



# Individual and combined effects of ochratoxin A and citrinin on viability and DNA fragmentation in cultured Vero cells and on chromosome aberrations in mice bone marrow cells

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## ABSTRACT

Ochratoxin A (OTA) and citrinin (CTN) are two common contaminant mycotoxins which can occur jointly in a wide range of food commodities. Both mycotoxins have several toxic effects but share a significant nephrotoxic and carcinogenic potential since OTA and CTN were reported to be responsible for naturally occurring human and animal kidney diseases and tumors. 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, in vivo and in vitro, whether DNA damage is 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 and DNA fragmentation in cultured *Vero* cells and in vivo by monitoring the induction of chromosome aberrations.

Our results clearly showed that cultured renal cells respond to OTA and CTN exposure by a moderate and weak inhibition of cell proliferation, respectively. However, when combined, they exert a significant increase in inhibition of cell viability. Similar results were found for the investigated genotoxicity endpoints (DNA fragmentation and chromosome aberrations). Altogether, our study showed that OTA and CTN combination effects are clearly synergistic. The synergistic induction of DNA damage observed with OTA and CTN taken concomitantly could be relevant to explain the molecular basis of the renal diseases and tumorigenesis induced by naturally occurring mycotoxins.

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## 1. Introduction

Mycotoxins are secondary metabolites of various moulds. They are naturally occurring contaminants encountered at high incidences in a wide variety of agricultural products intended for human and animal consumptions. The ingestion of mycotoxin-contaminated products can lead to serious health problems; several pathologies have been indeed associated to mycotoxins exposure and, investigations assessing the potential risk of mycotoxins to human health raised up. (For review, see Hussein and Brasel, 2001; Bennett and Klich, 2003). However, most of the conducted studies evaluate the effect of mycotoxins taken individually, thus, 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; Molinié et al., 2005). This is particularly true for ochratoxin A (OTA) and citrinin (CTN) (Fig. 1) which are quite common contaminants that occur jointly in a wide range

of food commodities. In fact, both OTA and CTN are, produced by *Penicillium* and *Aspergillus* families, which are worldwide in distribution (Martins et al., 2002; Speijers and Speijers, 2004). OTA and CTN constitute even one of the most frequently occurring combinations of mycotoxins in different plant products (Pohland, 1993; Vrabcheva et al., 2000; Speijers and Speijers, 2004).

OTA is a potent nephrotoxin and renal carcinogen in experimental animals (Elling, 1979; NTP, 1989; Boorman et al., 1992; Krogh, 1992). Epidemiological studies have provided evidence for its involvement in the pathogenesis of the human Balkan Endemic Nephropathy (BEN) associated with an increased frequency of urinary tract tumors (Pfohl-Leskowicz et al., 2002; Cosyns, 2003; Fuchs and Peraica, 2005; Stefanovic et al., 2006). Sufficient experimental evidence for carcinogenicity in animal studies has led to the classification of OTA as a possible human carcinogen (IARC, 1993). The mechanism of OTA toxicity and carcinogenicity is not well defined and controversial results regarding mode of action have been published. In fact, some authors proposed that genotoxicity plays a major role in OTA induced tumorigenesis and have postulated the formation of DNA adducts, induction of chromosome aberrations, micronuclei, etc. (Castegnaro and Dirheimer, 1998;

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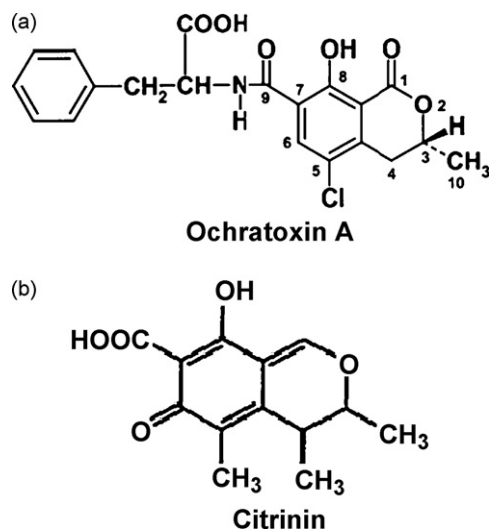


Fig. 1. Chemical structures of (a) ochratoxin A and (b) citrinin.

Lioi et al., 2004; Robbiano et al., 2004; Kamp et al., 2005; Russo, 2005; Simarro Doorten et al., 2006). Nevertheless, these data are in contrast to results from a number of studies which clearly (i) demonstrate that OTA does not form covalent DNA adducts (Zepnik et al., 2003; Dai et al., 2003; Mally et al., 2004, 2005) or (ii) support the hypothesis of a DNA non-reactive genotoxic mechanism for ochratoxin A (Arbillaga et al., 2007). Taking all the available evidence into consideration, results of genotoxicity studies do not suggest that OTA is mutagenic or a potent genotoxin.

Toxicity studies showed that CTN acted in animals as a nephrotoxin (Reiss, 1977; Betina, 1989), damaged the proximal tubules of the kidney (Aleo et al., 1991; Kogika et al., 1993; Phillips et al., 1980), and was implicated as a potential causative agent in human endemic Balkan nephropathy (Frank, 1991; IARC, 1986). Several studies showed that the compound had nephrotoxic properties and that it induced renal adenomas in rats (Betina, 1989; Arai and Hibino, 1983). While it is also known as a hepato-nephrotoxin in a wide range of species (Berndt, 1990; Bilgrami et al., 1980; Hanika et al., 1983), *in vitro* studies have demonstrated that CTN produced multiple effects on renal mitochondrial function and macromolecule biosynthesis that ultimately resulted in cell death (Chagas et al., 1992a,b, 1995). In addition, both positive and negative results have been reported on the mutagenicity and genotoxicity of CTN (Jeswal, 1996; Liu et al., 2003; Pfeiffer et al., 1998). CTN induced chromosomal aberrations, but not sister chromatid exchange (SCE), in V79-E cells in the presence of an exogenous metabolic system; this compound was not mutagenic in the *Salmonella typhimurium* assay (Thust and Kneist, 1979; Würzler et al., 1991). On the other hand, DNA single- and double-strand breaks were induced by CTN (Martin et al., 1986), showed aneuploidogenic potential in V79 cells (Pfeiffer et al., 1998) and induced chromosomal abnormalities in the bone marrow cells of treated mice (Jeswal, 1996). Furthermore, oral administration of CTN in the diets of male F344 rats induced the formation of renal adenomas in 70% of the fed rats (Arai and Hibino, 1983).

Considering the coincident production and taken into account their respective nephrotoxic and carcinogenic potential, OTA and CTN combination is particularly relevant. It is very likely that humans and animals are always exposed to the mixture rather than to individual compounds and that kidney cells are damaged by the concomitant action of the two toxins. In the present study, we aimed to investigate whether the combination of OTA and CTN would enhance their respective genotoxic potential. To this end, the

effect of OTA and CTN, combined or separate, was assessed *in vitro* on cell proliferation (kidney *Vero* cells) using MTT assay and DNA fragmentation and *in vivo* by monitoring chromosome aberration assay.

## 2. Materials and methods

### 2.1. 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), trypsin–EDTA, penicillin and streptomycin mixture, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), proteinase K, ribonuclease A (RNase A), *N*-lauryl sarcosine were from Sigma–Aldrich (France). Vinblastin (Gedeon Richter LTD) were dissolved in NaCl 0.9% solution. Giemsa was obtained from Fluka (France). Methanol and acetic acid (analysis grade) were from Prolabo and yeast powder was purchased from commercial local markets. All other chemicals used were analytical grade.

### 2.2. Cell culture and treatment

*Vero* cells, from green monkey kidney (Terasima and Yasukawa, 1988) (Biovalori, France) were routinely incubated in a humidified air/CO<sub>2</sub> 95:5 mixture at 37 °C. Cells were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum, 1% L-glutamine (200 mM) and 1% mixture penicillin (100 IU/ml) and streptomycin (100 µg/ml).

### 2.3. Animals

The mice used for the experiments were male white BALB/C, selected from mice of similar age and weight (20–25 g). The animals were kept for acclimatization 1 week under constant conditions of temperature and a cycle of light/dark 12/12 h. Animals had free access to standard granulated chow and drinking water. They were housed five animals per cage and randomly divided into four groups.

### 2.4. Cytotoxicity assay by MTT test

Cytotoxicity of OTA and CTN were 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<sup>4</sup> cells/well and treated with increasing concentrations of OTA or CTN and/or OTA + CTN combined in equimolar doses for 48 h at 37 °C. Then, 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 solubilize 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. IC50 values were defined as the concentration inducing 50% loss of cell viability.

### 2.5. DNA fragmentation assay in agarose gel electrophoresis

The cells were cultured (10<sup>6</sup> cells/ml) in 25 cm<sup>2</sup> flasks and left untouched for 24 h. Then, they were incubated with different concentrations of OTA (0.5, 1 and 5 µM), CTN (1, 6, and 12.5 µM) and CTN (0.5, 0.75 and 1 µM) combined to fixed concentration of OTA (0.5 µM) for 24 h at 37 °C. Controls were performed at the same time with ethanol:water (v:v). After 48 h incubation, the cell layer was rinsed twice with 2 ml of PBS. To extract DNA, the cells were lysed by incubation for 5 min with 2 ml of lysis buffer (5% *N*-lauryl sarcosine, 20 mM Tris–HCl pH 8.0, 5 mM EDTA) and the cell lysates were collected and transferred into 15 ml corning tubes. Proteins were digested overnight by incubation with 100 µg/ml proteinase K at 37 °C; then, 7.5 M ammonium acetate and phenol–Tris–HCl (pH 8)–chloroform (2:1, v:v) were added to the homogenate. After centrifugation for 5 min at 2200 × g at 20 °C, one volume (3.3 ml) of SEVAG (1 volume isoamyl alcohol: 2 volumes chloroform) was added to the aqueous supernatant and the mixture was centrifuged for 5 min at 2200 × g at 20 °C. The aqueous supernatant was transferred to Eppendorf tubes and incubated for 5 min at 37 °C to eliminate trace of chloroform. RNA was digested by incubation with 6 µg/ml RNase A for 30 min at 37 °C and then one volume of SEVAG was added. After centrifugation for 5 min at 2200 × g at 20 °C, the DNA in the aqueous supernatant was precipitated at –20 °C for 2 h with ethanol. The mixture was centrifuged for 45 min at 2200 × g at 4 °C, and the supernatant was removed. The pellet was rinsed with 70% ethanol, dried at room temperature for 2 h and resuspended in 100 µl TE 20-1 (20 mM Tris–HCl pH 8.0, 1 mM EDTA) for DNA quantification by UV spectrophotometry at 260 nm. Loading buffer (0.25% bromophenol blue, 40% saccharose) was added to 5 µg of DNA for each treatment, and the samples were analyzed by electrophoresis on a 1% agarose gel (1 h at 80 V/30 mA) with a Tris–Borate–EDTA running buffer (44 mM Tris–HCl, 44 mM boric acid, 50 mM EDTA, pH 8.0).

## 2.6. Quantification of DNA fragmentation

DNA fragmentation was measured according to the method described by Sandau et al. (1997). The cells were incubated in 25 cm<sup>2</sup> flasks and left untouched for 48 h. They were incubated with increasing concentrations of OTA, ranging from 0 to 80 μM, or CTN at concentrations ranging from 0 to 330 μM or OTA ranging from 25 to 80 μM combined with CTN at a fixed concentration (25 μM) for 24 h at 37 °C. The controls were performed with ethanol:water (v:v). After 24 h incubation, the cell layer was rinsed twice with 2 ml of PBS. Cells were then harvested by trypsination. After centrifugation (3000 × g, 20 °C), the cell pellet was resuspended in TTE lysis solution (Triton X-100 0.2%, Tris-HCl 10 mM, EDTA 1 mM,) and shaken vigorously. After centrifugation (5000 × g, 30 min), the pellet was resuspended in 1 ml TTE solution and the supernatant was transferred in new conical tubes. One milliliter of trichloro-acetic acid (TCA 25%) was added to the supernatant and to the resuspended pellet followed by incubation overnight at 4 °C. The tubes were then centrifuged (5000 × g, 10 min), 160 μl of 5% TCA was added to each tube content before the tubes were incubated for 15 min at 90 °C. 320 μl of diphenylamine solution (in 10 ml glacial acetic acid: 150 mg diphenylamine, 150 μl H<sub>2</sub>SO<sub>4</sub> and 50 μl acetaldehyde 16 mg/ml) was added to each tube, followed by incubation overnight at room temperature. The optical density was determined at 600 nm. DNA fragmentation was calculated as follows:

$$\text{Percent (\%)} \text{ of fragmented DNA} = \frac{\text{OD supernatant}}{\text{OD supernatant} + \text{OD pellet}} \times 100$$

## 2.7. In vivo chromosome aberration assay

### 2.7.1. Animal treatments

Animals were randomly divided into four groups:

1. Animals were given (ip) a dose of ethanol/water as negative control group.
2. Animals were given (ip) increasing doses of OTA: 0.6, 1.2, and 2.4 mg/kg bw, corresponding, respectively, to 2, 4, and 8% of the mouse LD50 (LD50 = 31 mg/kg bw) in a single injection and sacrificed 24 h later.
3. Animals were given increasing (ip) doses of CTN: 0.9, 1.8, and 3.6 mg/kg bw, corresponding, respectively, to 2, 4, and 8% of the mouse LD50 (LD50 = 45 mg/kg bw) in a single injection and sacrificed 24 h later.
4. Animals were given (ip) increasing doses of OTA/CTN combined at equimolar doses corresponding, respectively, to 2, 4, and 8% of the mouse (LD50 = 31 mg/kg bw for OTA and 45 mg/kg bw for CTN) in a single injection and sacrificed 24 h later.

### 2.7.2. Bone marrow cells preparation

Bone marrow cells were obtained according to the technique of Yosida and Amano (1965). Briefly, femur and tibia were removed immediately after animal sacrifice and bone marrow was flushed out with KCl solution (0.075 M, 37 °C) using a syringe. The bone marrow cell suspension was incubated for 20 min at 37 °C and centrifuged at 1200 rpm for 10 min. The supernatant was discarded, the pellet was resuspended in 5 ml of a fixative solution (acetic acid/methanol, 1:3, v/v), centrifuged (1200 rpm for 10 min) and the supernatant was discarded again. This step was repeated three times in order to clean the pellet. Finally, the pellet was resuspended in 1 ml of the above fixative solution and used for chromosome preparation.

### 2.7.3. Chromosome preparation

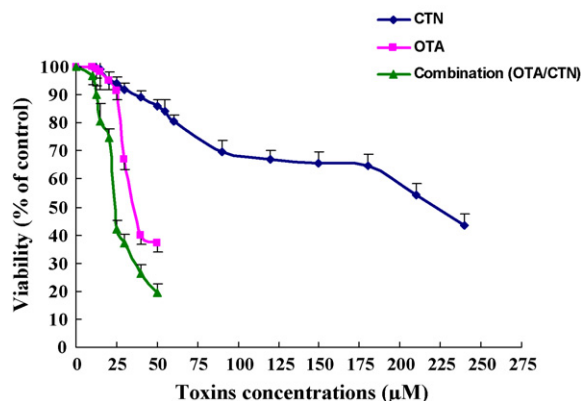
Chromosomes were prepared according to the technique of Evans et al. (1964). The cell suspensions were dropped on glass slides giving smears that were blazed on a flame for 5 s, then air-dried for conservation at room temperature and/or directly stained with Giemsa. Giemsa working solution was freshly prepared (4 ml in 100 ml) in phosphate buffer (0.15 M, pH 7.2). Slides were left for 15 min in this staining solution, then rinsed with water and allowed to dry at room temperature.

### 2.7.4. Slide analysis

The slides were examined under 100× magnification using an optical microscope (Carl Zeiss, Germany). Three hundred well-spread metaphases were analyzed per group for abnormalities. Metaphases with chromosome break, gap, ring and centric fusion (robertsonian translocation) were recorded and expressed as percentage of total metaphases per group.

## 2.8. Statistical analysis

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



**Fig. 2.** Cytotoxic effects of OTA and CTN (individually and combined) on kidney Vero cells after 48 h. Cell viability was determined using MTT assay and expressed as percentages of control, which was exposed to vehicle only. Control value was taken as 100%. Data are expressed as mean values ± standard deviation of independent experiments ( $n = 3$ ).

## 3. Results

### 3.1. Cytotoxicity assay

Cytotoxic effects of OTA and CTN on Vero cells after 48 h incubation as measured by the MTT assay are shown in Fig. 2. 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 has been already significant at low concentrations and the estimated IC50 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 IC50 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 IC50 of the combination was about 24 μM the interaction between the OTA and CTN seems to be of synergistic nature (see Table 1).

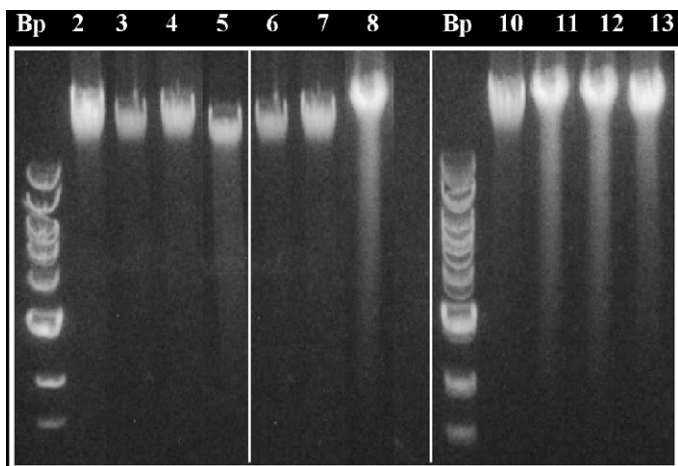
### 3.2. Detection and quantification of DNA fragmentation

Results of DNA fragmentation as evidenced by agarose gel electrophoresis are illustrated in Fig. 3. Our results showed no specific DNA fragments when cells were treated with increasing concentrations of OTA (0.5, 1 and 5 μM). For CTN, concentrations of 1 and 6 μM did not cause any DNA fragmentation, only the concentration of 12.5 μM (the highest tested concentration) induced DNA fragmentation as illustrated by the appearance of DNA ladders. Then, when we combined OTA (0.5 μM) and CTN at increasing concentrations of 0.50, 0.75 and 1.00 μM, a significant DNA fragmentation was observed and DNA fragments, showing varying sizes between 750 and 2000 base pairs, were clearly visible after electrophoresis. It is of note that both OTA and CTN concentrations used for the combination (0.50 μM and 0.50, 0.75 and 1.00 μM, respectively) are

**Table 1**

IC50 values of OTA, CTN and of their combination as determined by MTT assay

	IC50
OTA (μM)	37
CTN (μM)	220
Combination (OTA/CTN) (μM)	24



**Fig. 3.** Agarose gel electrophoresis of DNA extracted from Vero cells incubated with individual toxins, Lane 1: Ladder (Bp), Lane 2: control, Lanes 3–5: OTA (0.5, 1, and 5  $\mu\text{M}$ , respectively), Lanes 6–8: CTN (1, 6 and 12.5  $\mu\text{M}$ , respectively), Lane 9: Ladder (Bp), Lane 10: control, Lanes 11–13: combination of OTA at a fixed concentration (0.5  $\mu\text{M}$ ) and increasing CTN concentrations (0.5, 0.75 and 1  $\mu\text{M}$ , respectively).

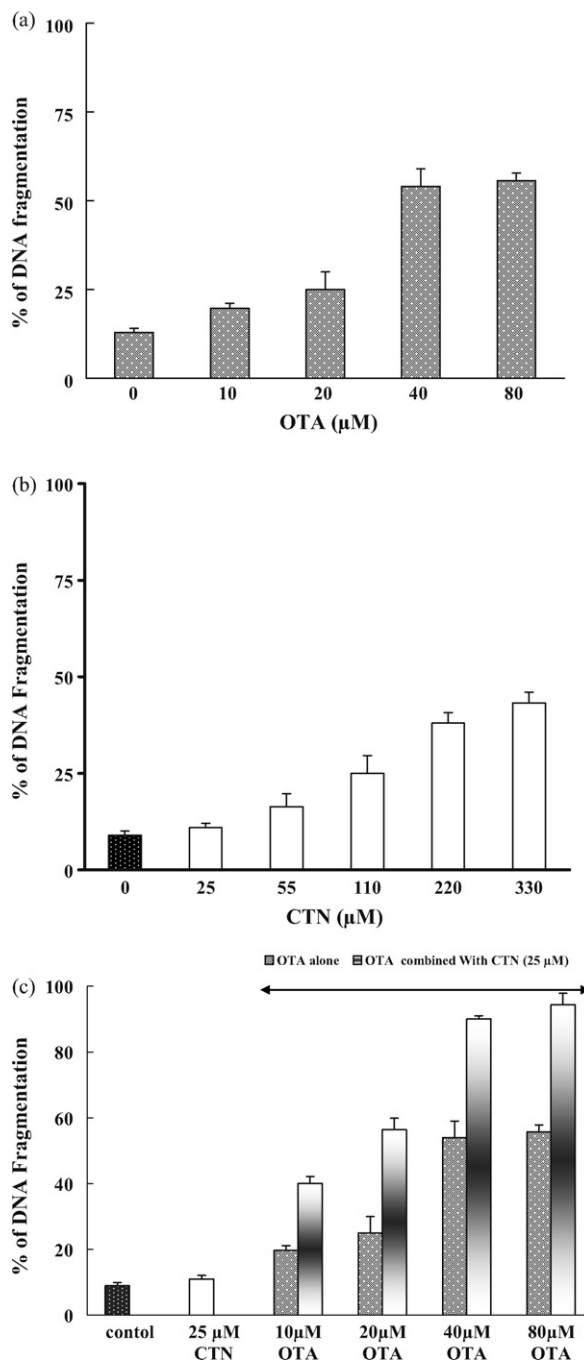
concentrations which did not induce DNA fragmentation when the toxins are taken separately.

To further confirm the effect on DNA damage of OTA and CTN taken separately or combined, we used a quantitative analysis of DNA fragmentation. A significant effect was observed in Vero cells after 24 h incubation in the presence of increasing concentrations of OTA ranging from 0 to 80  $\mu\text{M}$  (Fig. 4a) or CTN at concentrations ranging from 0 to 330  $\mu\text{M}$  (Fig. 4b). Thus, exposure to OTA and CTN separately led to a significant increase of fragmented DNA level ranging from  $19.66 \pm 1.41\%$  to  $55.66 \pm 2.12\%$  for OTA and ranging from  $11 \pm 1.1\%$  to  $43.21 \pm 2.79\%$  for CTN as compared to the control ( $8.95 \pm 1.08\%$ ). Then, to study the effect of the mixture of OTA and CTN, we combined increasing concentrations of OTA at 10, 20, 40 and 80  $\mu\text{M}$  to a unique concentration of CTN (25  $\mu\text{M}$ ). The combination of OTA and CTN significantly increased the percentage of DNA fragmentation up to 40.00, 56.33, 90.00 and 94.33%, respectively. It is of note that the concentration of 25  $\mu\text{M}$  of CTN is a concentration which did not induce any DNA fragmentation when taken alone (Fig. 4c).

Altogether, for both conducted assays of DNA damage (DNA fragmentation using agarose gel electrophoresis or DNA fragmentation quantification), the combination of OTA and CTN significantly increases the DNA damages as compared to the effect of the toxins taken separately. In addition, for both endpoints, we used concentrations of OTA and CTN that when taken alone did not induce any damage. We can conclude that for DNA damage investigations, the combination of OTA and CTN induces synergistic effects.

### 3.3. *In vivo* chromosome aberrations determination

It is of note that only structural aberrations were enumerated in our study, with special emphasis on gaps, rings, breaks and centric fusions. Percentages of total structural chromosome aberrations induced by each of OTA, CTN and their combination are presented in Table 2. Our results (Fig. 5) showed that OTA and CTN induced chromosome aberrations in mice bone marrow cells in a dose dependent manner. For OTA, the percentage of chromosome aberrations increased from  $12.00 \pm 1.90\%$  (at 2% LD50) to  $23.00 \pm 3.06\%$  (at 4% LD50) and to  $40.67 \pm 1.41\%$  (at 8% LD50) as compared to the control ( $8 \pm 1.52\%$ ). Concerning CTN, the percentage of chromosome aberrations also increased from  $12.00 \pm 2.00\%$



**Fig. 4.** Percentage of DNA fragmentation in Vero cells caused by (a) CTN, at concentrations ranging from 0 to 330  $\mu\text{M}$ , (b) by (OTA) at concentrations ranging from 0 to 80  $\mu\text{M}$  and (c) by CTN alone at 25  $\mu\text{M}$  and by combination of CTN at a fixed concentration (25  $\mu\text{M}$ ) with increasing OTA concentrations (0–80  $\mu\text{M}$ ) as determined by the diphenylamine assay.

(at 2% LD50) to  $14.00 \pm 1.50\%$  (at 4% LD50) and  $31.33 \pm 1.41\%$  (at 8% LD50) as compared to the control.

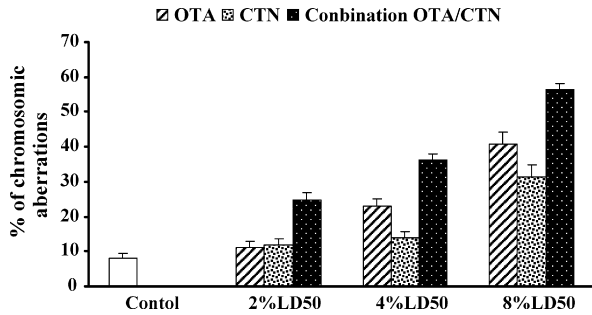
The combination of the two toxins at 2%, 4% and 8% of LD50 (LD50 for the mixture OTA/CTN, determined for an equimolar combination of the two mycotoxins) led to an important increase of chromosome aberrations as compared to each toxin taken alone ( $24.70 \pm 3.50\%$ ,  $36.30 \pm 2.10\%$  and  $56.3 \pm 1.60\%$ , respectively).

To define the type of interaction between OTA and CTN, we compared the percentage of total chromosome aberrations induced by the toxins taken individually to the percentage of aberrations when

**Table 2**

Comparison of the percentage of total chromosome aberrations induced by OTA and CTN taken separately or combined (at 2, 4 and 8% of their respective LD50)

	% of total chromosome aberrations		
	2% LD50	4% LD50	8% LD50
OTA	12 ± 1.9	23 ± 3.06	40.67 ± 1.41
CTN	12 ± 3.21	14 ± 4.28	31.33 ± 5.7
OTA/CTN	24.7 ± 4.16	36.34 ± 5.2	56.33 ± 3

**Fig. 5.** Percentage of chromosomal aberrations *in vivo* caused by OTA alone (2, 4, and 8% of LD50), CTN alone (2, 4, and 8% LD50) and combination of OTA and CTN (2, 4, and 8% LD50).

toxins are combined. The comparison between the effects of OTA and CTN on the induction of chromosome aberrations suggested that their combined effects are clearly more efficient (probably additive or synergistic) than their effects when considered individually. This comparison is summarized in Table 2.

It is of note that among different types of structural chromosome aberrations (Table 3), breaks and rings represent the majority of chromosome abnormalities; for OTA they are about 15.33 and 18%, respectively, about 16 and 7.67%, respectively for CTN and about 23.33 and 18.67%, respectively for the combination.

#### 4. Discussion

It is known for many years that several food items, derived from plants infected by fungi in the field during growing of the plant or during harvest and storage of the food item, can contain concomitantly different mycotoxins. As these combined mycotoxins occur simultaneously, consumption of the food will lead to a combined intake depending on the absorption rates of the different mycotoxins. Therefore, the question is justified whether such a combined intake of mycotoxins would lead to a possible higher risk for adverse health effects than the intake of one of these myco-

toxins alone. However, there is relatively little information on the interaction between concomitantly occurring mycotoxins and the consequence for the toxicity and subsequently its implication for food safety assessment is generally not known.

There are several combinations of mycotoxins that frequently occur in foodstuffs worldwide as established in analytical-chemical monitoring programs. Among the mycotoxins, OTA and CTN are very common and are reported to display several alterations and disorders in renal functions besides their tumorigenesis potency. Considering their nephrotoxic and cancerogenic potential as well as their co-occurrence, the present study was conducted to investigate whether genotoxicity of both molecules would be enhanced by their combination in renal cells as compared to their effects when taken individually.

In a first set of experiments, we have assessed the effect of OTA and CTN, either individually or concomitantly, on the inhibition of cell proliferation using cytotoxicity assay (MTT) in renal cells. Our results clearly showed that CTN alone was found weakly cytotoxic ( $IC_{50} = 220 \mu M$ ) as compared to OTA which has shown a moderate cytotoxicity ( $IC_{50} = 37 \mu M$ ) in *Vero* cells. The combination of the two toxins (OTA and CTN) led to an increased cytotoxicity ( $IC_{50}$  of the combination was of about  $24 \mu M$ ) (Fig. 2). This combination of OTA and CTN seems to be of synergistic nature.

In a second set of experiments, we have evaluated DNA fragmentation in *Vero* cells by gel electrophoresis and by diphenylamine assay. For both assays, we have monitored the effects of OTA and CTN taken individually or combined. DNA fragmentation quantification revealed that both mycotoxins induce DNA fragmentation in a dose-dependent manner (Fig. 4a and b). Furthermore, the combination of both mycotoxins at concentrations which do not exhibit any fragmentation (using agarose gel electrophoresis or diphenylamine assay) enhanced significantly DNA fragmentation level as compared to the effect of mycotoxins taken separately strongly suggesting a synergistic interaction between OTA and CTN (Figs. 3 and 4c).

To further assess OTA, CTN and OTA combined with CTN genotoxicity potential, we monitored the effect of both mycotoxins, either individually or concomitantly, on the induction of chro-

**Table 3**

Percentage of different types of structural chromosome aberrations induced by OTA and CTN taken separately or combined (at 2, 4 and 8% of their respective LD50) compared to total chromosome aberrations (CA)

	Centric fusions	Breaks	Rings	Gaps	% of CA
Control	5.33 ± 0.23	0.34 ± 0.58	2.33 ± 2.15	0 ± 0	8 ± 2.08
% of LD50 of OTA					
2%	3.66 ± 2.52	5 ± 4.58	3.34 ± 2.08	0 ± 0	12 ± 1.9
4%	4 ± 2.89	8.33 ± 10.07	10 ± 1.54	0.66 ± 1.15	23 ± 3.06
8%	6.66 ± 2.90	15.33 ± 6.8	18 ± 2.11	0.67 ± 1.16	40.67 ± 3.07
% of LD50 of CTN					
2%	3 ± 1.42	6 ± 3.2	2.67 ± 2.08	0.33 ± 0.7	12 ± 3.21
4%	3 ± 2.52	7.01 ± 2.1	3.66 ± 7.3	0.33 ± 0.3	14 ± 4.28
8%	7.67 ± 3.6	16 ± 4.58	7.33 ± 8.4	0.33 ± 0.58	31.33 ± 5.7
% of LD50 of OTA/CTN					
2%	7.04 ± 1.53	12.66 ± 9.5	4.33 ± 3.69	0.671 ± 1.15	24.7 ± 4.16
4%	9.33 ± 3.5	18.66 ± 4.6	7.33 ± 5.14	1.02 ± 0.3	36.34 ± 5.2
8%	12.67 ± 1.53	23.33 ± 8.2	18.67 ± 2.5	1.661 ± 0.8	56.33 ± 3

mosome aberrations *in vivo*. Our results clearly showed that the combination of the two toxins at 2% LD50, 4% LD50 and 8% LD50, led to an important increase of chromosome aberrations as compared to each toxin taken alone. For chromosome aberrations assay, the interaction is at list additive or even synergistic. It is acknowledged that an increase in the frequency of chromosomal aberrations in bone marrow cells and consequently in peripheral blood lymphocytes is associated with an increased overall risk of cancer (Hagmar et al., 1994, 1998). In fact, most of the chromosomal aberrations observed in the cells are lethal, but there are many other aberrations that are viable and cause genetic effects, either somatic or inherited (Swierenga et al., 1991). When cells with a small number of chromosome aberrations are allowed to survive, they will divide with possible occurrence of mutations; this will then give rise to tumor formation.

Generally, it is well established that in response to genotoxic stress and DNA damage, the cell may undergo an intricate network of multiple pathways including the arrest of the cell cycle progression and the activation of repair mechanisms. Then (i) in case of efficient DNA repair, cells activate the cell cycle and take up normal cell growth, (ii) if the repair process is overburdened, cells activate the apoptotic cascade leading to cell death otherwise (iii) an error-prone reparation can take place and promote the mutagenic pathway and enhance neoplastic transformation (Zhou and Elledge, 2000; Kastan and Bartek, 2004; Rowinsky, 2005; Siegel, 2006; Ayed-Boussema et al., 2007). As for OTA and CTN, both pathways apoptosis and cancerogenesis have been reported to be induced (Gekle et al., 2000; Pfeiffer, 1998; Yu et al., 2006).

Altogether, our study clearly demonstrates that the combination of OTA and CTN enhances their respective genotoxic potential as assessed by several endpoints including *in vitro* detection and quantification of DNA fragmentation and *in vivo* by monitoring chromosome aberrations. Interestingly, the concentrations chosen for the combinations did not display any effects when taken individually, however, when combined they showed a significant increase in the toxic responses. Our data strongly suggest that OTA and CTN when combined display synergistic DNA damage. It is worthwhile that in our previous investigation (Bouslimi et al., 2008), we have investigated the effect of OTA and CTN, taken individually or combined on the induction of oxidative damage *in vitro* using *Vero* cells. Our results showed that cultured renal cells respond to OTA and CTN exposure (taken separately) by induction of oxidative stress. Meanwhile, when combined, they exert a significant synergistic increase in the induction of MDA level and Hsp 70 expression, which are indicators of oxidative stress.

The synergistic induction of DNA damages and oxidative damages observed with OTA and CTN taken concomitantly could be relevant to explain the molecular basis of the renal diseases and tumorigenesis induced by naturally occurring mycotoxins (Riley, 1990; Bennett and Klich, 2003). This is particularly true, especially that several previous reports have also underlined a synergistic or additive interaction between OTA and CTN in different models and for different investigated endpoints (Bouslimi et al., 2008; Mayura et al., 1984; Siray et al., 1981; Kanisawa, 1984; Berndt and Hayes, 1979; Brown et al., 1986; Vesela et al., 1983).

In conclusion, 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 humans and animals feeding. Only the combination of two toxins is considered herein (OTA and CTN). It may happen that several congeners of these toxins are produced by the same genus *Aspergillus* and *Penicillium* (Dawlatana et al., 2002). However, an understanding of the mode of action in simple *in vitro* systems can provide a rational

basis for predicting interactions between mycotoxins. In this manner, it can also help setting priorities for specially designed studies to establish interactions, preferably those suspected to reveal synergistic actions. In addition, the study of cellular modes of mycotoxin actions can be very useful as a starting point to screen certain combinations for their possible synergistic or additive effects. A higher priority for further special research should then be given to those combinations of mycotoxins. Meanwhile, there is an emergent need to revise the estimated tolerated doses in foodstuffs to provide a solid base for estimating the associated health risk.

### Conflict of interest

None.

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