Low doses of ochratoxin A induce micronucleus formation and delay DNA repair in human lymphocytes.

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Abstract

Contamination of food and feed commodities by fungal mycotoxins has attracted great interest because many of these mycotoxins are responsible for different diseases, including cancer and other chronic illnesses. Ochratoxin A (OTA) is a mycotoxin naturally present in food, and long-term exposure to food contaminated with low levels of OTA has been associated with renal cancer. In the present study, the cytotoxicity, cytostaticity, and genotoxicity of OTA (0.075, 0.15, 1.5, 5.0, and 15 μM) in human lymphocytes were evaluated. The comet assay and the modified comet assay (DNA repair assay), which use N-hydroxyurea (NHU) to detect non-repaired lesions produced by OTA, and the cytokinesis-blocked micronucleus (CBMN) assay were used. Treatments with OTA were not cytotoxic but caused a cytostatic effect in human lymphocytes at 15 μM. OTA (0.075-5

μM) produced a slight increase in the percentage of DNA in the comets and a delay in the DNA repair capacity of the lymphocytes. Micronucleus (MN) induction was observed at OTA concentrations of 1.5 and 5 μM. In conclusion, our results indicate that OTA induces DNA stable damage at low doses that are neither cytotoxic nor cytostatic, and OTA delays DNA repair kinetics. These two findings indicate that OTA affects two pivotal events in the carcinogenesis pathway.

4.1 Introduction

Ochratoxin A (OTA) is a fungal toxin produced by species of the *Aspergillus* and *Penicillium* genera. It was first described by Van der Merwe et al. (1965). Foodstuffs for human and animal consumption are frequently contaminated with OTA worldwide, and the main contaminated foods are cereals, dried fruits, coffee, grapes, wine, and beer. The consumption of OTA-contaminated food is known to have a close relationship with the development of diseases, such as Balkan Endemic Nephropathy (BEN), a chronic tubule interstitial renal disease observed in settlements along the Danube River in the Balkan Peninsula (Stoev, 1998; Vrabcheva et al., 2004).

OTA consists of a dihydroisocoumarin with a para-chlorophthalic group that is linked to an L-β-phenylalanine by an amino bond (Moss, 1996). OTA is mainly nephrotoxic, but other toxic effects have been described, including teratogenicity, neurotoxicity, immunotoxicity, and genotoxicity. The nephrotoxic mechanisms of OTA have been studied *in vitro* and *in vivo*, but the mechanisms through which OTA exerts carcinogenic effects are not entirely understood (IARC, 1993; Benford et al., 2001; Abouzied et al., 2002). Two hypotheses are still discussed when dealing with the carcinogenicity of OTA. The first hypothesis suggests that the carcinogenicity of OTA is linked to its epigenetic nature because OTA produces effects in target cells that either indirectly lead to neoplasic transformation or facilitate the development of neoplasms from cytogenetically transformed cells (O'Brien and Dietrich 2005; Schilter et al., 2005; Turesky, 2005). The second hypothesis suggests that its carcinogenicity is due to genotoxic mechanisms (Manderville, 2005; Mantle et al., 2010a; Pfohl-Leszkowicz and Castegnaro, 2005). In any case, OTA has been classified as a possible group 2B human carcinogen by the International Agency for Research on Cancer (IARC, 1993). It has also been classified in the germ cell mutagen group 3B (MAK Value Documentation, 2006) and recommended to be regulated as a non-threshold carcinogen (Kuiper-Goodman et al., 2010).

Several *in vitro* studies using microbial and mammalian models have suggested that OTA does not act as a direct genotoxic compound. Some studies have reported that OTA shows no mutagenic capacity in the Ames assay, either with or without metabolic activation (Bendele et al., 1985). However, other studies have shown that OTA has mutagenic

capacity using the same Ames assay when OTA metabolites were formed by hepatocytes in culture (Hennig et al., 1991) or were generated by a kidney microsomal fraction (Obrecht-Pflumio et al., 1999).

Additionally, controversial results have been obtained with the Sister Chromatid Exchange (SCE) assay. Although an OTA treatment did not increase the SCE frequency in human peripheral blood lymphocytes (Cooray, 1984; Mosesso et al., 2008) or in the bone marrow of Chinese hamsters (Bendele et al., 1985), (Föllmann et al., 1995; Lioi et al., 2004) and Anninou et al. (2014) detected an increase in the SCE frequency with and without metabolic activation in response to treatment with OTA.

DNA adduct formation has been reported in many *in vitro* and *in vivo* studies and results from the direct covalent binding of OTA to DNA, which is related to the metabolic activation of OTA (El Adlouni et al., 2000; Mantle et al., 2010; Pfohl-Leszkowicz and Castegnaro, 2005; Hadjeba-Medjdoub et al., 2012). However, three different studies failed to identify DNA adducts following OTA exposure (Gautier et al. 2001; Gross-Steinmeyer et al., 2002; Mally et al., 2005).

Chromosomal instability caused by OTA has also been measured using the micronucleus (MN) assay. This assay is a tool to evaluate the clastogenic capacity of compounds that can induce chromosomal aberrations and aneugenicity through mitotic spindle dysfunction (Fenech, 2007). Some MN assays performed with OTA did not show a dose-response relationship. However, in most studies, the concentrations tested were higher than 12 µM (4.8 µg/mL) (Degen et al., 1997; Ehrlich, et al., 2002; Knasmüller et al., 2004; Fuchs et al., 2008).

The aim of this study was to evaluate the genotoxic potential of OTA in human lymphocytes through the comet and repair assays and through the cytokinesis-blocked micronucleus (CBMN) technique. This study also assessed the effects of low concentrations of OTA, which have not been frequently assayed in past research.

4.2 Materials and methods

4.2.1 Chemicals

The following products were obtained from Sigma-Aldrich (Steinheim, Germany): low-melting-point agarose, regular agarose type IIA, cytochalasin-B (Cyt-B) from *Drechslera dematioidea* (ref. C6762), ethylenediamine tetraacetic acid (EDTA) (ref. E9884), fluorescein diacetate (25 mg/mL) (ref. F7378), hydrogen peroxide (H_2O_2) at 30%, mitomycin C (ref. M4287), phytohemagglutinin (PHA) from *Phaseolus vulgaris* (red kidney bean) (ref. L4144), RPMI-1640 medium (ref. R4130), trypan blue solution (ref. T8154) and trypsin solution (1X) (ref. T3924).

Standard OTA (98% purity) was obtained from Chem Service® (ref. O1877) (West Chester, PA, USA), and ethanol 96° was obtained from Jalmek® (ref. A3380) (Nuevo León, México). The L-glutamine solution (100X) (ref. 25030) and 100X nonessential amino acid solution (ref. 11140-050) were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA).

Microscopy Hemacolor (ref. 111661) was obtained from Merck KGaA (Darmstadt, Germany). Ethidium bromide (ref. 161-0433), Tris (ref. 161-0716) and triton X-100 (ref. 1610407) were obtained from BioRad (Hercules, California. USA). Dimethyl sulfoxide (DMSO) for molecular biology (ref. D8418), glacial acetic acid, methanol, sodium hydroxide (NaOH), and sodium chloride (NaCl) were supplied by JT-Baker (Center Valley, PA, USA), and Petri dishes (1.9 cm^2) were obtained from Corning (ref. 3526) (New York, USA).

4.2.2 Cell culture and OTA treatments

Heparinized venous blood samples from healthy non-smoking male donors of 21-23 years of age were used for the experiments.

For the comet assay, a volume of 20 µL of whole blood in 1 mL of RPMI-1640 medium (without supplement) was treated with OTA for 3 h at 37 °C.

For the CBMN assay, a volume of 0.5 mL of whole blood was cultured in 7 mL of RPMI-1640 medium supplemented with 1% L-glutamine and non-essential amino acids (100x). The lymphocytes were stimulated with PHA (7.1 µg/mL) and cultured for 72 h at 37 °C. Cytochalasin B (6 µg/ml) was added in the last 24 h of culture to accumulate cells that had divided only once. OTA was added 24 h after the beginning of the PHA stimulation, and the OTA incubation lasted for the remaining 48 h of the assay. The OTA doses assayed were 0.075, 0.15, 1.5, 5.0 and 15 µM (ethanol: DMSO, 3:2) for both techniques. The percentage of solvent was less than 0.05% of the final volume.

4.2.3 Cell viability tests

4.2.3.1 Double staining with fluorescein diacetate and ethidium bromide

The cytotoxicity was evaluated using the fluorescent dyes fluorescein diacetate (FDA) and ethidium bromide (EtBr). The OTA-treated cells were resuspended, and 100-µL aliquots of each treatment group were transferred to new tubes and centrifuged at 3000 rpm for 2 min. The supernatant was removed, and the cell pellet was maintained on ice until use. For the cell viability analysis, a fresh staining solution was prepared with 30 µL of FDA in acetone (5 mg/mL), 200 µL of EtBr in phosphate buffer saline (200 µg/mL), and 4.8 mL of PBS. The cell pellet was resuspended in 20 µL of the FDA/EtBr solution, placed on a slide, and covered with a coverslip. Cell counting was performed with an Axio Scope A1 fluorescence microscope (Carl Zeiss, Gottingen, Germany) using the 20X objective. The living cells could be visualized in green, whereas the dead cells could be visualized in red; a total of 200 cells were counted for each treatment.

4.2.4 Comet assay

The alkaline comet assay was performed as described by Tice et al. (2000). After OTA treatment, button cells were mixed with 150 µL of low-melting-point agarose (0.5%) at 37 °C, placed on slides that had been precoated with a layer of regular agarose (0.5%) and allowed to polymerize at 4 °C. Another layer of low-melting-point agarose was added and allowed to solidify. The slides were placed for 24 h in Coplin jars with 50 mL of lysis solution (2.5 M NaCl, 100 mM EDTA-Na_2 and 10 mM Tris, pH>10) at 4 °C, 5 mL of DMSO and 0.5 mL of Triton X-100. Prior to electrophoresis, the slides were incubated for 20 min

in an alkaline buffer (10 M NaOH, 200 mM EDTA-Na_2, pH>13), which is capable of detecting DNA damage, including single-strand breaks (SSB), alkali-labile sites (ALS), and DNA-DNA/DNA-protein cross-linking. After alkali unwinding, the slides were run by electrophoresis at 25 V and 300 mA for 20 min, neutralized, and fixed. The slides were stained with EtBr for analysis and prepared in duplicate per treatment. Slides were randomized and coded to blind the scorer. A total of 100 individual cells were screened per treatment (50 cells from each slide). The scoring was performed in a fluorescence microscope Carl Zeiss Axio Scope A1 with 20X objective. The percentage of DNA was determined using the *Image Comet software version 2.2.*

The results are presented as the means from three independent experiments. To determine the kinetics of DNA repair in the lymphocytes exposed to OTA, a modified comet assay was conducted. Four Eppendorf tubes (T0 (DNA damage produced by a second known exposition); T30 and T60 (two repair times in minutes) and TNHU (10 µL of N-hydroxyurea was used as positive control for DNA repair) were used for each of the following conditions: whole blood lymphocytes treated with OTA (1.5 and 5 µM), a positive control (10 µL, 30% H_2O_2) and a negative control (ethanol:DMSO <0.05%). All of the tubes were incubated for 3 h at 37 °C. After incubation, the samples were treated with 10 µL of H_2O_2 (30%) for 10 min at room temperature, centrifuged, washed with 1 mL of culture medium and centrifuged again. Slides were prepared for the T0 sample as described above. The T30 and T60 samples were incubated for their respective repair times, and the tubes were then centrifuged prior to slide preparation. TNHU was also incubated for 60 min.

4.2.5 Cytokinesis-block micronucleus assay (CBMN)

4.2.5.1 Nuclear index (NI)

To evaluate the effect of OTA on the mitogenic response of lymphocytes, the NI was evaluated according to a method described by Eastmond and Tucker (1989). The viable cells (n=200) were scored to determine the frequency of cells with one, two, three, or four nuclei, and the NI was calculated according to NI = (M1+2(M2)+3(M3)+4(M4))/N, where M1 to M4 represent the number of viable cells with one to four or more nuclei and N represents the total number of viable cells scored.

4.2.5.2 CBMN technique

The evaluation of MN was performed using the CBMN technique with criteria established through the work conducted by Fenech *et al.* (2003). Mitomycin C was used as a positive control for MN induction. The slides were codified, and the examination of the slides was performed blindly. One thousand binucleated cells per slide were evaluated, and the resulting MN frequency was expressed as the number of MN in 1000 binucleated cells.

The mitogenic response of lymphocytes and the frequency of MN were evaluated using a light microscope (Carl Zeiss Axiostar Plus, Gottingen, Germany) at 1000X magnification.

4.2.6 Statistical analysis

Statistical analysis were conducted using the GraphPad Prism 5.01 and Stata 8.0 programs (Stata statistical software, Stata Corporation, College Station, TX, USA). The effects of OTA on cell viability, percentage of binucleated cells and NI were analysed using the analysis of variance (ANOVA) and the Bonferroni post-test. The results of the MN and comet assays were analysed with Mann Whitney and Dunn's tests. P values of $p<0.05$ were considered to be statistically significant.

4.3 Results

4.3.1 Cytotoxicity and cytostaticity

At the doses assayed (0.075-15 µM OTA), the viability of the lymphocytes was approximately 80% or slightly higher, and no significant differences were noted between the treated and the solvent control cells. In addition, OTA did not statistically decrease NI or the percentage of binucleated cells at concentrations lower or equal to 5 µM compared with the solvent control. However, a cytostatic effect was observed at a concentration of 15 µM (Table 1).

Table 1. Citotoxicity and citostaticity in human lymphocytes treated with ochratoxin A

	Treatment	Viability (%)	P-value	Nuclear index	P- value	Binucleated cells (%)	P-value
OTA (μM)	0.075	88.3 ± 9.3	0.647	1.9 ± 0.2	0.89	55.2 ± 11.8	0.92
	0.15	89.0 ± 9.1	0.525	1.8 ± 0.2	0.85	50.7 ± 19.1	0.53
	1.5	89.0 ± 6.8	0.419	1.8 ± 0.1	0.47	54.1 ± 13.2	0.80
	5	90.6 ± 9.3	0.290	1.7 ± 0.1	0.10	54.7 ± 7.5	0.85
	15	88.0 ± 7.6	0.645	ND		ND	
Mitomycin C (1 μM)	(+)	90.0 ± 4.7	0.016	1.4 ± 0.24	<0.0001	15.8 ± 10.4	<0.0001
Control	(-)	87.3 ± 6.8	0.831	1.8 ± 0.1	0.56	60.3 ± 20.3	0.21
Solvent (DMSO %)	0.05	86.6 ± 4.8		1.9 ± 0.2		61.1 ± 10.2	

Data represent the means ± SD of three independent experiments run in triplicate. P-values are significantly different than the dissolvent control ($p<0.05$). ND: No data, the amount of cells was not sufficient to complete one thousand binucleated cells per slide.

4.3.2 Comet Assay

The results obtained from the comet assay showed that although no differences were found in the tail length in the concentration range assayed (data not shown), a slight increase was observed in the percentage of DNA at the 0.075, 1.5 and 5 µM treatments (Fig. 1) without a dose-response pattern. In addition, according to the repair assay results, although the lymphocytes repaired their DNA within 30 min following oxidative treatment (H_2O_2), co-exposure to H_2O_2 and OTA delayed their repair capacity for a period of 60 min, until then, the DNA percentage was similar to control ($p>0.05$), suggesting that OTA can alter the capacity of lymphocytes to repair their DNA (Fig. 2).

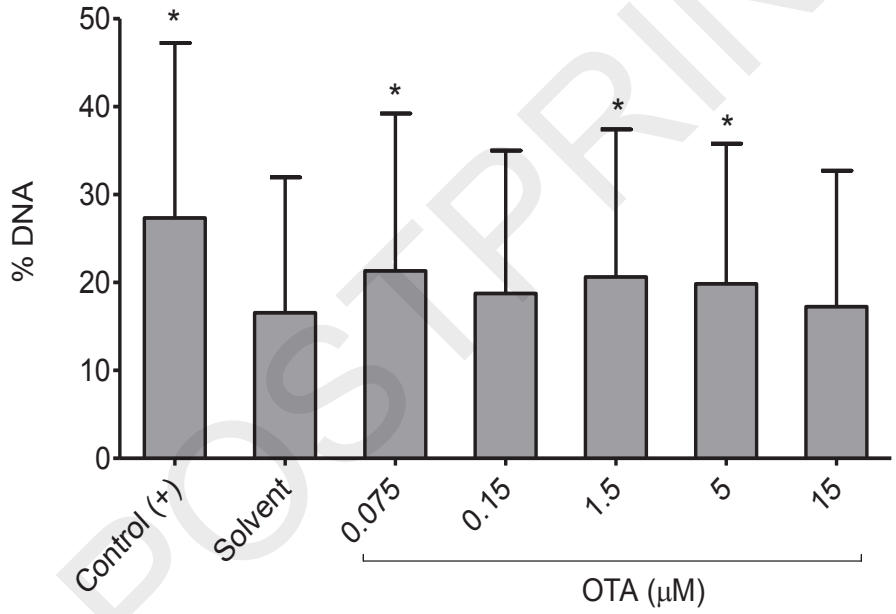

Figure 1. DNA damage (measured as DNA percentage, %DNA) in human lymphocytes after 3 h exposure to OTA. Positive control: 10 µL H_2O_2 (30%), solvent control: 0.05% ethanol:DMSO (3:2). Each value represents the mean ± SD of three independent experiments run in duplicate. *Significantly different than the solvent control ($p<0.05$).

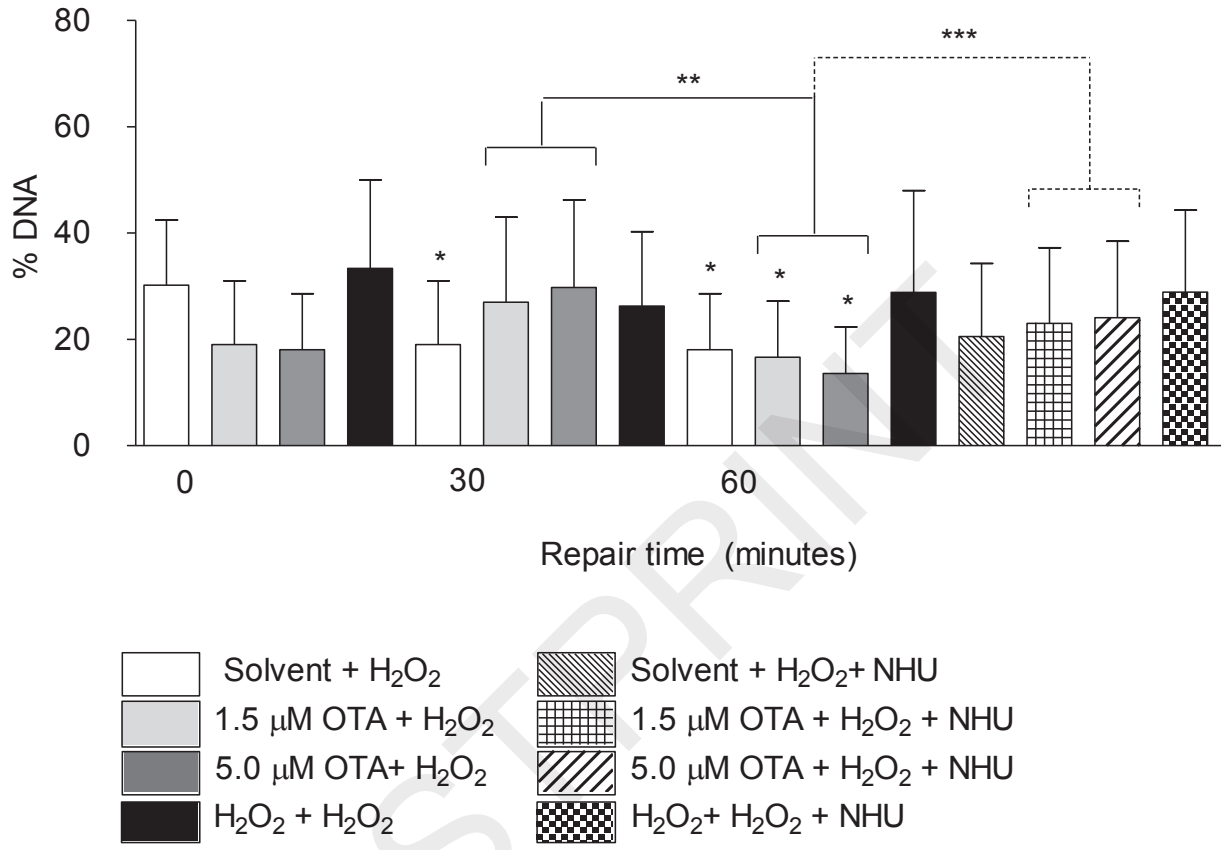

Figure 2. Kinetics of DNA repair in human lymphocytes treated with OTA (1.5 and 5 μM) measured as DNA percentage at 0, 30, and 60 min. The response of lymphocytes to 10 mM NHU (N-hydroxyurea) was determined at 60 min. Each value represents the mean ± SD of three independent experiments run in duplicate. *Significantly different from the solvent control at time 0, and ** Repair effect at time 60 (min) is significantly different from the OTA + H_2O_2 treatment at time 30 (min) ($p<0.05$). ***OTA treatments with NHU are different from the OTA + H_2O_2 treatments at time 60 (min) without NHU ($p<0.05$).

4.3.3 CBMN assay

Fig. 3 shows the formation of MN in binucleated human lymphocytes after OTA exposure. OTA induced a slight but significant increase in the MN frequency in cells treated with OTA concentrations of 1.5 µM and 5 µM compared with the solvent control. MN induction was not scored accurately in the cells treated with OTA at a concentration of 15 µM due to the cytostatic effect observed.

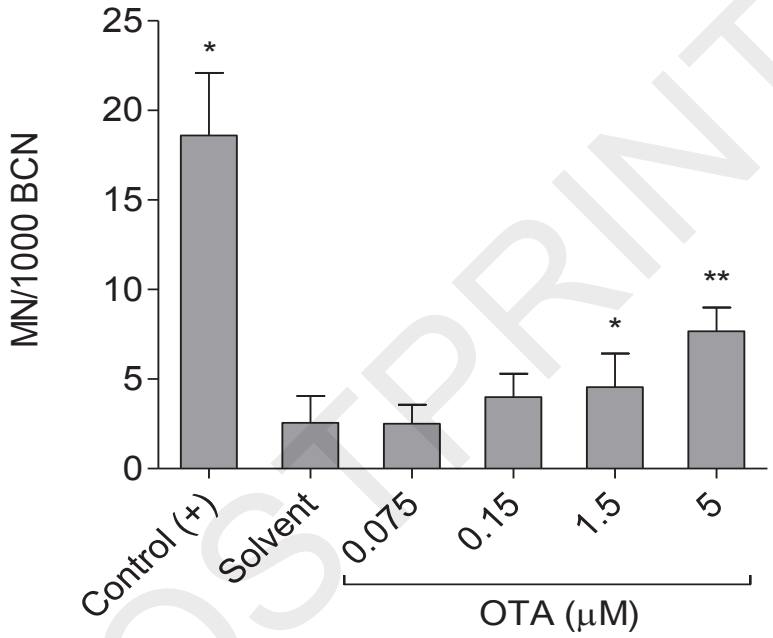

Figure 3. Effect of OTA on the formation of MN in binucleate human lymphocyte cells. Values are means ± SD of three independent experiments run in triplicate (1000 binucleated cells were analysed per treatment). Positive control: 1 µM mitomycin C; solvent control: 0.05% ethanol:DMSO (3:2). *Significantly different than the solvent control ($p<0.05$). **Significantly different than the 1.5 µM treatment ($p<0.05$).

4.4 Discussion

Several studies have shown that the levels of OTA found in food are often in the range of µg/kg (ppb). In foods intended for direct human consumption, such as grape juice, wine, or beer, the mean contamination levels in the EU were 0.56 µg/kg, 0.36 µg/kg and 0.03 µg/kg, respectively (Jørgensen, 2005), making these items important sources of OTA in the diet. OTA is associated with chronic toxicity following long periods of exposure to relatively low levels of this mycotoxin, and therefore toxicity tests must necessarily be carried out at low OTA levels. For this reason, lower levels of OTA than those commonly referenced in the literature were included in this study.

OTA has been reported as either a carcinogen or non-carcinogen compound (IARC, 1993; JECFA, 2006; 2010), and diverse tests and cell types have been used to investigate its genotoxic potential. Among them, the CBMN assay is a well-known comprehensive system that provides data from the DNA damage measures of cytostaticity and cytotoxicity (Fenech, 2007). In contrast, the comet assay measures DNA damage by assessing strand breaks in single cells. In this study, we performed the alkali version to detect single- and double-strand breaks and the comet assay to assess DNA repair. These studies provide data on the mechanism of damage and on the capacity of cells to block the base excision repair system, which can allow the accumulation of breaks for longer periods of time.

The results of the cytotoxicity assay performed in this study are consistent with other reports, in which the viability was above 70-80% when the OTA concentration was lower than 20 µM (Golli-Bennour et al., 2010; Ali et al., 2011), indicating that the viability values were higher than the recommended level (cytotoxic limit) for performing the *in vitro* CBMN assay (OECD, 2010) and for the *in vitro* comet assay (Tice et al., 2000). Regarding the cytostatic effect of OTA, our data showed that this mycotoxin, at a concentration of 15 µM, decreased the NI in human lymphocytes. Dönmez-Altuntaş et al. (2003) reported that OTA did not decrease the percentage of binucleated cells at concentrations ranging from 100 pM to 10 µM; however, the authors also reported the opposite results at higher concentrations (25 µM), and these latter results were consistent with our findings.

In our study, the OTA treatments did not appear to increase the tail length (data not shown) but did increase the percentage of DNA at concentrations of 0.075, 1.5 and 5 μM, although not in a dose-dependent manner. In addition, when an oxidative stress (co-incubation with H_2O_2) was applied in conjunction with the OTA treatments to evaluate the repair capacity of the lymphocytes, the DNA repair of the lymphocytes was found to be delayed to a time point of 60 min. Our results thus agree with those previously reported by Lebrun and Föllmann (2002), who found that OTA induced single-strand breaks in Madin-Darby canine kidney (MDCK) cells at high concentrations (up to 100 μM) in a dose-dependent manner. The authors also observed an increase in the genotoxic effect of OTA when an external metabolizing enzyme system (S9-mix from rat liver) was added, although the damage was completely repaired within 2 h. Additionally, in two studies performed by Ali et al. (2011; 2014), a non-significant increase in the DNA damage (% tail intensity, 4 h of treatment, without S9 treatment) was found with 5 μM OTA in three different cell lines (TK6, CHO and L5178Y tK+/-). However, L5178Y tK+/- cells were more sensitive than the other two cell lines at 10 μM OTA. A dose-response pattern was observed in the range of 20 to 50 μM OTA in the TK6 and CHO cells and in the range of 10 to 100 μM OTA in the L5178Y tK+/- cells.

Russo et al. (2005) found that human fibroblasts treated with OTA for 72 h (6-50 μM) exhibit an exposure time-dependent increase in the percentage of damaged DNA. This study also suggested the involvement of oxidative stress (due to an increase in ROS) in the OTA genotoxicity, which is also consistent with the work of Schilter et al. (2005) and Zheng et al. (2013). This finding is further supported by a study conducted by Kamp et al. (2005) in the V79 and CV-1 cell lines and in primary rat kidney cells, which revealed a slight increase in the basic DNA damage (percent of DNA) without treatment with DNA repair enzymes (formamidopyrimidine-DNA glycolyase and endonuclease III) in both cell lines. However, this study was performed using only a short exposure time (1 h) and a high OTA concentration (≥500 μM).

Based on their work with human leukocytes, Klarić et al. (2010) reported that OTA does not cause genotoxicity at concentrations of 1 and 5 μM (1 h of exposure), as measured by the tail length extension. However, the tail intensity and tail moment parameters were significantly higher, which is consistent with our results.

Although the mechanism that leads to OTA genotoxicity, as measured through the comet assay, is not fully understood, evidence from *in vitro* (with different cell types) and *in vivo* studies suggests the role of oxidative stress (Lebrun and Föllmann, 2002; Arbillaga et al., 2007; Hadjeba-Medjdoub et al., 2012; Aydin et al., 2013; Hibi et al., 2013a; Hibi et al., 2013b; Ali et al., 2014; Yang et al., 2014). It has also been reported that OTA biotransformation produces metabolites that are more genotoxic than the original toxin. However, it is important to note that this type of DNA damage can be effectively repaired (Lebrun and Föllmann, 2002; Simarro et al., 2006).

The results obtained in the present study show that OTA produces a slight but significant MN induction at concentrations as low as 1.5 μM (1.8 times more MN than in the solvent control) and 5 μM (3 times more MN than in the solvent control). Similar results were observed in seminal vesicle cell cultures treated with 12 μM OTA for 6 h (Degen et al., 1997).

Several studies have reported that the OTA-based MN induction is dependent on the concentration of OTA at doses higher than 5 μM (Degen et al., 1997; Dopp et al., 1999; Knasmüller et al., 2004; Fuchs et al., 2008; Klarić et al., 2008). In contrast, studies conducted at low OTA concentrations have reported MN induction but have not shown a dose-response pattern (Ehrlich et al., 2002; Dönmez-Altuntaş et al., 2003; Ali et al., 2011). Unlike the studies previously mentioned, our results with the CBMN assay showed that OTA is genotoxic at concentrations as low as 1.5 μM. The mechanism underlying OTA-induced genotoxicity has been the subject of controversy. Some studies have suggested that genotoxicity results from the direct covalent binding of OTA to DNA (El Adlouni et al., 2000; Pfohl-Leszkowicz and Mandeville, 2012). In contrast, Dopp et al. (1999) determined that 3 h of exposure to 10 μM OTA causes an induction of MN production in Syrian hamster embryo (SHE) fibroblasts. Ehrlich et al. (2002) reported that only 1 h of OTA treatment (61.9 μM) is sufficient to increase the MN frequency in HepG2 cells, with an effect that was approximately 30% lower than when the cells were exposed for 24 h at the same concentration.

In the case of human lymphocytes, Mosesso et al. (2008) reported that OTA did not cause an increase in the MN frequencies at concentrations ranging from 5.3 to 53.2 μM in both

the absence and presence of S9 metabolism. However, it is important to consider the high cytotoxicity (32-67%) and the low values of the cytokinesis block proliferation index (CBPI) (1.17-1.08) reported in this study. Additionally, Dönmez-Altuntaş et al. (2003) treated a lymphocyte culture (48 h) with OTA at concentrations of 100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM and 25 µM. At the highest concentration, the authors reported that OTA induced MN but also led to a clear decrease in the percentage of binucleated cells observed.

The available evidence suggests that OTA increases MN production by inducing clastogenic events (60-70%) more commonly than aneugenic events due to chromosomal breakage (Degen et al., 1997; Dopp et al., 1999; Knasmüller et al., 2004). To this end, Dopp et al. (1999) reported that OTA is an effective inducer of DNA damage and serves to disrupt intracellular calcium homeostasis and actin stress fibres. The study concluded that OTA has a predominantly clastogenic mode of action.

In contrast, the mechanisms through which OTA can cause a clastogenic effect in both comet and MN assays have not been well established. Some studies have reported that OTA can induce cell cycle arrest in the G1 phase by down-regulating the expression of CDK4 and the cyclin D1 protein and by limiting apoptosis in human peripheral blood mononuclear cells (hPBMC) *in vitro* (Liu et al., 2012). Kuroda et al. (2014) demonstrated that OTA also produces an increase in γ-H2AX expression and was capable of inducing double-strand breaks (DSBs) in DNA in a rat model. OTA also increases the mRNA and protein expression levels of homologous recombination (HR) repair-related genes (Rad51, Rad18 and Brip1) and genes involved in G2/M arrest (Chek1 and Wee1) and the S/G2 phase (Ccna2 and Cdk1) in a dose-dependent manner.

It is important to note that inconsistencies in the OTA genotoxicity results could be due to differences in the concentrations of OTA, the characteristics of the particular cell lines, the exposure time, the specific genotoxicity endpoint analysed, and the criteria used to assess a valid study and/or a positive response (Ali et al., 2011). Furthermore, it is important to remember that OTA is known for its rather high affinity to serum proteins. It is therefore possible that the serum content of the cell cultures under different culture conditions could affect the bioavailability and toxicity of OTA in *in vitro* systems (Degen et al., 1997).

Human lymphocytes are frequently exposed to low amounts of OTA under *in vivo* conditions. The amount of OTA in the plasma/serum of healthy volunteers from 23 European countries and several additional countries from around the world ranged from 0.15 to 9.15 ng/mL, with a mean of 0.45 ng/mL (74% of positive samples) (Coronel et al., 2010). Although these levels appear low, OTA has been associated with the induction of adverse effects in humans, especially in some specific cell types, such as urothelial cells. However, questions regarding the OTA genotoxicity at low concentrations remain unsolved (JECFA, 2006; 2010).

In conclusion, our results indicate that OTA induces DNA stable damage at low doses, which are neither cytotoxic nor cytostatic, and delays the DNA repair kinetics, thus inducing pivotal events in the carcinogenesis pathway of xenobiotics.

Conflict of interest

The authors declare that they have no conflicts of interest.

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