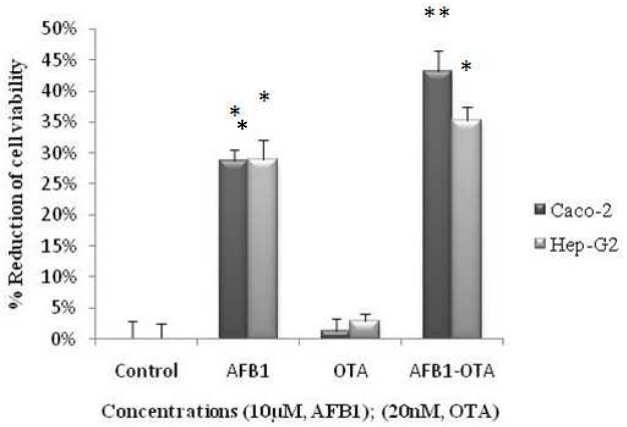

Figure 1. *Cytotoxicity effect of AFB_1 (10µM) or OTA (20nM) or their mixture on Caco-2 or HepG2 cells after 72 h incubation evaluated by MTT test. Results are given as mean ± SD from three independent experiments. *Different from control at p < 0.05 and **p < 0.001.*

3.1.2. Cell Viability Measured by Lysosome Integrity

"Fig.2" summarized viability of both HepG2 and Caco-2 cells as measured by Neutral Red test after incubation of mycotoxin AFB_1 (10µM) or OTA alone (20nM) or their mixture. AFB_1 (10µM) alone reduced cell viability in order to 24% and 28% in both Caco-2 and HepG2 cells respectively. In contrast, OTA (20nM) did not reduce cells viability. Interestingly, the addition of AFB_1 (10µM) to OTA (20nM) leaded to reduction of cell viability at 38% and 43% in Caco-2 and HepG2 cells respectively so an increase of AFB_1 toxic effect by OTA.

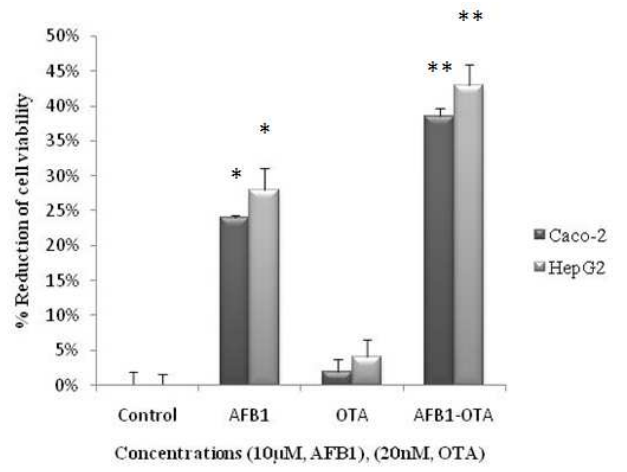

Figure 2. *Cytotoxicity effect of AFB_1 (10µM) or OTA (20nM) or their mixture on HepG2 or Caco-2 cells after 72 h incubation evaluated by Neutral Red test. Results are given as mean ± SD from three independent experiments.*Different from control at p < 0.05 and **p < 0.001.*

3.2. Cell Death Evaluated by LDH Depletion

"Fig.3" showed that OTA (20nM) alone did not increase leakage of LDH into the culture medium when compared to controls. However, AFB_1 (10µM) increased leakage of LDH which was more intensive when associated with OTA (20nM). Moreover, AFB_1 (10µM) had been found more potent in HepG2 cells.

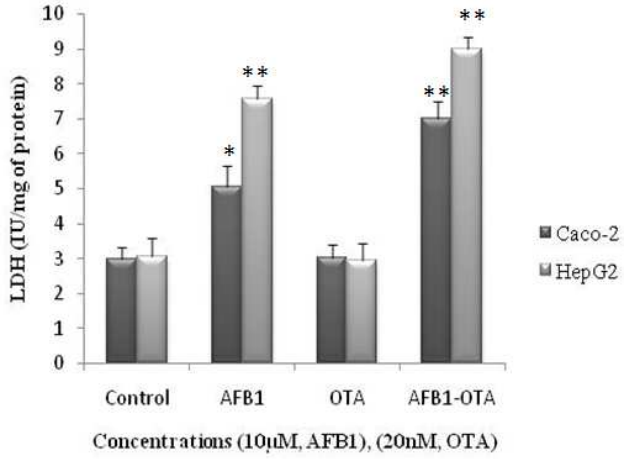

Figure 3. *Increasing of LDH leakage into the cell culture medium after incubation of Caco-2 or HepG2 cells with OTA (20nM) or AFB_1 (10µM) or their mixture by 24h. Results are given as mean ± SD from three independent experiments.*Different from control at p<0.05 and **p<0.001.*

3.3. Lipid Peroxydation as Measured by MDA Production

"Fig.4" showed that AFB_1 (10 µM) alone increased MDA production by 23% and 40.3% in Caco-2 and HepG2 cells respectively while OTA (20nM) did not elevate MDA production when compared to control. The mixture of AFB_1 (10µM) and OTA (20nM) increased MDA production more intensively when compared to AFB_1 (10 µM) alone at level by about 70.6% and 150% in Caco-2 and HepG2 cells respectively.

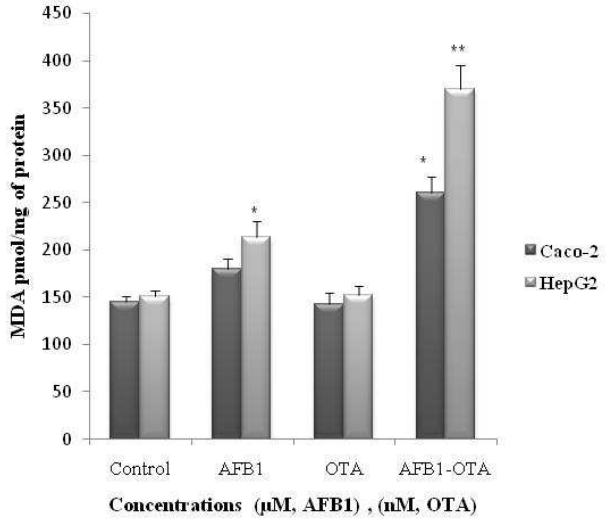

Figure 4. *Lipid peroxidation as measured by MDA production (complex formed with thiobarbiturate) in Caco-2 or HepG2 cells incubated for 24 h with AFB_1 (10µM) or OTA (20nM) alone or mixtures of these mycotoxins. Results are given as mean ± SD from three independent experiments. *Different from control at p<0.05 and **p<0.001.*

Table 1 presented deductions from a rapid comparison between experimental values of MDA increases and theoretical values calculated from observed individual ones. The addition of OTA and AFB_1 increased MDA production in a additive, synergistic or potentiation way.

Table 1. *Mean values of cells viability reduction, cells death and MDA production in Caco-2 and HepG2 cells line after of single or combined treatment expressed by % of control.*

HepG2	AFB_1	OTA	AFB_1 + OTA	Expected values	Conclusion
MTT test	29%	3%	35%	29%	Additive
NR test	28%	4%	43%	32%	Synergistic
LDH leakage	37.3%	0%	49%	37.3%	Potentiation
MDA incresing	40.3%	0%	150%	40.3%	Potentiation
Caco-2	**AFB_1**	**OTA**	**AFB_1 + OTA**	**Expected values**	**Conclusion**
MTT test	28%	1.5%	43%	29.5%	More than additive
NR test	24%	2%	38%	26%	More than additive
LDH leakage	25.8%	0%	40.1%	25.8%	Potentiation
MDA incresing	23%	0%	74.3%	23%	Potentiation

4. Discussion

According to the literature, more than one mycotoxin may occur together in the same grain and furthermore, the manufacturing process mixes together different raw materials, yielding totally new matrixes with a new risk profile [3]. Thus, there is a higher probability of being exposed to low doses of several mycotoxins at the same time throughout life than to a high level of only one mycotoxin. Logically, several studies were performed on varied combinations of mycotxins such as binary or tertiary combination on cells line in the aim to evaluate their simultaneous cytotoxic or genotoxic effect [5, 7, 14-16]. Previously, it had been reported that the interaction between OTA and AFB_1 tested both at high levels leaded always to additive effect [15, 16]. Surprisingly, when OTA was tested at very low or non toxic level, OTA decreased AFB_1 genotoxicity [16]. This finding poses an interesting question. Could OTA tested at non cytotoxic level decrease also AFB_1 induced - cytotoxic effect? In this study, we attempted to provide adequate response to this question insofar as numerous surveys carried out on presence of mycotoxins in foods from Côte d'Ivoire revealed high concentrations of AFB_1 but low concentrations of OTA [21, 22].

Concentrations of toxins OTA (20nM) and AFB_1 (10µM) used in the present study have been determined in order to associate OTA at non toxic concentration and AFB_1 at evident toxic concentration. Thus, it has been possible to evaluate the possible potentiating effect of OTA in AFB_1 toxic action. Binary OTA (20nM) and AFB_1 (10µM) were incubated in both Caco-2 and HepG2 cells lines and their interactive had been observed effect through cell endpoints such as lysosomes and mitochondria integrities, cell lactate dehydrogenase (LDH) leakage and MDA production. OTA alone tested at 20nM was not cytotoxic in both Caco-2 and HepG2 cells lines, but, associated to AFB_1 (10µM), OTA enhanced markedly toxic effect induced by AFB_1. Such results have been observed for all cell endpoints studied in the present study. The mitochondria impairment and lysosome damage were more intensive with the mixture of mycotoxins when compared to AFB_1 alone. Similarly, OTA enhanced cells necrosis and lipid peroxydation (measured by MDA production) induced by AFB_1 alone. Since OTA (20nM) was not significantly cytotoxic so not different to negative control, we can admit that a possible potentiating of OTA in AFB_1 toxic action. Indeed, potentiation occurs when one substance does not have a toxic effect on a certain organ or system but when added to another chemical makes that chemical much more toxic (example: 0 + 2 = 10) [25]. So, the elevation of AFB_1 – cytotoxic effect caused by OTA tested at non toxic level (20nM) could be the consequence of such chemical characteristic interactive. Thus, ours results supported the findings reported on the capably of OTA to influence cell pathways involved in AFB_1 toxic action [16]. Indeed, OTA has complex mechanisms of action that include evocation of oxidative stress, bio-energetic compromise, mitochondrial impairment, inhibition of protein synthesis, production of DNA single strand breaks and formation of OTA-DNA adducts [18, 26-28]. The bio-energetic compromise induced by OTA may be responsible for the generation of reactive oxygen species (ROS) that leaded to global oxidative damage to DNA and lipids and damage to proteins [29, 30]. Despite, its cytotoxic effect was not perceptible at level of 20nM, but, through its mechanism of action namely mitochondrial impairment and generation of ROS as measured in the present study by mitochondria succinate dehydrogenase activity and MDA production respectively, OTA could create suitable intracellular conditions which may favorable to AFB_1 cytotoxic action. Indeed, it had been reported that, as a secondary route of toxic pathway, AFB_1 may yield oxygen free radicals forms that react with guanine residues, forming 8-oxodG or others cytotoxic effects [31] namely lipids cell or lysosome membrane peroxydation. These cytotoxic pathways could lead to lysosome membrane destroyed or cells necrosis which release the LDH in extracellular medium. In addition, previous studies reported that AFB_1 could also cause mitochondria impairment by the decreasing activities of mitochondria enzymes such as succinate dehydrogenase, malatedehydrogenase and isocitrate dehydrogenase [32]. Thus, since, OTA and AFB_1 could exert their toxic action on the same cellular targets or through the same cell pathways, their interaction appear evident. Therefore, the influence exerted by OTA, despite non toxic, on AFB_1 cytotoxicity leading to additive, synergistic, potentiation or antagonism is plausible. The potentiation of AFB_1 toxic effect by varied compounds such as ethanol or *Fusarium* toxins zearalenone (ZEA) and deoxynivalenol (DON) had been previously revealed [14, 33]. Indeed, ethanol pretreatment of rats potentiated AFB_1-induced

hepatotoxicity by increasing MFO enzymes, aniline hydroxylase and p-nitroanisole-O-demethylase activities and lipid peroxydation, and decreasing in cytochrome b5, epoxide hydrolase activity and hepatic glutathione content [33]. More recently, it has been reported that AFB_1 + ZEA or DON showed synergetic effects on cytotoxicity measured by mitochondria enzyme succinate succinate dehydrogenase activity. Low levels of AFB_1 were antagonistic to ZEA, but high doses of AFB_1 displayed synergistic effects with ZEA or DON on oxidative damage. ZEA also ameliorated AFB_1-induced apoptosis [14].

On the other hand, the hepatic cells HepG2 have been found more sensitive to mycotoxins (AFB_1 and OTA + AFB_1) when compared to intestinal cells Caco-2 particularly in cells necrosis and more high in oxidative damage. That confirmed that not only liver as main target of AFB_1 but also the activation of ROS production with consequences DNA, proteins and lipid oxidation, is one of the keys molecular bases of the liver diseases induced by AFB_1 [3, 9, 33, 34]. Moreover, the apparent resistance of human intestinal cells Caco-2 to AFB_1 could be explained by its structure which may confer to membrane selective permeability to toxicants passage into intestinal cells [35, 36].

In conclusion, the interactive effect between AFB_1 and very low level of OTA have revealed an enhancement of AFB_1 in both human intestinal (Caco-2) or hepatic (HepG2) cell lines suggesting a synergistic effect or potentiation of AFB_1 cytotoxity by OTA used at non toxic level (20nM). On the other hand, the human hepatic cells (HepG2) have been found very sensitive at toxic effect mycotoxins particularly in cells necrosis and more high in lipids oxidative damage.

Competing Interests

The authors declare that we have no competing interests.

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