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Oxidative DNA damage induced by Ochratoxin A in the HK-2 human kidney cell line: evidence of the relationship with cytotoxicity

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Ochratoxin A (OTA) is a mycotoxin produced by species of the genera *Aspergillus* and *Penicillium*. The kidneys are the target organ of this mycotoxin and it is considered a potent renal carcinogen in male rats. The mechanisms of its genotoxicity and carcinogenicity have been studied thoroughly, but controversial results have been published. The aim of this study was to evaluate the ability of OTA to produce single-strand DNA breaks and oxidative DNA damage in the human renal proximal tubular epithelial cell line (HK-2), due to the fact that there is no study on human kidney cells as the toxic target. In addition, we attempted to determine if biotransformation processes mediate OTA genotoxicity. Therefore, single-cell gel electrophoresis assay (comet assay) was performed after 3h- and 6h-treatments using different OTA concentrations, both cytotoxic and non-cytotoxic, in order to be able to distinguish a genotoxic effect of the mycotoxin from an indirect effect derived from its general cellular toxicity. No effect was shown where no cytotoxicity was found, both in the presence and in the absence of metabolic activation (10% rat liver S9-mix). However, oxidative DNA damage was shown at cytotoxic concentrations when formamidopyrimidine DNA glycosylase (FPG) and endonucleaseIII (EndoIII) were introduced in the assay with or without metabolic activation. Furthermore, at these concentrations, an elevation of reactive oxygen species was measured and pre-incubation with *N*-acetyl-L-cysteine was able to produce a slight protective effect on OTA-induced oxidative DNA damage as well as cytotoxicity. These data suggest that OTA is not acting as a direct genotoxic carcinogen and that oxidative stress is implicated in the genotoxicity and cytotoxicity observed in these human renal cells.

Introduction

Ochratoxin A (OTA, Figure 1) is a mycotoxin produced by several fungi of *Aspergillus* and *Penicilium* species, often found in cereals and agricultural products. The target organ of OTA is the kidney. In humans, it has been related to the Balkan endemic nephropathy (1), although this hypothesis has not been completely demonstrated. In farm animals, chronic exposure to OTA produces a nephropathy, which has been well described (2). Rodent studies have demonstrated that OTA is one of the most potent renal carcinogens in rats and mice (3–5). Sufficient experimental evidence for carcinogenicity in animal studies has led to the classification of OTA as a possible human carcinogen by the International Agency for Research on Cancer (6,7). Nevertheless, with regard to the mechanism of toxicity, the data from the literature are not conclusive.

The genotoxic and mutagenic activity of OTA has been assessed in a variety of standard tests, in order to evidence direct DNA damage that could be the origin of the mechanism involved in chemical carcinogenesis, but the results that have been published are controversial. OTA was originally regarded as a non-mutagenic compound because most bacterial assays gave negative results (8–12). Positive results using *Salmonella typhimurium* Ames strains have only been reported when the test substance was OTA-exposed hepatocyte culture medium (13) or in the presence of kidney microsomal fractions, instead of hepatic, as the metabolic activation system (14). These results suggested an important role on the part of the metabolism in the genotoxicity of OTA and/or a selective toxicity in target kidney cells. In assays that detect unscheduled DNA synthesis due to repair processes, contradictory results have also been found. Bendele *et al.* (10) did not detect DNA repair synthesis induction in primary rat hepatocyte cultures; Mori *et al.* (15), found a weak DNA repair synthesis induction in mice and rat hepatocytes; more pronounced effect was found in porcine urinary bladder epithelial cells by Dörrenhaus and Föllmann, (16). These results pointed to a selective toxic effect of OTA in target cells.

On the other hand, using the ^{32}P-postlabelling method for the detection of DNA adducts, several studies have reported the generation of DNA adducts in a time and dose dependent manner in several mouse tissues after *in vivo* exposure to OTA (17–19); and also in Vero cells from monkey kidney (20). Furthermore, adduct formation was correlated with tumour formation (4). These results contrast with others, where no evidence was found of reactivity between ^{3}H-OTA or its metabolites and the DNA of rat or human hepatocytes (21) or in rat target and non-target tissues (22,23). These later results indicate that covalent binding of OTA or some of its metabolites to DNA is not produced, and therefore, would exclude the possibility of a genotoxic mechanism in the carcinogenicity of OTA. The ^{32}P-postlabelling method cannot distinguish between adducts that contain the OTA itself or metabolites and those caused by the products of oxidative stress and cytotoxicity. These data suggest that the ^{32}P-postlabelled lesions are not due to binding of OTA or its metabolites, but rather, they occur through products derived from OTA-mediated toxicity. Furthermore, DNA adducts could not be corroborated using HPLC-MS or LC-MS, and these authors concluded that the biotransformation of OTA occurs at very low rates and it does not produce reactive intermediates that are capable of binding to DNA (22,24). Nevertheless, new data suggest the idea that

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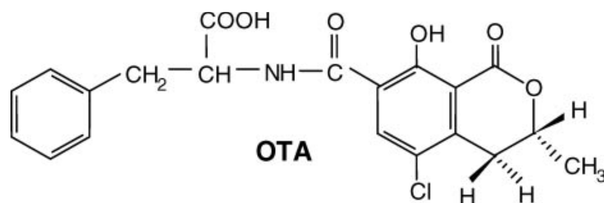

Fig. 1. Chemical structure of Ochratoxin A.

the phenoxyl radical of OTA can have a role in DNA adduction *in vivo* (25).

In the different assays that measure DNA fragmentation and chromosome aberrations, OTA has always given positive results. It has been shown to cause single-strand DNA breaks in rat and mice kidney and liver (23,26,27), and also in target and non-target cell lines of different species (28–31). Many studies have been performed with the comet assay and a few have evaluated oxidative DNA damage (23,30). It has also been found that OTA induces micronuclei in cell lines of different origins, Hep G2, SHE (Syrian Hamster embryo) and OSV (ovine seminal vesicle) (28,32,33). Nevertheless, to our knowledge there is no study on human kidney cells derived from proximal tubular cells, which are the toxic target.

For this reason, in this work, the ability of OTA to cause DNA strand breaks and oxidative DNA damage was assessed using the alkaline comet assay in the human renal proximal tubular epithelial cell line (HK-2). The proximal tubular origin of this cell line was confirmed by immunocytochemistry, and the cytocrome P450 activities CYP1A1, CYP1A2 and CYP2C9 were measured by luminescence. The study was designed following the recommendations made by the expert panel regarding the development of guidelines for the use of the comet assay (34). Cells were treated with OTA concentrations during 3 or 6 h, under the presence or absence of an external metabolic activation (10% rat liver S9-mix). After exposure, cell viability and DNA damage were evaluated in parallel. Apart from detecting DNA strand breaks at the level of single cells, oxidative DNA damage was also evaluated by using different digestion enzymes: endonucleaseIII (EndoIII) for the detection of oxidized pyrimidines, and formamidopyrimidine DNA glycosylase (FPG) for the detection of the principal purine oxidation product 8-oxoguanine as well as other altered purines (35). In addition, in order to evaluate the mechanisms underlying cytotoxicity and DNA damage produced by OTA, the protective capability of *N*-acetyl-L-cysteine (NAC), a well-established antioxidant and intracellular reactive oxygen species (ROS) scavenger, was investigated.

Materials and methods

Chemicals

Ochratoxin A (OTA) and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich (Steinheim, Germany). OTA was dissolved in 0.1 M $HNaCO_3$ (pH 7.4), aliquoted and maintained at −20°C until use; NAC was diluted in ethanol/H_2O (1:1) just before its addition.

All organic solvents were obtained from Sigma-Aldrich in the highest purity available.

Cell culture

HK-2 cells (CRL-2190), which are human renal proximal tubular epithelial cells immortalized by transduction with human papilloma virus 16 *E6*/*E7* genes (36), were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Prat de Llobregat, Barcelona, Spain)/Nutrient Mixture F-12 (HAM) (F-12, Gibco) (1:1) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% antibiotic (10 000 U/ml penicillin and 10 000 μg/ml streptomycin, Gibco) and L-glutamine (Gibco). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2, adding fresh growth medium every 2 days until confluence.

Immunocytochemistry

The proximal origin of the HK-2 cells was confirmed by staining with an antibody against a specific proximal tubular antigen, *N*-cadherin (A-CAM). *N*-cadherin is a member of a family of Ca^{2+}-dependent intracellular adhesion molecules that is specific for proximal tubular cells, but not for other kidney cell types, such as distal tubular cells, collecting duct cells and glomerulum-derived cells (37).

HK-2 cells were cultured on microscope slides (Menzel-Glaser, Braunschweig, Germany) until confluence, fixed with formol during 15 min at room temperature, and stained for *N*-cadherin. The following antibody was used for the immunocytochemical analysis: anti-*N*-cadherin (3B9) (class, IgG1 mouse monoclonal) (Zymed Laboratories, San Francisco, CA, USA), diluted 1/200. The EnVision™ signal enhancement system was performed as the indirect method for the immunocytochemical technique. Paraffin-embedded rat kidney sections were also included as positive control. As negative control, kidney sections and cells were processed in an identical way with omission of the primary antibody.

Characterization of cytochrome P450 activity

Cytochrome P450 (CYP450) activity was measured by a luminescent method using a kit, P450-Glo™ assay (Promega, Madison, WI, USA). The assay is performed by incubating the samples (source of CYP450 isoenzymes) and a luminogenic cytochrome P450 substrate. The substrates are converted to luciferin by CYP450, which in turn reacts with luciferasa to produce a luminescent signal, which was measured by Luminoskan Ascent (Thermo-Labsystem). The amount of light produced is directly proportional to the activity of the CYP450. Three different isoforms were assessed: CYP1A1 (Ref. V8751/2), CYP1A2 (Ref. 8771/2) and CYP2C9 (Ref. 8791/2). Determinations were performed in HK-2 cells and in rat liver and kidney S9-mix, obtaining luminescence arbitrary units/μg protein. Mean values ± standard deviations were obtained in three independent experiments.

Protein concentrations were measured by the method of Lowry *et al*. (38).

Cytotoxicity assay

The determination of cell viability was measured by assessing the reduction of MTT to formazan by the mitochondrial enzyme, succinate dehydrogenase, as described by Mosmann (39). Cells were seeded in 96-well plates at 4.5×10^3 cells/well, and maintained at 37°C in 5% CO_2 for 48 h, until confluence. OTA was then added to serum-free medium at 50, 100, 200, 400 and 800 μM, in order to avoid serum binding of OTA during the treatment period; the treatments were carried out in the presence and in the absence of an external metabolizing enzyme system, 10% liver S9-mix obtained from male rats pretreated with Aroclor 1254. A negative control was included in which cells were incubated only with the solvent (0.1 M $HNaCO_3$, pH 7.4). After 3 and 6 h treatments, cells were washed with phosphate-buffered saline (PBS), and 25 μl MTT (5 mg/ml; Sigma-Aldrich) in PBS was added to 225 μl of fresh medium to each micro well. After an incubation of 3 h at 37°C, the supernatant was removed, the insoluble formazan crystals were dissolved in 100 μl of DMSO and the absorbance was determined at 540 nm using a spectrophotometer reader (Titertek Multiskan® MCC/340 MK II). The results were expressed as the percentage of viability (%) with respect to the control (solvent treated cells), according to the following formula: [(absorbance treated cells − absorbance blank)/(absorbance control cells − absorbance blank)] × 100.

Comet assay

Cells were seeded in 24-well plates at 3×10^4 cells/well and maintained for 48 h until treatment with 50, 100, 200, 400 and 600 μM OTA in serum-free medium. The treatments were performed again during 3 and 6 h and with and without metabolic activation. A negative control with cells incubated with the solvent (0.1M $HNaCO_3$, pH 7.4) was included. In order to check the performance of the comet assay, positive controls were also included: a treatment with 50 μM H_2O_2 during 5 min on ice for the experiments without metabolic activation, and a treatment with B(*a*)P 10 μg/ml for the experiments with metabolic activation. Immediately following the treatment with OTA, the comet assay was carried out. The technique was applied according to Singh *et al*. (40) and Tice *et al*. (34), with some modifications. The cells were trypsinized, counted in a haemocytometer (Neubauer Improved, Paul Marienfeld, Lauda-Koenigshofen, Germany), centrifuged at 175 *g*, and resuspended to get a final concentration of 6.25×10^5 cells/ml in PBS.

80 μl of cell suspension were mixed with 260 μl of 1% low-melting-point agarose (Sigma-Aldrich), and 80 μl of this mixture was spread onto microscope

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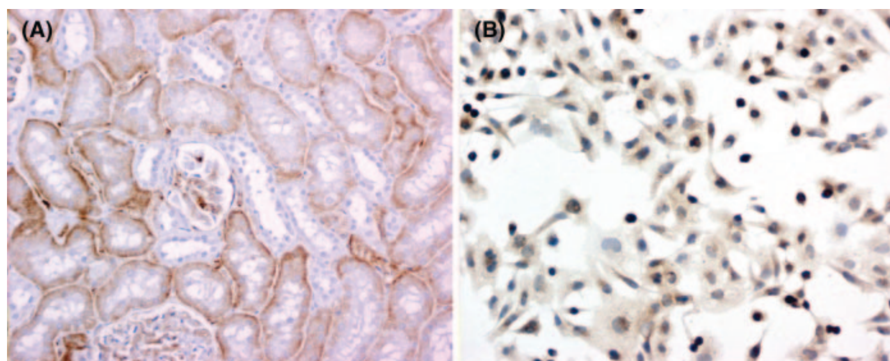

Fig. 2. Immunocytochemistry of *N*-cadherin. (**A**) Expression of *N*-cadherin in normal rat kidney section (X20). (**B**) Expression of *N*-cadherin in HK-2 confluent culture (×20).

slides precoated with 0.5% of normal-melting-point agarose (Sigma-Aldrich). Three slides were prepared for each condition, Slides 1, 2 and 3, one slide in order to observe single-strand DNA breaks and the other two, in order to obtain information on the presence of oxidized DNA bases using a digestion with the enzymes, FPG (Slide 1) and EndoIII (Slide 2). Glass cover slips (Menzel-Glaser) were placed on the gels, which were allowed to set for 5 min at 4°C. Then the cover slip was removed, and the cells embedded in agarose were lysed for 1 h by immersing the slides in 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Trizma–HCl, pH 10 and 1% Triton X-100 at 4°C. After that, the slides were washed three times (5 min each time) with enzyme buffer (0.1 M KCl, 0.5 mM Na_2-EDTA, 40 mM HEPES–KOH, 0.2 mg/ml BSA, pH 8.0) and incubated for 45 min at 37°C with FPG in the enzyme buffer (Slide 1), or EndoIII in the enzyme buffer (Slide 2), or with buffer alone (Slide 3). Both enzymes were kindly supplied by Dr A. Collins (Institute for Nutrition Research, University of Oslo, Norway). The slides then were placed on a horizontal gel electrophoresis tank and the DNA was allowed to unwind for 40 min in freshly prepared alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na_2-EDTA, pH $>$ 13). Electrophoresis was carried out in the same buffer for 30 min at 25 V ($\sim$0.8 V/cm across the gels and $\sim$300 mA) in an ice bath condition. The slides were gently rinsed 3 times (5 min each time) with 400 mM Trizma (pH 7.5) in order to neutralize the excess alkali. Then the slides were washed in water and dried overnight.

Gels were stained with 25 µl of DAPI (Sigma-Aldrich), covered with a cover slip and coded before microscopic analysis. DAPI stained nuclei were evaluated with a Nikon Eclipse TE 300 fluorescence microscope (Nikon, Tokyo, Japan). A total of 100 comets on each gel were visually scored and classified as belonging to one of five classes according to the tail intensity (41). Each comet class was given a value between 0 and 4: 0 = no damage and 4 = maximum damage. The total score was calculated by the following equation: (percentage of cells in class 0×0) + (percentage of cells in class 1×1) + (percentage of cells in class 2×2) + (percentage of cells in class 3×3) + (percentage of cells in class 4×4). Consequently, the total comet score was within the range of 0–400 arbitrary units.

Dihydrodichlorofluorescein (H_2DCF) oxidation assay

ROS were determined by using fluorescent probe dihydrodichlorofluorescein diacetate (H_2DCF-DA), according to Wang and Joseph (42). H_2DCF-DA is enzimatically hydrolized by intracellular esterases to form non-fluorescent H_2DCF, which is then rapidly oxidized to form highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity parallels the amount of intracellular ROS. Then, cells were seeded in 96-well plates at 4.5×10^3 cells/well and incubated for 30 min at 37°C in serum-free and phenol red lacking DMEM medium containing 100 µM H_2DCF-DA. Afterwards, cells were washed once with PBS and exposed to 50, 100, 200, 400 and 800 µM OTA in serum-free and phenol red lacking DMEM medium. ROS were measured after 6 h using a microplate fluorometer Fluoroskan Ascent (Thermo Labsystems) at an emission wavelength of 538 nm and an excitation wavelength of 485 nm. ROS level was expressed as the fluorescence of the treated samples compared to the fluorescence of the control samples (solvent treated cells) = [(fluorescence treated cells/fluorescence control cells) × 100].

Pre-treatment with NAC

In order to evaluate the possible protective effect of NAC, this antioxidant was added to the cultures exposed to OTA for 6 h. Cells at confluence were pre-treated with 4mM NAC in serum-free medium for 1h, after which the medium was replaced with a new one containing different OTA concentrations; cytotoxicity, oxidative DNA damage and intracellular ROS level were assessed as previously described.

Statistical analysis

Statistical analysis was performed by using the software, SPSS 13.0. Data is presented by descriptive analysis [mean ± standard deviation (SD) for three independent experiments]. Comparisons were performed by the non-parametric Mann–Whitney *U*-test. The $P \leq 0.05$ probability was accepted as the level of significance.

Results

Expression of N-cadherin in proximal tubular cells

N-cadherin staining was observed in every proximal tubule in normal rat kidney section (Figure 2A). In contrast, distal tubules were negative for the staining. In the HK-2 cell culture, a strong positive staining was observed (Figure 2B), thereby clearly confirming its proximal tubular epithelial origin.

Cytochrome P450 activity

HK-2 cells showed very low rates of cytochrome P450 activity: CYP1A1 (2.45 ± 0.70), CYP1A2 (0.84 ± 0.07) and CYP2C9 (1.82 ± 1.88), evidencing the poor metabolizing capacity of these renal cells.

Rat liver and kidney S9-mix were also assessed for CYP1A1 isoform. Liver S9-mix showed a higher activity (7.02 ± 0.23) than the kidney S9-mix (0.05 ± 0.01). Therefore, liver S9-mix was chosen for the addition to be made to the cell culture as an external metabolizing enzyme system.

Cytotoxicity of OTA

MTT assay was performed in order to determine cell viability, measuring the conversion of tetrazolium salt to formazan, the amount of which is proportional to the number of living cells.

The viability of HK-2 cells was not affected ($>$80%) by OTA at concentrations of up to 400 µM after 3 h incubation. At 800 µM, a strong decrease was observed. In contrast, after 6 h of treatment, viability decreased significantly in a concentration- dependent manner, between 50 and 400 µM OTA, and 50% of viability was observed at 800 µM (Figure 3). Therefore, concentrations between 50–600 µM were chosen for performing the comet assay under the same conditions.

The presence of an external metabolizing enzyme system (10% rat liver S9-mix) did not show any variation in the percentages of survival of the HK-2 cells treated with OTA (data not shown). Viability of the cells was not altered with the treatment of the positive controls used in the comet assay. The percentages of survival obtained with H_2O_2 at 50 µM was 98 ± 1, and 83 ± 5 and 75 ± 2 with B(*a*)P at 10 µg/ml for 3 and 6 h, respectively.

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Oxidative DNA damage

The effects of OTA on HK-2 cells after 3 h of treatment, with and without metabolic activation (10% rat liver S9-mix), did not show a significant increase in the DNA damage score. The cells treated with all of the concentrations of OTA and without any external metabolic activation showed similar total comet scores (Figure 4A). When 10% S9-mix was added, the total comet score increased slightly but no significant damage was evidenced (Figure 4C). When the digestion with FPG and EndoIII was incorporated into the assay to detect oxidized bases, there were not additional breaks in the DNA (Figure 4B and D).

When HK-2 cells were exposed to OTA during 6 h, there was no significant difference between the total comet score of the solvent and the total comet score of the cells treated with 50, 100 and 200 μM OTA in DNA strand breaks (Figure 5A and C). Only at 400 and 600 μM was slight but significant damage observed, when the viability of the cells was between 50 and 60%.

In contrast, a significant increase was observed in oxidative DNA damage detected by both enzymes, FPG and EndoIII, in all the concentrations tested ($P < 0.05$) (Figure 5B). Treated cells post-digested with FPG showed total comet scores higher than those post-digested with EndoIII. In the presence of metabolic activation, the increase was higher with FPG, but not significant with EndoIII (Figure 5D).

Effect of NAC on intracellular ROS level, cell viability and oxidative DNA damage

Under the same conditions where this significant oxidative DNA damage was detected, after 6 h OTA exposure, an increased level of ROS was also observed (Figure 6A). The protective effect of NAC was also evaluated. NAC pre-treatment showed a significant decrease in ROS levels at 200, 400 and 800 μM OTA (Figure 6A), resulting in a significant increase in cell viability at all concentrations tested (Figure 6B).

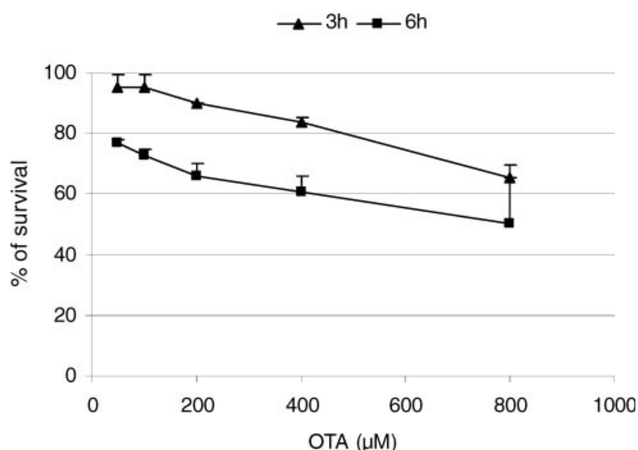

Fig. 3. Viability of HK-2 cells after 3 and 6 h of incubation with 50, 100, 200, 400 and 800 μM OTA obtained with MTT assay. Mean values (SD) were obtained in three independent experiments.

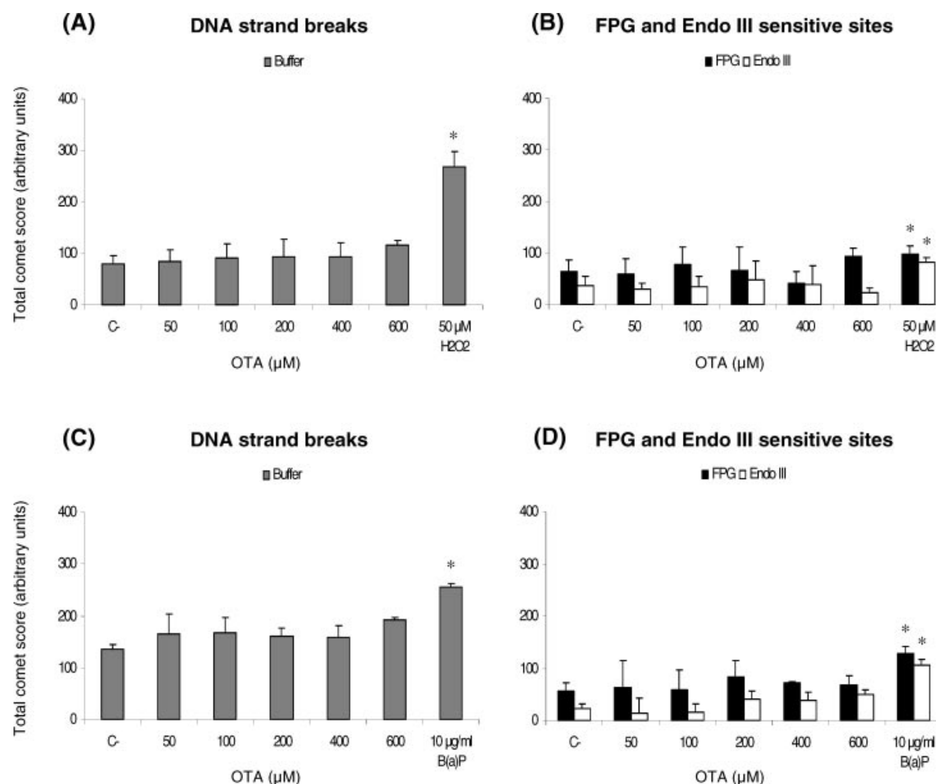

Fig. 4. DNA strand breaks and oxidative damage in HK-2 cells after 3 h of incubation with 50, 100, 200, 400 and 600 μM OTA in the absence of external metabolic activation (**A** and **B**) and in the presence of 10% rat liver S9-mix (**C** and **D**) by the comet assay. Grey bars represent nuclei incubated with the buffer without enzymes (buffer). Black bars represent nuclei post-digested with FPG, and white bars represent nuclei post-digested with EndoIII, after having subtracted the comet score values obtained with the buffer. Mean values (SD) were obtained in three independent experiments. In order to see the significant effect of OTA in DNA strand breaks and oxidative DNA damage, each concentration was compared with the one of the cells treated with the solvent (C-). $^*P \leq 0.05$.

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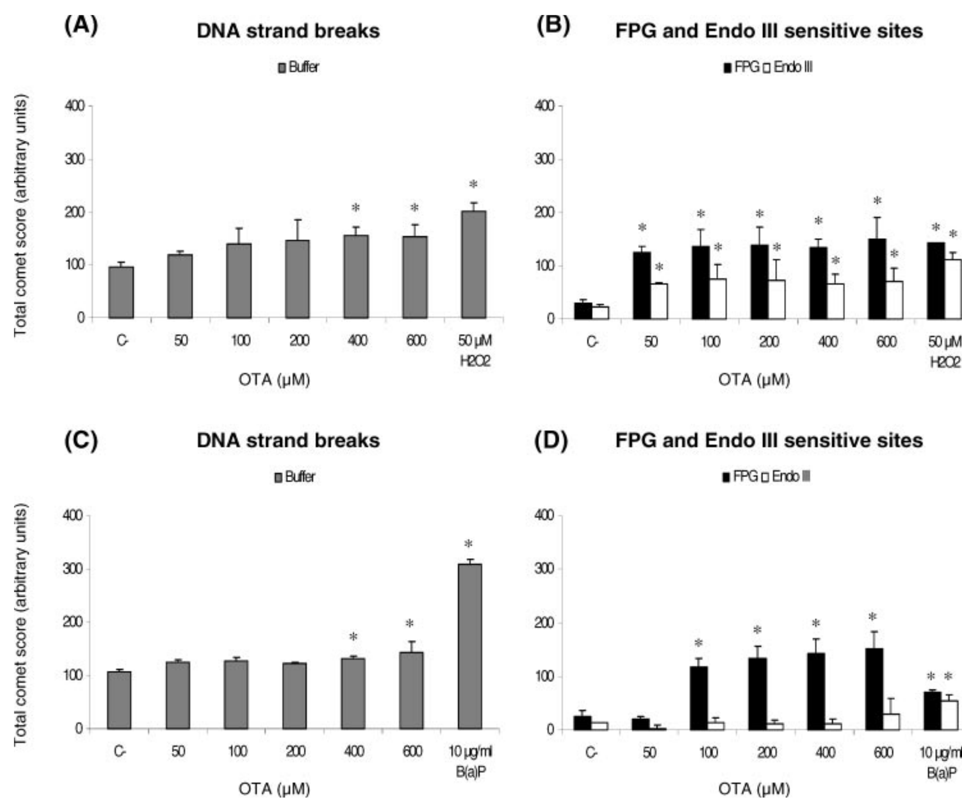

Fig. 5. DNA strand breaks and oxidative damage in HK-2 cells after 6 h of incubation with 50, 100, 200, 400 and 600 μM OTA in the absence of external metabolic activation (**A** and **B**) and in the presence of 10% rat liver S9-mix (**C** and **D**) by the comet assay. More details in legend corresponding to Figure 4. Mean values (SD) were obtained in three independent experiments. *$P \leq 0.05$.

The oxidative DNA damage detected by FPG in the comet assay was also reduced by NAC, but only at the low concentrations of OTA. NAC was not able to reduce the damage at 400 and 600 μM OTA (Figure 6C). Nevertheless, in none of the cases was NAC able to completely protect the cells, because the level of ROS and oxidative DNA damage continued to increase in comparison with solvent treated cells (C-) (Figure 6A and C).

Discussion

Regarding human health, the genotoxic and carcinogenic effects of OTA today are the aspects that cause the most concern to scientists due to the possibility of inducing tumours. It was suggested that these effects were a result of the ability of OTA to form adducts and single-strand breaks with DNA, directly or through the generation of reactive species that may interact with it. Although very interesting works addressing these aspects have been recently published (23,25,43), in this study, we have tried to distinguish both possibilities by using the modified comet assay that uses the FPG and EndoIII enzymes for detecting DNA oxidative damage.

The single-cell gel electrophoresis (SCGE) or comet assay has become one of the standard methods for assessing DNA damage due to its simplicity, sensitivity, versatility, speed and economy (41). The alkaline (pH > 13) comet assay detects both single- and double-strand breaks, cross-links, incomplete excision repair sites as well as apurinic or apyrimidinic sites, which are alkali labile and therefore appear as breaks under the alkaline conditions of the assay (40). Moreover, using different enzymes, more specific damage, as base oxidation, can be detected. Nevertheless, there is not one experimental procedure, which has been validated for the evaluation of the genotoxicity of chemical products; only some general recommendations have been made and they are not always followed. Consequently, there is a great variation in the experimental design, and the results are sometimes difficult to interpret and to compare. In this study, special care has been taken in the design of the experiment, following the recommendations of Tice *et al*. (34), and also in the control of the general cytotoxicity generated by the OTA in order to be able to distinguish a genotoxic effect of the mycotoxin from an indirect effect derived from its general cellular toxicity. In accordance with the aforementioned, two short treatments of 3 or 6 h were chosen because they seem to be sufficient so as to evidence a genotoxic effect caused by a chemical product; longer treatment may affect the culture conditions (osmolarity, pH) and can give false positive results or make the interpretation of the results more difficult. Moreover, the visual scoring method has been applied to evaluate the amount of DNA damage because it has been shown that there is a clear relationship between visual scoring and the frequency of DNA strand breaks (44,45).

After the 3 h treatment, no significant increase in the total comet score was detected. Therefore, under the standard conditions established for the exposure concentrations, covering a range from little or no toxicity to toxic doses that decrease viability by <30% compared with the control cultures, DNA damage was not detected by this sensitive assay (Figure 4A and C). But after a 6 h treatment, a slight significant increase of damage was observed at concentrations with high levels of cytotoxicity (viability percentages <60%) (Figure 5A and C).

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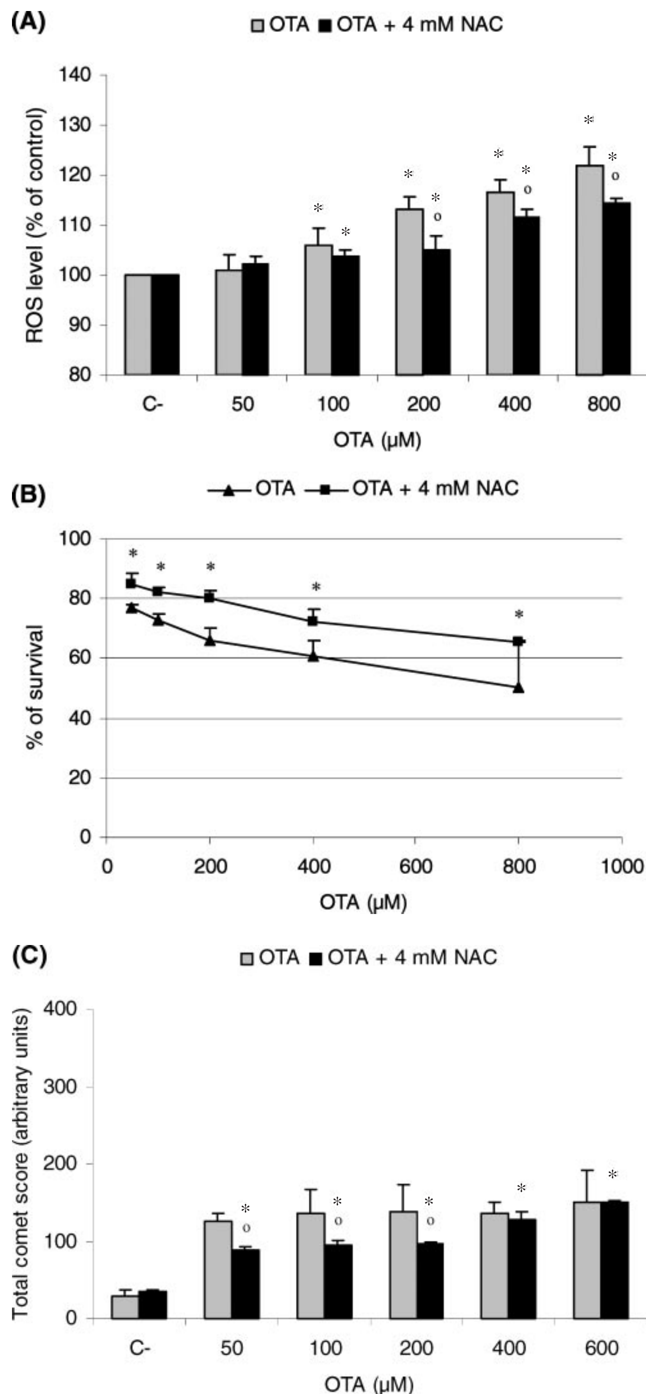

Fig. 6. The effect of 1 h pre-incubation with 4 mM NAC on intracellular ROS level, viability, and oxidative DNA damage in HK-2 cells treated with OTA during 6 h. The data are mean values (SD) of three independent experiments (**A**) Intracellular ROS level. *Significantly different from solvent treated cells (C-) ($P \leq 0.05$). °Significantly different from OTA treated cells ($P \leq 0.05$). (**B**) Cell viability. *Significantly different from OTA treated cells ($P \leq 0.05$). (**C**) Oxidative DNA damage. The protective effect was detected by the FPG enzyme in the comet assay. The bars represent nuclei post-digested with FPG, after having subtracted the comet score values obtained with the buffer. *Significantly different from solvent treated cells (C-) ($P \leq 0.05$). °Significantly different from OTA treated cells ($P \leq 0.05$).

Short assay times and percentages of survival >80% are the good conditions for performing the comet assay in order to be able to reflect a direct genotoxicity. In our opinion, these results would support the hypothesis that OTA does not have the ability to cause DNA damage directly in target cells, but rather does it indirectly and as a consequence of its cellular toxicity.

Ehrlich *et al*. (28) were the first to show positive results with the comet assay applied to OTA-exposed human HepG2 cells (24 h exposure); at concentrations ranging from 7 to 62 μM in serum-free medium, they reported percentages of viability >80% and a significant increase in the tail length, although the dose–response relationship was not evident. Apparently, these data do not coincide with our results, but the different conditions of the experiments with respect to cell line, exposure time, concentrations tested, cytotoxicity assay used (Trypan blue exclusion method) and the endpoint measured (tail length) make comparisons difficult to carry out. A long exposure time at low concentrations of OTA could produce intracellular ROS which are able to damage the DNA but not significant enough to degrade the membrane, which is the parameter evaluated by the Trypan blue exclusion method (46).

Many reports have suggested that oxidative damage to the DNA also can cause cancer, and 8-oxo-7,8-dihydroguanine (8-oxo-Gua) has been measured as the most commonly oxidation product. In order to characterize the OTA-induced DNA oxidative damage, a comet assay was also performed in the presence of FPG and EndoIII, bacterial endonucleases involved in DNA repair and which have specificity for certain kinds of oxidative base damage, converting the oxidised base to DNA break, detectable with the assay. FPG and EndoIII-based methods are extremely sensitive assays for the detection of oxidative lesions (47). In our study, no significant increase in the total comet score was detected at 3 h incubation of HK-2 cells with all the concentrations tested (Figure 4B and D), but a significant increase of oxidative DNA damage was observed after 6 h of treatment with 50–600 μM OTA (Figure 5B and D). At this time point, the viability of the cells was <80%, in which 50% viability was shown at the highest concentration tested. These results showed evidence of the relationship between the oxidative DNA damage and the cytotoxicity induced by OTA. Total comet score was higher after FPG digestion than after EndoIII digestion, in all the conditions and concentrations tested. These results suggest that OTA treatment induces the formation of 8-oxo-Gua, the major purine oxidation product.

Furthermore, under these conditions, increased levels of ROS were detected. The H_2DCF can be oxidized to the fluorescent DCF by different oxygen species; consequently, the increase in intracellular DCF fluorescence is an overall oxidative stress index in cells (42). In order to corroborate that the oxidative stress is responsible for the OTA-induced cytotoxicity and oxidative DNA damage detected after 6h, cells were pre-treated with NAC. NAC is a well-established antioxidant and intracellular ROS scavenger (48); it has also been considered to be a precursor of GSH synthesis (49). In our experiment, NAC had a protective effect, being able to significantly reduce the level of ROS and also to increase cell viability under the same experimental conditions. Oxidative DNA damage detected by the comet assay after 6 h was also diminished, but only at low OTA concentrations. In order to reduce the damage at 400 and 600 μM OTA, it can be assumed that a higher NAC concentration is needed, but further investigations would be necessary. These results clearly show that the link between OTA-induced cytotoxicity and oxidative DNA damage is the intracellular oxidative stress caused by OTA.

These data are in line with that of Schaaf *et al*. (50), who demonstrated that primary rat proximal tubular cells and

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continuous proximal tubular cells (LLC-PK1) responded to OTA treatment by elevation of ROS, depletion of GHS levels and an increase in the formation of 8-oxo-Gua. The production of ROS preceded the loss of cell viability, and the NAC pre-treatment completely protected the cells against the OTA-induced loss of viability. The highest ROS production was observed after 24 h treatment with 100 μM OTA, where the cell viability was $\leq$50%. In non-target human fibroblasts (HF1 cell line), longer exposures (48–72 h) to OTA 50 μM were needed in order to detect a significant increase in intracellular ROS production and damage to genomic DNA (31). Kamp *et al*. (30) have also shown that OTA-induced an increase in oxidative DNA damage detected by FPG post-digestion, in CV-1 (African green monkey kidney) cells and in primary rat kidney cells, after 24 h of treatment, without any effect on basic DNA breakage. In CV-1, these results were found at concentrations below the IC_{50} value determined by the Trypan blue assay or by the sulforhodamin B assay that measures protein content. Due to the long exposure times and the different parameters used to evaluate the cytotoxicity or the comet assay (fluorescence intensity of tail, *T* I%), it is difficult to carry out comparisons, but a dose-related increase in DNA breakage, that would be expected if OTA caused a direct DNA damage, was not observed under any condition. Moreover, *in vivo* it has been demonstrated that in OTA treated rat liver and kidney, the extent of DNA damage detected by the comet assay was enhanced in the presence of the FPG enzyme (23). All these results suggest that OTA may cause genetic damage regardless of direct covalent binding to DNA.

Other recent studies (51–53) using different cell lines -Hep G2, Caco 2, MDCK (Madin Darby canine kidney), BME-UV1 (bovine mammary epithelium)- also demonstrated that anti-oxidants such as α-TOCO, retinol, rosmarinic acid and cyaniding-3-0-β-glucopyranoside, can protect the cells from OTA-induced ROS increase, loss of cell viability and protein and DNA synthesis inhibition.

The role of the bioactivation in the production of the genotoxic and carcinogenic metabolites is not resolved yet; although some authors observed genotoxic effects in the presence of some CYP450 isoforms (54–57), others suggested that OTA is poorly metabolized by CYP450 (21,22,24). The HK-2 cells showed very low activities of CYP 450 isoforms 1A1, 1A2 and 2C9. When 10% rat liver S9-mix was added as an external metabolizing enzyme system, no differences were shown on DNA strand breaks and oxidative DNA damage (Figures 4C and D, and 5C and D). Therefore, under these conditions, the metabolism of OTA was not involved in the DNA damage observed in these human renal cells at cytotoxic doses. Simarro-Doorten *et al*. (58), in NIH/3T3 cells transfected with human *CYP2C9* gene, have found a significant increase in DNA damage with respect to non-transfected control cells. These differences may be explained by the interspecies variations in cytochrome P450 activities.

In summary, no DNA damage was shown at non-cytotoxic concentrations, both in the presence and in the absence of metabolic activation; at the cytotoxic concentrations (50–80% cell viability) oxidative DNA damage was evident and an increased level of ROS was also observed. Furthermore, NAC was able to produce a slight protective effect against OTA-induced ROS increase, cytotoxicity and DNA damage. Therefore, these results suggest that oxidative stress precedes cytotoxicity and genotoxicity, and plays an important role regarding the mechanisms involved in OTA nephrotoxicity and carcinogenicity. Consequently, they support the hypothesis that OTA is not a direct genotoxic carcinogen and that its genotoxic effects appear to occur through indirect mechanisms (59).

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