**Cytotoxicity, apoptosis, DNA damage and methylation in mammary and kidney epithelial cell lines exposed to Ochratoxin A**

Running heater: **Ochratoxin A *in vitro* mode of action**

*Carlotta Giromini*, Raffaella Rebucci, Eleonora Fusi, Luciana Rossi, Francesca Saccone & Antonella Baldi*

Department of Health, Animal Science and Food Safety, Università degli Studi di Milano, Via D. Trentacoste 2, 20134, Milan Italy; E-mails: raffaella.rebucci@unimi.it (R.R.);

***** Author to whom correspondence should be addressed; E-Mail: carlotta.giromini@unimi.it; Tel.: +39 250.315.727; Fax: +39 250.315.746

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**Abstract**

This study aimed to investigate the *in vitro* damage induced by Ochratoxin A (OTA) using BME-UV1 and MDCK epithelial cells. Both cells lines were treated with OTA (0 up to 10 μg/mL) and cell viability (MTT assay), membrane stability (LDH release assay), and apoptotic cell rate (Tunel assay) were investigated. Further, the effect of the incubation with OTA has been evaluated at DNA level by the determination of DNA integrity, by the quantification of DNA adduct formation (8-OHdG) and by the assessment of the global DNA methylation status (5-mC). The obtained results showed that after 24 hours of OTA treatment, BME-UV1and MDCK cell viability was reduced in a dose-dependent way. OTA significantly ($P$<0.05) increased LDH release in BME-UV1 cells at all concentrations tested. OTA (1.25 μg/mL) induced 35% LDH release in MDCK cells ($P$<0.05). A significant ($P$<0.05) percentages of 10±0.86 and 24.86±0.88 of apoptotic BME-UV1 and MDCK cells respectively were calculated when the cells were co-incubated with OTA. The level of 8-OHdG adduct formation was significantly ($P$<0.05) increased in BME-UV1 cells treated with 1.25 μg/mL of OTA. The results of the present study suggest that a different mechanism of action may occur in these cell lines.

## 1. Introduction

Mycotoxins are ubiquitous in nature and widespread distributed in food and feedstuffs due to fungi contamination in susceptible agricultural commodities. On global level, 81% of food and feed samples were found to be positive to at least one mycotoxin with associated negative effects mainly on crop and livestock production with overall economic losses (Cheli *et al*., 2014).

Ochratoxin A (OTA) is produced by filamentous mold species belonging to the genera *Aspergillus* and *Penicillium.* This toxin aroused public concern due to its wide range of toxicological effects and its widespread distribution in feed and food (EFSA, 2006).

OTA is considered a potent nephrotoxic, hepatotoxic and teratogenic substance in different animal species, with the longest half-life in human blood (Stojkovic' *et al.,* 1984). After small intestinal absorption, OTA enter in the bloodstream where it strongly binds serum proteins and it is subsequently distributed to different tissues and organs, with kidney as the primary target ( Hagelberg et al., 1989; Pfohl-Leszkowicz & Manderville, 2007; Palli et al., 1999). However, lower OTA concentrations could be detected in different tissues, such as liver, adipose tissue and muscles. In addition, detectable amounts of OTA have been found in milk of several species, such as humans, rabbits, rats and ruminants, suggesting that the mammary gland could be one of the potential target of this mycotoxin ( Fink-Gremmels, 2008; Sorrenti et al., 2013).

At cellular level, several OTA mechanisms of action have been proposed for OTA and cell viability, cell membrane damage, oxidative stress and the DNA damage have been studied. However, the mechanism by which OTA elicits its cytotoxic action is different from a tissue to another. OTA could express its toxicity through the covalent binding of OTA metabolites to DNA or with an indirect mechanism of action (Pfhol-Leszkowicz & Manderville 2012; Ali et al., 2011). Accumulating evidence has demonstrated that *in vitro*, OTA expresses its toxicity through an indirect mechanism, which mainly consists in the overproduction of Reactive Oxidative Species (ROS) causing oxidative

stress (Palma et al., 2007; Zheng et al., 2013). Overproduction of ROS lead to a wide array of oxidative cell lesions at membrane, mitochondria and DNA level, which may induce cell death. In fact, according to the cell type considered and the time of exposure, the oxidative effects of OTA may cause apoptosis, necrosis or cancer (Costa et al., 2007). ROS production has been correlated with DNA oxidation and, subsequently, with the formation of DNA adducts. One of the major OTA-induced oxidative DNA damage product is 8-hydroxy-2′-deoxyguanosine (8-OHdG). 8-OHdG is an abundant base modification in mammalian DNA that is associated with oxidative stress ( Cheng et al., 1992) and commonly used as an *in vitro* biomarker for the assessment of OTA-induced oxidative damage (Zheng et al., 2013). However, a comprehensive knowledge of the mechanism of OTA toxicity at DNA level in mammalian cells is still a matter of debate.

Therefore, in the present study the cytotoxicity of OTA was first investigated by the evaluation of cell viability, membrane stability and apoptotic cell rate in BME-UV1 and MDCK cell lines. Further, the effect of the addition of OTA has been evaluated at DNA level by the determination of the DNA integrity, by the quantification of DNA adduct formation (8-OHdG) and by the assessment of the global DNA methylation status (5-mC) in order to improve the available data regarding OTA mechanism of action *in vitro*.

## 2 Material and methods

### *2.1 Chemicals*

Ochratoxin A and all other chemicals were purchased by Sigma-Aldrich (St. Louis MO, USA), unless otherwise indicated.

### *2.2 Cell culture and treatments*

BME-UV1 and MDCK were obtained from University of Vermont and European Type Culture Collection, respectively, and routinely cultivated into 75 $cm^2$ tissue culture flasks (Costar, Corning, NY, USA) in complete medium, specific for the cell line. BME-UV1 (passage number 39-42) were cultured in DMEM-F12 supplemented with RPMI-1640, NCTC-135 medium, supplemented with 10% Fetal Bovine Serum (FBS), (BioWhittaker, Cambrex, Belgium), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 1 μg/ml insulin, 5 μg/ml transferrin, 1 μg/ml hydrocortisone, 0.5 μg/ml progesterone, 10 μg/ml L-ascorbic acid and antibiotics (penicillin 100 IU/ml; streptomycin 100 μg/ml). MDCK cells (passage number 20-24) were cultured in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% Glutamax (Gibco, Invitrogen), 1% Non-Essential Amino Acid (NEAA), and 0.2% penicillin/streptomycin.

Cell lines were maintained at 37°C in a humidified 5% $CO_2$ incubator until sub-confluence. Medium was replenished every second day for both cell lines and cells were passaged for exposure to treatment media while in the logarithmic growth phase.

OTA was dissolved in methanol (100%) to obtain a stock solution of 10,000 μg/mL. The treatment concentrations of OTA were composed of serum free basal medium (DMEM for BME-UV1 and EMEM for MDCK cells) contained OTA at several concentrations (from 0.15 μg/mL up to 10 μg/mL). Preliminary experiments showed that the concentration of methanol employed in the serum free OTA dilutions had no effect on cell viability.

Portion (150 μl) of cell suspension ($2.5 \times 10^5$ cell/mL for BME-UV1 and $1 \times 10^5$ cell/mL for MDCK) were dispensed into 96 well-plates (NuncClon Surface, Nunc, Denmark). Under these conditions, a sub-confluent monolayer was observed after 24 hours of incubation at 37°C in an atmosphere of 5% $CO_2$. At sub-confluence, complete medium was removed from each well and BME-UV1 and MDCK

cells were exposed to treatment concentrations of OTA (150 µL/well) for an incubation time of 24 hours.

### *2.3 Cell viability: MTT test*

Cell viability was determined after incubation with OTA by measuring the production of the chromophore formazan from 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT). Formazan is produced in viable cells by the mitochondrial enzyme succinate dehydrogenase.

After 24 hours of OTA treatment, medium was removed and 150 µL MTT stock solution (5 mg/ mL) in Phosphate Buffer Saline (PBS, Euroclone) were added to each well and the plate was incubated for 3 h at 37°C in a humidified chamber. After incubation time MTT solution was removed to stop the reaction and 150 µl of dimethyl sulfoxide was added to dissolve the formazan. The optical density of the dimethyl sulfoxide solution at 570 nm was determined on a Biorad 680 microplate reader (Biorad, Veenendaal, The Netherlands). The percentage cytotoxicity was calculated as follows: Mitochondrial activity = (mean optical density in presence of OTA/ mean optical density of negative control) x 100.

From MTT assay results, the Half Lethal Concentration 50 ($LC_{50}$) of OTA was calculated for each cell line.

### *2.4 Membrane stability: LDH assay*

BME-UV1 and MDCK cell lines were treated with selected concentrations of OTA (0.3, 0.6 and 1.25 µg/mL) chosen on the basis of $LC_{50}$. In order to examine cell membrane damage induced by OTA treatments Lactate dehydrogenase enzyme release (LDH) assay was performed using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) as instructed by the manufacturer. Briefly, after 24 hours of OTA treatment, supernatants were removed and centrifuged for 5 minutes at 1500 × g (4 °C). 50 µL of each supernatant was transferred to a 96 well plate.

Cellular monolayers were lysed by adding 15 µL of 9% Triton X-100 solution in water per 100 µL of basal medium (EMEM), followed by an incubation for 1 h at 37 °C. Cells debris were removed by centrifugation for 5 min at 1500 × g (4 °C) and 50 µL of each cell lysate was transferred to 96 well plate. Then, 50 µL of LDH substrate was added to the supernatants and cell lysates. After the incubation for 30 minutes at room temperature in the dark, the enzymatic assay was stopped by adding 50 µL of 1 M acetic acid and the plate was read at 490 nm using a Biorad 680 microplate reader. The percentage of LDH release was calculated as the amount of LDH in the supernatant over total LDH from both supernatant and cell lysate.

### *2.5 Apoptosis: Tunel (TdT-mediated dUTP nick end labeling) assay*

BME-UV1 and MDCK cells were seeded at a density of $0.3 \times 10^6$ and $0.2 \times 10^6$ cells/mL, respectively, in a two-well chamber slides (Nunc Lab-Tek, Nunc, Denmark) and maintained for 24 hours. Afterward, the medium was removed and the cell monolayers washed twice with PBS. Based on results obtained from previous assays, the concentrations of 0.8 µg/mL and 1 µg/mL of OTA were selected and added to BME-UV1 and MDCK cells respectively for the following 24 hours.

At the end of the incubation, the medium was removed and the cells fixed with refrigerated methanol for 10 min at -20°C. The Tunel assay was performed using the DeadEnd Colorimetric Apoptosis Detection kit (Promega, Madison, WI, USA). The monolayers were washed twice with PBS and permeabilized by immersing the slide in 0.2% Triton X-100 solution in PBS for 5 min at room temperature. After washing with PBS, cells were incubated with biotinylated nucleotide mixture together with terminal deoxynucleotidyl transferase enzyme. Horseradish peroxidase–labeled streptavidin (streptavidin HRP) was then added to bind to these biotinylated nucleotides, which are detected using the peroxidase substrate hydrogen peroxide and the stable chromogen diaminobenzidine (DAB). Afterward, to distinguish and calculate the apoptotic and non apoptotic

cells, haematoxylin staining was performed. Images (20X) were captured under an Olympus BX51 microscope. For each experiment, ~500 cells were counted in randomly selected fields, and the percent of Tunel-positive cells were calculated.

### *2.6 DNA damage: agarose gel electrophoresis and 8OH-dG adduct formation*

BME-UV1 and MDCK cells were cultured in 25 $cm^2$ flasks (Sarstedt, Germany) at the seeding density of $1x10^6$cells/mL and left untouched for 24 hours at 37°C and 5% of $CO_2$. Cells were treated with the treatment medium containing the selected concentrations of OTA (0.3-1.25µg/mL) and the subsequent day cells were collected for purification and quantification of the DNA.

DNA from BME-UV1 and MDCK cells was extracted using the Wizard® genomic DNA purification kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. The concentration and the quality (λ260/280) of the total DNA was determined by UV spectrophotometry at 260nm and by agarose gel electrophoresis followed by Image Lab™ software analysis (BioRad). Specifically, loading buffer was added to 5µg DNA for each treatment and to 5µg of DNA ladder (Molecular Weight Marker II, Roche) and the samples were analysed by electrophoresis on a 1% agarose gel (1.30 hour at 65V/200mA) with 1X Tris/Boric acid/EDTA (TBE) buffer (BioRad). DNA was subsequently used for the further analysis investigating DNA damage induced by OTA treatments.

To assess DNA damage induced by OTA treatment, HT 8-oxo-dG ELISA kit II (Trevigen®) was employed. After DNA collection and quantification, a concentration of 500 µg/mL of BME-UV1 and MDCK cell DNA was fragmented in single nucleotides and processed according to the manufacturer's protocol. Results are expressed as [8-OHdG] (nM) / DNA (µg/mL).

### *2.7 Global DNA methylation: 5-methyl-Cytosine (5-mC)*

The same DNA samples were employed for the analysis of 5-mC in BME-UV1 and MDCK cell lines.

In particular, in order to investigate if OTA treatment and the DNA damage could be linked to change in the global DNA methylation, MethylFlash Methylated DNA 5-mC quantification (Epigentek) was performed as indicated by the manufacturer. The percentage of 5-mC was calculated in 100ng DNA extracted BME-UV1 and MDCK cells treated with OTA using the second-order regression equation of the standard curve that was constructed with negative control and positive controls in the same experiment. Results are expressed as 5-mC percentage in total DNA.

### *2.8 Statistical analysis*

Results are expressed as mean value ± standard error of three independent experiments (n=3) in triplicate wells (three technical replicates for each OTA concentration).

Statistical differences between control and OTA-treated cells for all experiments were evaluated by one way analysis of variance (ANOVA) using the General Linear Model procedure (proc GLM) of SAS 9.4 [33]. The level of significance at $P < 0.05$ was considered statistically significant.

## 3 Results

### *3.1 Cell viability: MTT test*

Cytotoxicity of OTA was first assessed by the determination of cellular viability in BME-UV1 and MDCK cells. In particular, the incubation with increasing concentrations (ranging from 0 up to 10 µg/mL) of OTA for 24 hours determined a dose dependent inhibition of cell viability in both cell lines (Figure 1A and B). The $LC_{50}$ calculated in BME-UV1 and MDCK cells were 0.8 µg/mL and 1.0µg/mL, respectively. Considering the $LC_{50}$ values, the range of OTA concentrations for the further experiments was determined in 0.3 -1.25µg/mL.

**Figure 1**

*3.2 Membrane stability: LDH release assay*

The cell membrane damage induced by OTA on BME-UV1 and MDCK cells was investigated by the release of LDH, a biomarker of membrane integrity. As showed in **Figure 2A** and **2B**, 24 hours incubation with OTA induced a dose dependent LDH release in both cell lines, indicating that OTA caused cell membrane damage. OTA significantly ($P$<0.05) increased LDH release in BME-UV1 cells at all the concentrations tested, compared with control cells (0 μg/mL OTA). In particular, a percentage of 46% of LDH release has been observed in BME-UV1 cells treated with 1.25 μg/mL of OTA. In MDCK cells, 1.25 μg/mL of OTA induced 35% ($P$<0.05) LDH release, compared with control cells (0 μg/mL of OTA).

**Figure 2**

*3.3 Apoptotic cell rate: Tunel (TdT-mediated dUTP nick end labeling) assay*

The number of apoptotic cell nuclei induced by OTA treatment on BME-UV1 and MDCK cells was investigated by Tunel assay. Representative photos of BME-UV1 and MDCK cells morphology incubated with complete medium alone (control cells, 0 μg/mL of OTA) for 24 hours are showed in **Figure 3 A** and **C**, respectively.

In the absence of OTA, the uniformity of monolayers, the typical cell-cell interactions in cultures were evident in both cell lines tested, with only a small amount of apoptotic nuclei detected. The average percentage of apoptotic nuclei calculated in the selected fields for BME-UV1 control cells was of 1.37± 0.88 (**Figure 3A**), while for MDCK control cells was 2.2±0.88 (**Figure 3C**).

In BME-UV1 cells co-incubated with 0.8 μg/mL of OTA for 24 hours, the average percentage of apoptotic nuclei was 10± 0.86 ($P$<0.05). In addition the uniformity of the monolayers was greatly compromised and floated cellular debris have been detected in all the observed fields (**Figure 3B**).

In MDCK cells co-incubated with 1 μg/mL of OTA for 24 hours, the average percentage of apoptotic nuclei was 24.86±0.88 ($P$<0.05), as demonstrated by the high presence of dark brown nuclei in the **Figure 3D.**

**Figure 3**

*3.4 DNA damage: agarose gel electrophoresis and 8OH-dG adduct formation*

The DNA was extracted from BME-UV1 and MDCK cells incubated with selected concentrations of OTA and a qualitative analysis was performed by agarose gel electrophoresis. OTA incubation caused a dose-dependent DNA degradation in BME-UV1 cells, as illustrated by the appearance of DNA ladders in **Figure 4A**. No specific DNA degradation was detected in MDCK cells treated with OTA. Further, the effect of OTA on the formation of DNA adduct was investigated in the DNA of BME-UV1 and MDCK cells. The formation of 8-OHdG was significantly increased ($P$<0.05) in BME-UV1 cells treated with specific OTA concentrations, compared with control cells (**Figure 4B**). In particular, a 24 hours exposition of 1.25μg/mL OTA led to a concentration of 9.03nM of 8-OHdG adduct formation in BME-UV1, whereas in the BME-UV1 control cells (0 μg/mL OTA) the concentration of 8-OHdG was 2.23 nM. No difference was detected in MDCK cells treated with OTA compare with control, suggesting a different pathway of OTA toxicity in this cell line.

**Figure 4**

*3.5 Global DNA methylation: 5-methyl-Cytosine (5-mC)*

Methylation of cytosine in the DNA of BME-UV1 and MDCK cells was investigated in order to determine whether the cytotoxic action of OTA and the subsequent DNA damage induction could also affect the pattern of global DNA methylation in these cell lines. In both BME-UV1 and MDCK control cells (0 μg/ml OTA) the average percentage of 5-mC was below 6%. No detectable changes

in the global DNA methylation pattern have been observed after OTA treatment in BME-UV1 and MDCK cells (**Figure 5**).

**Figure 5**

## 4 Discussion

The MTT assay was performed to measure the cell viability after OTA treatment, the quantification of the LDH release was used as a biomarker of the integrity of the cell membrane. According to $LC_{50}$, both cell lines cells showed a higher sensitivity to OTA compared with other cell models. Hepatocellular (Hep3B) cells exhibited a $LC_{50}$ of 104μM after 24 hours of OTA treatment (Anninou et al., 2014). In OTA treated-PK15 cells the $LC_{50}$ obtained by MTT and Trypan Blue assays were 14 And 20 μM, respectively (Klaric' et al., 2012). The sensitivity of BME-UV1 cells is significant considering that OTA and its metabolites were detected in milk of several species (Sorrenti et al., 2013; Pfhl-Leszkowicz & Manderville, 2012), even if their carry over in ruminant milk is limited, due to the rumen microflora hydrolysis. However, rumen detoxification capacity is saturable and can vary with changes in the diet, the duration of OTA exposure, and the health and production status of the animal (Fink-Gremmels, 2008; Cheli et al., 2015) leading to a more consistent OTA transfer to milk in ruminants.

In previous studies, preliminary data about OTA-induced cell damage were reported considering cell viability and apoptosis in different *in vitro* models (Baldi et al., 2004; Fusi et al., 2008). In the present study, the Tunel assay, widely used for detecting apoptotic cells at earlier stages, demonstrated that the cellular monolayers observed were compromised and the cellular debris were present, underlining the OTA-induced cytotoxicity in both BME-UV1 and MDCK cells. Tunel-positive apoptotic nuclei were observed in MDCK cells and this finding is consistent with the kidney as the main target of OTA and in line with previous findings (Genkle et al., 2000). After OTA incubation, an increased

number of apoptotic cells has been described in different kidney cell-based models (Schwerdt et al., 1999; Kamp et al., 2005) and in cells from different sources (Cui et al., 2010). In this study, a lower but consistent number of apoptotic cells was also detected in BME-UV1 cells incubated with 0.8µg/mL OTA. However, given the amount of detached cells and cellular debris floated in the observed fields we can assume that a different mechanism of death intervened in OTA-treated BME-UV1 cells, also considering the high level of membrane damage previously described by LDH release assay in this cell line. Moreover, since the cell death is a multi-stage procedure, quantifying and characterize the apoptotic process need several techniques and remains to be elucidated in BME-UV1 cell lines.

The capability of OTA and its metabolites to bind DNA is still conflicting and OTA-DNA damaging potential was mainly related to free radical overproduction and oxidative stress. Previous studies demonstrated the ability of OTA to induce DNA damage *in vitro*, such as by the chromosomal aberration and the formation of DNA strand breaks, DNA adducts and DNA-DNA cross links (Mally & Dekant, 2005; Yang et al., 2015; Rutigliano et al., 2015). OTA-induced adduct formation has been investigated in different cell lines. Zheng et al. (2013) demonstrated the toxic effect of 25µM of OTA in HepG2 cells with a significant increase in the formation of 8-OHdG and an induction of global DNA hypo-methylation. Further, DNA degradation and 8.8ng/mL 8-OHdG adduct formation was found in kidney LLC-PK1 cells after 60µM of OTA treatment (Costa et al., 2008). OTA caused the formation of 8-OHdG also in HPBMCs cells, as demonstrated by Liu et al.

However, biological data associated to the activity of OTA on the mammary gland tissue are rather limited, especially those regarding the induction of DNA damage. The results here obtained demonstrated the genotoxic effect induced by OTA *in vitro* with a significant DNA degradation and 8-OHdG adduct formation in the DNA of BME-UV1 cells after 24 hours of treatment. Considering the *in vitro* condition, the 8-OHdG formation in BME-UV1 cells may occur independently of metabolic activation. The investigation of the mechanism of adduct formation, by the covalent

binding of OTA metabolites to DNA or by an indirect mechanism (ROS-mediated DNA damage) in BME-UV1 cells is complex and requires further qualifiers and different endpoint to measure as HPLC techniques and 32P-postlabelling assays (Obrecht-Pflumio & Dirheimer, 2000). However, a different scenario turned up when MDCK cells were treated with OTA, with no DNA degradation detected by the agarose gel-electrophoresis and no significant 8-OHdG adduct formation occurred. This may be owed to a different cell response to the mycotoxin activity, as also suggested by the LDH release and Tunel assay results. In addition, although 8-OHdG is one of the most studied oxidative biomarker and widely considered a simple and a valid method to estimate the cellular oxidative damage related to the genotoxic potential of OTA (Kasai, 1997; Faucet et al., 2004; Zheng et al., 2013), different forms of DNA damage are known to be produced *in vitro.* Therefore, it becomes important to make effort in the investigation of other cell biomarkers in BME-UV1 and MDCK cells to describe and characterize OTA-induced damages at DNA level.

The methylation of the 5$^{th}$ position of cytosine to form 5-mehtyl Cytosine is one of the most important epigenetic biomarker in mammalian cell genome, and it is commonly associated with DNA integrity. Specifically, if the DNA adduct occurs in a methylated portion of the DNA it could alter the cytosine methylation profile at this site. Adduct formation by OTA may impair the global methylation of the DNA as suggested for other mycotoxins (Kouadio et al., 2007). Zheng *et al*. (2013) demonstrated a link between OTA treatment and the decrease of 5-mC percentage, leading to a global DNA hypo-methylation in hepatic cells.

However, in the present study the global DNA methylation appears not to be affected in BME-UV1 and MDCK cells treated with OTA. The 8-OHdG adduct formation observed in BME-UV1 incubated with OTA was not associated with global DNA methylation alteration. This finding suggests that the *in vitro* relationship between OTA incubation, adduct formation and DNA methylation is not resolved yet. Other epigenetic markers and different *in vitro* models need to be developed in order to have a complete understand of OTA mechanism of action at DNA level.

## 5 Conclusions

Overall, the results obtained in this study contributed to the characterization of OTA toxic mechanism *in vitro.* Cytotoxicity potential of OTA impaired BME-UV1 and MDCK cell viability, cell membrane integrity, DNA integrity and increased the number of apoptotic cells. The major difference in the OTA-induced toxicity was a more consistent formation of DNA adduct in BME-UV1 cells, compared with MDCK cell line. However, the high apoptotic cell number detected in MDCK cells is consistent with the kidney as the primary target of OTA *in vivo*. These findings could help in ameliorate the characterization of OTA mode of action *in vitro,* suggesting that a different mechanism of toxicity may occur in these cell lines and merits a deeper characterization. Given the high OTA cytotoxicity in MDCK cells together with the high number of apoptotic cells detected, we can conclude that a predominant cytotoxic mechanism rather than a genotoxic mechanism of action occurs, with marked effects on cell viability, membrane and tight junction's integrity.

## Conflicts of Interest

The authors declare no conflict of interest.

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**Figure legends**

**Figure 1**: Effect of increasing concentrations of OTA added to the culture medium for 24 hours on cell viability (MTT test) in BME-UV1 cells (**A**) and MDCK cells (**B**). Results are from three independent experiments (n=3) with triplicate wells and presented as least square means ± SEM relative to cell viability in basal medium (0 µg/mL OTA).

**Figure 2**: Effect of increasing concentrations of OTA added to the culture medium for 24 hours on membrane stability (LDH release assay) in BME-UV1 cells (**A**) and MDCK cells (**B**). Results are from three independent experiments (n=3) with triplicate wells and presented as least square means ± SEM relative to membrane stability in basal medium (0 µg/mL OTA). Values significantly different from membrane stability obtained in basal medium are indicated by * ($P$<0.05).

**Figure 3**: Representative photos of TUNEL-stained BME-UV1 and MDCK cells. The TUNEL-positive nuclei, indicating apoptotic cells, are stained in dark brown, while the vital nuclei are stained violet (haematoxylin). (**A**) BME-UV1 cells in culture for 24 h (0 µg/mL OTA); (**B**) BME-UV1 cells incubated with 0.8 µg/mL OTA for 24 h; (**C**) MDCK cells in culture for 24 h (0 µg/mL OTA); (**D**) MDCK cells incubated with 1 µg/mL OTA for 24 h. Bars 100 µm

**Figure 4**: (**A**) Effect of increasing concentrations of OTA evaluated on the integrity of DNA extracted from BME-UV1 and MDCK cells and loaded on agarose gel electrophoresis. Line 1A:DNA Ladder; Line 2A DMEM (0 µg/mL OTA); Line 3: 0.3 µg/mL OTA; Line 4: 0.6 µg/mL OTA; Line 5: 1.2 µg/mL OTA; Line 1B: MEM (0 µg/mL OTA); Line 2B: 0.3 µg/mL OTA; Line 3B: 0.6 µg/mL OTA; Line 4B 1.25 µg/mL OTA; Line 5B:DNA Ladder. (**B**)Effect of increasing concentrations of OTA on 8-OHdG adduct formation in BME-UV1 cells and MDCK cells. Results are from three independent experiments (n=3) with triplicate wells and presented as least square means ± SEM relative to 8-OHdG adduct formation in basal medium (0 µg/mL OTA). Values significantly different from 8-OHdG adduct formation obtained in basal medium are indicated by * ($P$<0.05).

**Figure 5:** Effect of increasing concentrations of OTA on DNA global methylation in BME-UV1 cells (**A**) and MDCK cells (**B**). The level of global DNA methylation is indicated by the percentage of 5-mC in the total cytosine. Results are from three independent experiments (n=3) with triplicate wells and presented as least square means ± SEM relative to membrane stability in basal medium (0). Values significantly different from membrane stability obtained in basal medium are indicated by * ($P$<0.05).