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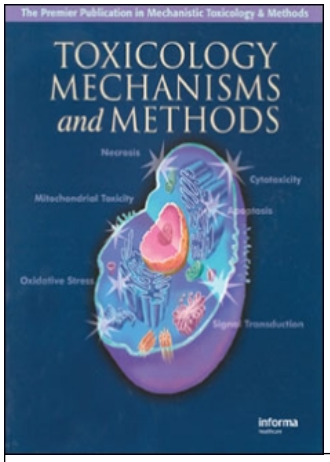

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Cytotoxicity and Oxidative Damage in Kidney Cells Exposed to the Mycotoxins Ochratoxin A and Citrinin: Individual and Combined Effects

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ABSTRACT Ochratoxin A (OTA) and citrinin (CTN) are two mycotoxins, quite common contaminants, that can occur jointly in a wide range of food commodities. Both mycotoxins have several toxic effects but both share a significant nephrotoxic potential since OTA and CTN were reported to be responsible for naturally occurring human and animal kidney diseases.

Considering the concomitant production of OTA and CTN, it is very likely that humans and animals are always exposed to the mixture rather than to individual compounds. Therefore, the aim of the present study was to investigate, using kidney cell culture (Vero cells), whether cytotoxicity and essentially oxidative cell damage (a key determinant of renal diseases) are enhanced by combination of both mycotoxins as compared to their effect separately. To this end, we have assessed their effects individually or combined on cell proliferation using three different cell viability assays (MTT, Trypan Blue, and Neutral Red). In addition, the role of oxidative stress was investigated by measuring the malondialdehyde (MDA) level and the expression of the heat shock protein Hsp 70.

Our results clearly showed that cultured renal cells respond to OTA and CTN exposure by a moderate and weak inhibition of cell proliferation and induction of oxidative stress, respectively. However, when combined, they exert a significant increase in inhibition of cell viability as well as the induction of MDA level and Hsp 70 expression. OTA and CTN combination effects are clearly of synergistic nature. The enhanced induction of oxidative stress observed with OTA and CTN simultaneously could be relevant to explain the molecular basis of the renal diseases induced by these mycotoxins.

KEYWORDS: Mycotoxins; Ochratoxin A; Citrinin; Cytotoxicity; Oxidative Damage; Renal Toxicity; Combined Effects

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INTRODUCTION

In human health risk assessment, ingestion of food is considered a major route of exposure to many industrial or environmental contaminants. Mycotoxins constitute an example of naturally occurring contaminants that have been found in a wide variety of agricultural products destined for human and animal feeding. They are secondary metabolites produced by three main genera of fungi (*Aspergillus*, *Fusarium*, and *Penicillium*) and the ingestion of mycotoxin-contaminated products can lead to serious health

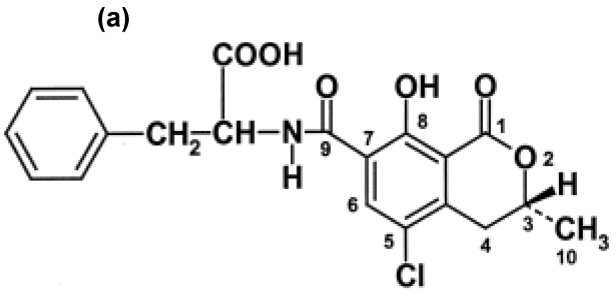

FIGURE 1 **Chemical structures of (a) ochratoxin A and (b) citrinin.**

problems. Several pathologies associated with mycotoxin exposure in humans and animals have been evidenced; therefore, mycotoxins have become a worldwide preoccupation (for review, see Hussein and Brasel 2001; Bennett and Klich 2003).

In recent years, investigations assessing the potential risk of mycotoxins to human health arose; however, most of the conducted studies evaluate the effect of mycotoxins taken individually. Therefore, in our understanding, the real risk to human health is decreased since food items generally contain concomitantly different mycotoxins produced by the same species (Abbas et al. 1989; Yiannikouris and Jouany 2002; Molinié et al. 2005). This is particularly true since several studies have demonstrated that consumption of food items containing concomitantly different mycotoxins has a greater degree of damage to health (Sedmikova et al. 2001; Speijers and Speijers 2004; Wangikar et al. 2005).

Among different mycotoxins, ochratoxin A (OTA) and citrinin (CTN) (Fig. 1) are quite common contaminants that can occur jointly in a wide range of food commodities; both are, in fact, produced by *Penicillium* and *Aspergillus* families, which are worldwide in distribution. OTA and CTN constitute one of the most frequently occurring combinations of mycotoxins in different plant products (Pohland et al. 1992; Vrabcheva et al. 2000). This mycotoxin combination is particularly relevant since although both OTA and CTN have many toxic effects in humans and animals, they share an important nephrotoxic potential (for review, see Hussein and Brasel 2001; Bennett and Klich 2003). Indeed, OTA and CTN have been identified to be nephrotoxic and associated with alterations of renal functions and the development of renal pathologies in in vivo studies (Krogh et al. 1973 ; Plestina 1992; Stoev et al. 1998; Petkova-Bocharova and Castegnaro 1991; Krogh 1992; NTP 1989; Kogika et al. 1993; IARC 1986). In addition, some evidence pointed out these mycotoxins as causal agents of the human Balkan endemic nephropathy (BEN). BEN is a slowly progressing and chronic nephropathy becoming overt usually in the fourth or fifth decade of life and leading eventually to renal failure and death (Fillastre 1997; Peraica et al. 1999; Pfohl-Leszkowicz et al. 2002; Stoev 1998; Tatu et al. 1998).

Considering the coincident production of OTA with CTN, it is very likely that humans and animals are always exposed to the mixture rather than to individual compounds. This fact leads to the question of whether these mycotoxins interact with each other and whether this interaction would enhance their respective nephrotoxic potential. In this context, the aim of the present study was to evaluate using kidney cell culture (Vero cells) whether cytotoxicity and essentially oxidative cell damage, a key determinant of renal disease, are enhanced by combination of both mycotoxins as compared to their effect separately (Riley 1998; Kasiske and Keane 1999; Goldstein and Schnellmann 1998).

To this end, the effect of OTA and CTN combined or separate was assessed on cell proliferation using three different cell viability assays–MTT assay, Trypan Blue assay (TB), and Neutral Red assay (NR)–in kidney Vero cells. In addition, the role of oxidative stress as a specific factor in OTA- and CTN-mediated nephrotoxicity was investigated by measuring (i) an index of lipid peroxidation, the malondialdehyde (MDA) level, presumed late marker of oxidative stress, and (ii) the expression of the heat shock protein Hsp 70, an early marker of oxidative damage.

MATERIALS AND METHODS

Chemicals

OTA and CTN were obtained from Sigma Chemical Company (St. Louis, MO) and were dissolved in ethanol/water (v:v). Phosphate buffer saline (PBS); Trypsine-EDTA; penicillin and streptomycin mixture; 3–4,5-dimethylthiazol-2-yl, 2,5-diphenyltetrazolium bromide (MTT); Neutral Red; Trypan Blue; Goat antimouse alkaline phosphatase-conjugated antibody; Nitro Blue Tetrazolium (NBT); and 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) were from Sigma–Aldrich (France). Mouse anti-Hsp 70 monoclonal antibody (SPA-80) was from Stressgen (USA).

Cell Culture

Vero cells, from green monkey kidney (Terasima and Yasukawa 1988) (Biovalori, France), were routinely incubated in a humidified air/CO_2 95:5 mixture at 37 °C. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM), and 1% of mixture penicillin (100 IU/mL) and streptomycin (100 μg/mL).

Cell Viability Assays

To examine the effect of xenobiotics in cellular toxicity, the most frequently used cytotoxicity endpoints are based on the breakdown of the cellular permeability barrier and reduction of mitochondrial functions (dor review, see Eisenbrand et al. 2002). In the present study, we choose to monitor three cytotoxicity endpoints that evidenced either alteration of mitochondrial

functions (MTT assay) or membrane permeability changes (TB and NR assays).

MTT Assay

Cytotoxicity of OTA and CTN was determined using the colorimetric method described by Mosmann (1983). This method assesses the ability of viable cells to convert MTT into formazan by the mitochondrial enzyme succinate dehydrogenase. Cells were seeded on 96-well culture plates (Polylabo, France) at 2.10^4 cells/well and treated with increasing concentrations of OTA, ranging from 0 to 50 μM, or CTN at concentrations ranging from 0 to 250 μM or OTA and CTN simultaneously (0–50 μM) combined at equimolar doses for 48 h at 37 °C. Then, the culture medium was replaced by 200 μL fresh medium containing 0.5 mg/mL MTT and the plates were incubated for 3 h at 37 °C. The medium was then removed and replaced by 100 μL of 0.04 M HCl/isopropanol to dissolve the converted purple dye. The absorbance was measured with spectrophotometer microplate reader (Stat Fax 3200 Awareness Technology) at a wavelength of 560 nm.

Cell viability was expressed as the relative formazan formation in treated samples as compared to control cells. IC_{50} values were defined as the concentration inducing 50% of loss of cell viability and determined from the corresponding viability curves.

Neutral Red Assay

This second cytotoxicity assay was carried out using NR dye. This assay is based on the incorporation of the supravital dye NR into the lysosomes of viable cells only. Compounds that injured the plasma or lysosomal membranes decreased the uptake and the subsequent retention of the dye (Garret et al. 1981). The NR assay was performed as described by Borenfreund and Puerner (1985).

Vero cells were seeded in 96-well plates at a density of 2.10^4 cells/well. After 24 h, the growing cells were treated with different concentrations of OTA (0–50 μM), CTN (0–250 μM), and OTA with CTN simultaneously combined at equimolar proportions (0–50 μM) for 48 h. The solutions were removed from the plates and the cells were washed with 200 μL PBS/well. Then, cells received 150 μL of the NR solution (50 mg/mL NR in RPMI) and were incubated for 3 h and washed three times with PBS. The dye within viable cells was released by extraction with a mixture of acetic acid, ethanol, and water (1:50:49). Absorbance of NR was measured using a spectrophotometric microplate reader (as previously described) at 540 nm. NR uptake, which is proportional to the number of viable cells, was expressed as a percentage of uptakes as compared to control.

Trypan Blue Assay

TB assay is a dye that penetrates both viable and dead cells; however, only viable cells can reject the dye and appear brilliant; due to an irreversible membrane damage, dead cells are unable to reject the dye and appear blue.

Cells (5.10^5 cells/well) were cultured in 24-well multidishes plates (Polylabo, France) for 24 h at 37 °C, and then cultures were incubated in the presence of OTA ranging from 0 to 50 μM, CTN ranging from 0 to 250 μM, and OTA with CTN simultaneously combined at equimolar proportions (0–50 μM). After 48 h, the cells were washed with PBS and trypsinized. The cells were then collected and centrifuged at 1800 rpm for 10 min, and the cell pellet was suspended in 1 mL RPMI and aliquots of 40 μL were added to 160 μL of a TB solution (0.4%). The number of noncolored cells (viable cells) was scored in a Malassez chamber.

Type of Interactive Effect

In order to determine what kind of interaction is occurring between OTA and CTN (additive, synergistic, antagonist, etc.), we have calculated the "V" value for the different undertaken viability assays.

According to Brown (2000), The "V" value can be calculated as follows:

$$v = \frac{\text{Expected } ED_{50} \text{ of } (A + B)}{\text{Observed } ED_{50} \text{ of } (A + B)}$$

V = interactive effect, ED_{50} = effective dose, A = OTA, and B = CTN.

V < 0.7: there is antagonism between the two mycotoxins.

0.7 < V < 1.3: an additive effect is occurring between them.

1.3 < V < 1.8: the effect is more than additive, indeed synergistic.

Lipid Peroxidation Determination

Lipid peroxidation was assayed by the measurement of the MDA level according to the method of Ohkawa et al. (1979). The cells were exposed to different concentrations of OTA (12.5 and 25 μM), CTN (60 and 120 μM), and OTA (12.5 and 25 μM) combined to fixed CTN (60 μM) for 12 h, followed by addition of 1 mM H_2O_2 for 2 h. The cells were then washed with cold PBS, scraped, and homogenized in ice-cold 1.15% KCl. Samples containing 100 μL of cell lysates were combined with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid adjusted to pH 3.5, and 1.5 mL of 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4 mL with distilled water and heated to 95°C for 120 min. After cooling to rt, 5 mL of the mixture of n-butanol and pyridine (15:1, v/v) was added to each sample and the mixture was shaken vigorously. After centrifugation at 15000 rpm for 10 min, the supernatant fraction was isolated and the absorbance was measured at 532 nm.

Hsp 70 Expression

Cells (10^6 cells/well) were cultured in 6-well multidishes plates (Polylabo, France) for 24 h at 37°C, and then cultures were incubated in the presence of increasing doses of OTA (0–50 μM), CTN (0–100 μM), and a combination of the two mycotoxins–CTN at a fixed concentration (0.75 μM) and OTA (0.05–1 μM)–for 48 h at 37°C and were directly processed for Hsp 70 analysis. After incubation, cells were rinsed with ice-cold PBS, scraped, collected in a lysis buffer (Hepes 0.5 M containing 0.5% Nonidet-P40, 1 mM PMSF, 1 μg/mL aprotinin, 2 μg/mL leupeptin, pH 7.4), and incubated in ice for 20 min before centrifugation. Protein concentrations were determined in cell lysates using Protein BioRad assay (Bradford 1976). Equal amounts of proteins (20 μg) were separated

by 10% SDS–polyacrylamide gel electrophoresis. Separated proteins were then electroblotted on nitrocellulose membrane in a transfer buffer (10 mM Tris–base, pH 8.3, 96 mM glycine, and 10% methanol). The membrane was then blocked in TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 5% of bovine albumin serum and washed in TTBS (TBS containing 0.3% Tween 20) and probed with an antibody for Hsp 70 at a 1:1000 dilution for 12 h at rt. After that the membrane was washed and incubated with goat antimouse alkaline phosphatase-conjugated antibody at a 1:3000 dilution for 1h. The membrane was then washed and the chromogenic substrate BCIP/NBT was added to localize antibody binding. The levels of Hsp were obtained by scanning densitometry.

Hsp Cytoprotective Potential Assay

This assay was performed to check the ability of induced Hsp to the ability of an Hsp to protect against OTA- and CTN-induced oxidative stress and to prevent cell death. Vero cells seeded on 96-well culture plates at 2.10^4cells/well were first heat shocked (1 h, 43 °C) and allowed to recover at 37 °C for 24 h to have maximum Hsp expression. After heat shock and recovery, cells were exposed to different concentrations of OTA (varying from 0 to 1 μM) either alone or combined with CTN at a fixed concentration (0.75 μM) for 48 h at 37 °C. Cell viability was assessed by the MTT assay, as previously described. Finally, inhibition of cell viability was compared between cells treated with OTA and CTN

Statistical Analysis

The data are expressed as mean ± standard deviation (SD) for at least three independent determinations (triplicate) for each experimental point. Statistical differences between controls and treated groups were determined by χ^2 test. Differences were considered significant at $p < 0.01$ or $p < 0.005$ as noted.

RESULTS

Inhibition of Cell Proliferation

MTT Assay

Cytotoxic effects of OTA and CTN on Vero cells after 48 h of incubation were measured by the MTT assay. OTA treatment caused a marked decrease of cell viability in a dose-dependent manner at concentrations ranging from 0 to 50 μM. Reduction in the viability of Vero cells by OTA was significant already at low concentrations and the estimated IC_{50} was about 37 μM.

Concerning CTN, at concentrations ranging from 0 to 250 μM, a decrease of cell viability was observed, however, with a much higher IC_{50} value, around 220 μM. In fact, up to a concentration of 60 μM, no significant change in cell viability was observed. OTA appears to be much more cytotoxic than CTN.

The combination of the two toxins at equimolar concentrations ranging from 0 to 50 μM led to an important increase of cytolethality as compared to each toxin taken alone; the IC_{50} of the combination was about 24 μM.

TABLE 1 **IC_{50} values of OTA, CTN, and the combination as determined by MTT, Neutral Red, and Trypan Blue assay**

	IC_{50}		
	MTT Assay	Neutral Red Assay	Trypan Blue
OTA (μM)	37	12	11
CTN (μM)	220	175	160
combination (OTA/CTN) (μM)	24	5.8	8.5
(V) of combination OTA/CTN	1.7	1.6	1.7

"V" value of the combination as determined by Brown (2000).

Neutral Red Assay

Similar results were obtained with the NR assay. A dose-dependent decrease of cell proliferation was clearly observed with both mycotoxins OTA and CTN, with IC_{50} values of 12 μM and 175 μM, respectively. When cells were exposed to OTA and CTN simultaneously, cell viability decreased significantly as evidenced by the IC_{50} value of the mixture found around 5.8 μM.

Trypan Blue Assay

The IC_{50} values determined for OTA and CTN using the TB assay were approximately 11 μM and 160 μM, respectively. The IC_{50} value of combined toxins is 8.5 μM. IC_{50} values of the different cytotoxicity assays are summarized in Table 1.

Induction of Lipid Peroxidation

The results of lipid peroxidation are illustrated in Figure 2. After 12 h of incubation of Vero cells in the presence of two concentrations of OTA of 12.5 and 25 μM, the MDA level increased from a basal value of 0.87 μM in controls to reach

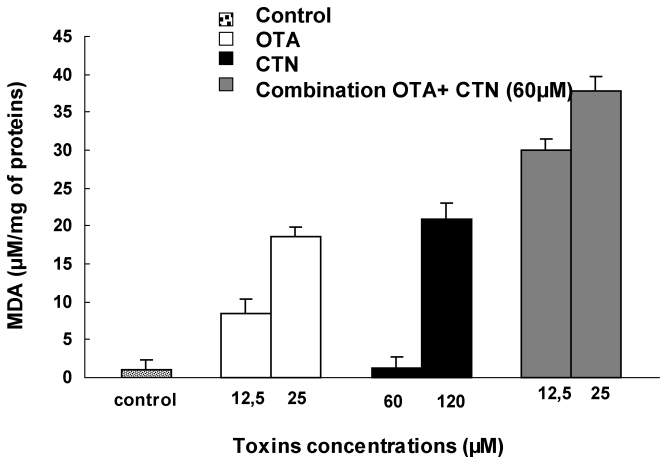

FIGURE 2 **Induction of lipid peroxidation as measured by MDA level in Vero cells treated with OTA alone (12.5 and 25 μM), CTN alone (60 and 120 μM) and combined (CTN 60 μM + OTA 12.5 μM or CTN 60 μM + OTA 25 μM). Data are expressed as mean values ± standard deviation of independent experiments (n = 3).**

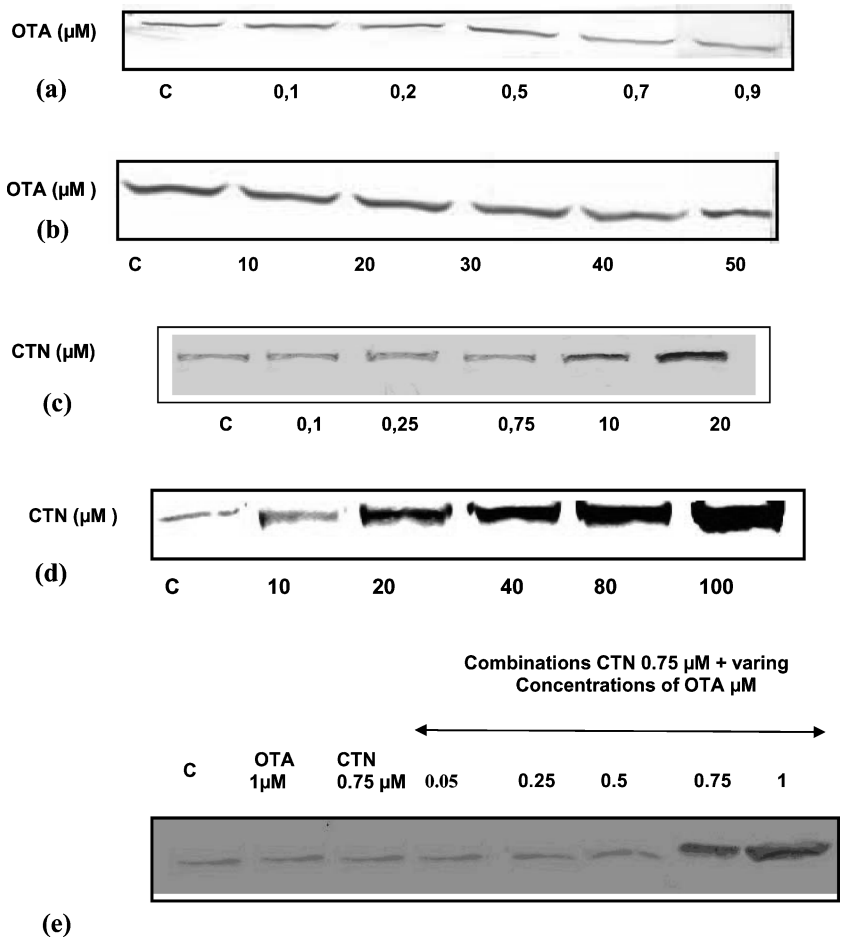

FIGURE 3 **Induction of Hsp70 expression in the presence of mycotoxins: (a) OTA (0.1–0.9 μM), (b) OTA (0–50 μM), (c) CTN (0–20 μM) (d) CTN (0–100 μM), and (e) combination of CTN at a fixed concentration (0.75 μM) and increasing OTA concentrations (0.05–1 μM).**

8.59 μM and 20 μM at 12.5 μM and 25 μM, respectively. For CTN, similar results were found. In fact, at 60 μM, the MDA level was nearly identical to the control (1.2 μM); however, when cells were treated with CTN at 120 μM, the MDA level increased to 21 μM.

When CTN is added at a fixed concentration of 60 μM (a concentration that doesn't induce MDA formation) in addition to OTA at 12.5 μM and at 25 μM, MDA production increased up to 29.36 μM and to 38.03 μM, respectively.

Hsp 70 Expression

Although several OTA concentrations were tested (from 0 to 50 μM), no change in Hsp 70 expression could be seen as illustrated by the example of immunoblotting (Fig. 3a and b). CTN at concentrations ranging from 0 to 20 μM displays a dose-dependant induction of the level of Hsp 70 as indicated by the immunoblotting (Fig. 3c), which was maintained at concentrations up to 100 M (Fig. 3d). To investigate the combined effects of both mycotoxins, we chose concentrations of OTA and CTN that do not display any Hsp 70 induction. Thus, at OTA concentrations varying from 0.05 μM to 1 μM (Fig. 3a) combined with a fixed concentration of CTN (0.75 μM concentration not inducing Hsp 70, Fig. 3c), an OTA dose-dependent increase in Hsp 70 level was observed (Fig. 3e). Induction of Hsp 70 level when cells were exposed to both mycotoxins simultaneously was further confirmed by results of scanning densitometry (Fig. 4).

Cytoprotection

An increase of cell viability was observed when cells are first heat shocked and then exposed to different OTA or CTN individually or combined. Thus, when cells were treated with increasing concentrations of OTA (0–1 μM) alone, with prior heat shock, cell viability decreased only about 2% for OTA concentration, whereas under the same conditions but without prior heat shock, cell viability decreased about 10%.

When OTA (0–1 μM) and CTN (0.75 μM) were combined, with prior heat shock, cell viability decreased about 15%. Without prior heat shock, and under the same conditions, cell viability fell from 100% to 72%. We can note that at noncytotoxic concentrations of OTA and CTN, the Hsp induced by the heat shock exhibits a significant cytoprotective effect, which was more evident when OTA and CTN were combined (Fig. 5).

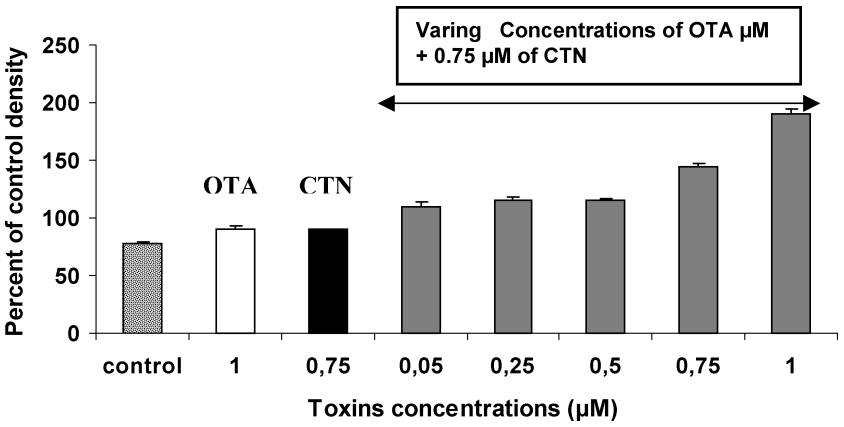

FIGURE 4 **Hsp70 level as determined by densitometry scanning induced by OTA (1 μM) alone, CTN alone (0.75 μM), or combination of a fixed CTN concentration (0.75 μM) and varying OTA concentrations (from 0.05 to 1 μM).**

DISCUSSION

Oxidative stress is a term commonly used to denote the imbalance between the concentrations of reactive oxygen species (ROS) and the antioxidative defense mechanisms of the body. Compelling evidence suggests that an excess of ROS production, to the extent that cellular defenses are overwhelmed and the cell is injured, is largely considered as playing a key role in a variety of human diseases (Halliwell and Gutteridge 1990; Calabrese et al. 1998), particularly renal diseases (Riley 1998; Speijers and Speijers 2004; Kasiske and Keane 1999; Goldstein and Schnellmann 1998).

Among the mycotoxins occurring in foodstuffs worldwide, OTA and CTN are very common and are reported to display several alterations and disorders in renal functions. Considering their nephrotoxic potential as well as their co-occurrence, the present study was conducted to investigate the role of cytotoxicity and mainly oxidative stress in the toxic response of renal cells following exposure to mycotoxins OTA and CTN taken individually or combined.

In the first set of experiments, we have assessed the effect of OTA and CTN, either individually or combined, on the inhibition of cell proliferation using different cytotoxicity assays (NR, TB, and MTT). Cell viability assays have shown that CTN alone was found weakly cytotoxic as compared to OTA, which has shown a moderate cytotoxicity in Vero cells as evidenced by the values of IC_{50} (Table 1). The combined effect of OTA and CTN are clearly above additive effects. In fact, the combination of OTA and CTN increased their cytotoxic potential and the cytolethality increased severalfold, which was further confirmed by the calculated V value found about 1.7 for the three different tests (MTT, TB, NR), confirming the synergistic effect (Table 1). Obviously, MTT, NR, and TB cytotoxicity assays evidenced cytolethality using different mechanisms that explain the different IC_{50} values.

In the second set of experiments, we have evaluated the involvement of oxidative stress in OTA- and CTN-induced toxicity using kidney cells. To assess oxidative stress, several methods are available. In the present study, we chose to measure the stable peroxidation products (mainly lipid peroxidation

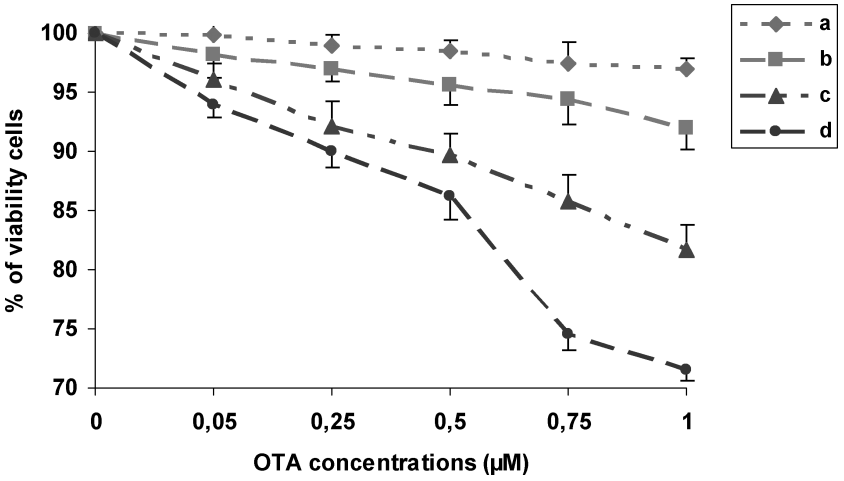

FIGURE 5 **Cytoprotective effects by Hsp 70 synthesized after a sublethal heat shock (1 h, 43°C) against OTA, and OTA combined with CTN: (a) Cytoprotective effect against mortality induced by OTA after a sublethal heat shock, (b) cytoprotective effect against mortality induced by OTA without a sublethal heat shock, (c) cytoprotective effect against mortality induced by OTA combined with CTN (varying OTA concentration + fixed concentration of CTN) after a sublethal heat shock, (d) cytoprotective effect against mortality induced by OTA combined with CTN (varying OTA concentration + fixed concentration of CTN) without sublethal heat shock.**

products) and the induction of a set of cellular cytoprotective proteins, Hsp 70.

In fact, the most common group of indices used to assess oxidative stress is that of peroxidation products of lipids, usually polyunsaturated acids, which are susceptible to attack by free radicals. The initial products of lipid peroxidation are conjugated dienic hydroperoxides that decompose into various aldehydes (Dotan et al. 2004). All these products of degradation and decomposition are used in assessing oxidative stress; however, the most widely used index is the end product of lipoperoxidation and considered as a late biomarker of oxidative stress and cellular damage, the MDA (Vaca et al. 1988; Kim et al. 2000; Draper et al. 1993; Yeo et al. 1999). Lipid degradation and consequently MDA production alter the structure and function of the cellular membrane and block cellular metabolism leading to cytotoxicity (Ennamany et al. 1995).

In the present study, we have monitored lipid peroxidation (MDA level) induced by OTA, CTN, and OTA with CTN simultaneously. Our findings have shown that both mycotoxins induced oxidative damage by enhancing lipid peroxidation in Vero cells since OTA and CTN, taken individually, increased MDA formation in a concentration-dependent manner (Fig. 2). We have noticed, however, that OTA's effect on MDA induction was more pronounced than CTN's. The combination of both mycotoxins at concentrations that do not exhibit significant MDA induction (CTN at 60 μM and OTA at 12.5 and 25 μM) enhanced the MDA level severalfold as compared to the mycotoxins taken separately. Interestingly, with regard to concentrations that display MDA induction, OTA- and CTN-induced oxidative stress seem to precede the loss of cell viability in Vero cells, indicating that the oxidative stress response may contribute to their cytotoxicity and is not a consequence of it.

To further assess OTA, CTN, and OTA with CTN cytotoxic- and oxidative-induced damage in Vero cells, the expression of Hsp 70 was monitored. In fact, nonspecific cellular oxidative damage is often observed during toxicity (Okada et al. 1999), and it is difficult, based on the analysis of MDA only, the presumed late biomarker of oxidative damage, to determine if the oxidative stress is the cause or the consequence of cellular toxicity. For this reason, it is relevant to check early biomarkers, such as Hsp. In fact, after oxidative injury, a set of cellular cytoprotective proteins known as heat shock proteins are induced and play a key role in cell protection and repair (Ritossa 1962; Welch 1993). This induction is triggered by structural damage caused to cell proteins, mainly thiol oxidation, and on general perturbations of the cellular redox status level by oxidative stress (Voellmy 1996; Zou et al. 1998; Freeman et al. 1999). Several published data have reported that many sources of oxidative stress can lead to the up-regulation of the Hsp 70 (Goldbaum and Richter 2001; Fehrenbach and Northoff 2001). In addition, the ability of a number of induced Hsp to protect against oxidative stress was proved (Gautier et al. 2001; Beyersmann and Hechtenberg 1997; Arrigo 1998). Hsp induction is even considered as an early marker of oxidative stress since several of these proteins have been shown to be induced by oxidative stressors at levels where overt oxidative damage and toxicity are not observed (Goering et al. 1992; Beyersmann and Hechtenberg 1997).

In this regard, we chose to monitor the effect of both mycotoxins either individually or combined on the expression of Hsp 70. Our results clearly showed that while CTN induced a sharp elevation in the expression level of Hsp 70 at concentrations ranging from noncytotoxic to sublethal (0–100 μM), in a dose-dependent manner (Fig. 3c and 3d), no significant change in Hsp 70 expression was observed after treatment with OTA (Fig. 4a and 4b). Interestingly, the combination of CTN (a fixed and low concentration that doesn't induce Hsp 70 [0.75 μM]) (Fig. 3c) with increasing doses of OTA (0.1–0.9 μM) showed a significant induction of Hsp 70 in a OTA dose-dependent manner. Thus, the observed Hsp 70 induction is strikingly due to the combination of both mycotoxins (Fig. 3e and Fig. 4).

Previous studies have shown that exposure to a heat shock may provide protection against other types of subsequent stresses through the induction of Hsp. On the other hand, it is possible that the induction of Hsp 70 during toxins (OTA and CTN) is a cytoprotective response of the cell; however, it may also be a by-product of cellular damage and serve no cytoprotective function. To examine these possibilities, the effect of heat shock on OTA and CTN cytotoxicity was measured. Heat shock diminished OTA cytolethality; approximately 10% of cells escaped the death at very low concentrations (0–1 μM). Cell viability was improved by 30% when cells were heat shocked then treated with the combination of OTA and CTN (Fig. 5).

The cytoprotection afforded by the heat shock and high levels of Hsp against OTA- and CTN-induced cytotoxicity argues for the fact that Hsp may constitute an important cellular defense mechanism. This protection is likely due to Hsp ability to protect cells from induced oxidative injury through an antioxidant mechanism, perhaps by improving protein stability of endogenous antioxidants (Polla et al. 1996).

In conclusion, cultured renal cells respond to OTA and CTN exposure by inhibition of cell proliferation and induction of oxidative stress. OTA-induced cytolethality and oxidative damage were found more pronounced than for CTN. However, when combined, both mycotoxins exerted a significant increase in inhibition of cell viability as well as the induction of MDA level and Hsp 70 expression, which are indicators of oxidative stress. Our results demonstrated that OTA and CTN combination effects are clearly of synergistic nature. Interestingly, the concentrations chosen for the combinations don't display any effects when taken individually (e.g., MDA assay, Hsp 70 expression); however, when combined, they showed a significant increase in the toxic response. The enhanced induction of oxidative stress observed with OTA and CTN simultaneously could be relevant to explain the molecular basis of the renal diseases induced by naturally occurring mycotoxins (Riley 1998; Bennett and Klich 2003).

Only the combination of two toxins is considered herein. It may happen that several congeners of these toxins are produced by the same genus *Aspergillus* and *Penicillium*. Up to five mycotoxin families have been found in the same commodities in Bangladesh (Dawlatana et al. 2002). Thus, there is an emergent need to revise the estimated tolerated doses in foodstuffs to provide a solid base for estimating the associated health risk to the general public.

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