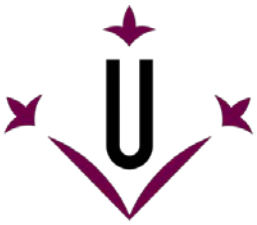

Cytotoxicity of the mycotoxins deoxynivalenol and ochratoxin A on Caco-2 cell line in presence of resveratrol

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Abstract

Exposure to mycotoxins through dietary food intake involves a highly complex scenario where co-contamination of different mycotoxins has been frequently demonstrated. On the other hand, the effect of the interaction of mycotoxins with other generally considered beneficial food components, as the antioxidants, has been scarcely studied. The main goal of the present work was to assess the cytotoxic effects on Caco-2 cells of the mycotoxins deoxynivalenol (DON) and ochratoxin A (OTA), alone or combined, and to explore potential protective effects of resveratrol (RES), an antioxidant frequently found in wine. In parallel, reactive oxygen species (ROS) production has also been studied as a first approach to understand the underlying mechanism of cytotoxicity. Results indicate a higher toxic effect of the mycotoxins when they are co-exposed. This increase in cytotoxicity was not accompanied by an increase in ROS production. Opposite to our expectations, the co-exposure with RES didn't result in a protection for the cell line from the mycotoxins, resulting in an increase in cytotoxicity at sub-toxic doses unrelated with an increase in ROS production.

Keywords: Antioxidant; mycotoxin co-exposition; *in vitro* cytotoxicity; oxidative stress; ROS production.

1. Introduction

Ochratoxin A (OTA) (Fig. 1a) is a fungal secondary metabolite produced by some species of the genera *Aspergillus* and *Penicillium*. Sources of human exposure to OTA are mainly foodstuffs of vegetal origin, such as cereals and derivatives, grapes, musts and wines, coffee, beer, nuts and dried fruits, spices and, to a minor extent, animal by-products. OTA is a potent kidney toxin and has been classified by the IARC as a 2B cancer compound (possibly carcinogenic to humans) (IARC, 1993). It is among the strongest carcinogenic compounds in rats and mice, and its toxicological profile includes teratogenesis, nephrotoxicity and immunotoxicity (Fernández-Cruz et al., 2010). Reported *in vitro* toxic effects of OTA include inhibition of cellular proliferation, apoptosis and impairment of barrier function and increasing membrane permeability (McLaughlin et al., 2004). OTA has been found to induce oxidative damage *in vitro* (Schaaf et al., 2002; Kamp et al., 2005; Mally et al., 2005) and *in vivo* (Petrik et al., 2003; Hsuuw et al., 2013) and to be genotoxic (Lebrun and Föllmann, 2002; González-Arias et al., 2014).

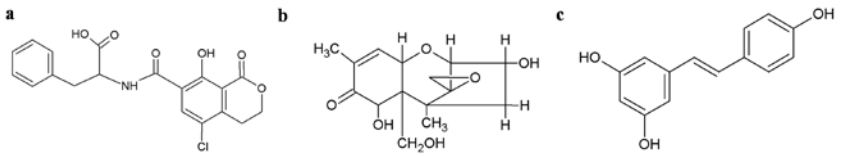

Figure 1. Molecular structure of (a) ochratoxin A, (b) deoxynivalenol and (c) resveratrol

Deoxynivalenol (DON) (Fig. 1b), is one of the most common fungal contaminants of cereals worldwide. This mycotoxin is produced by different species of *Fusarium*, thus a wide range of cereal-based foods have been reported to be contaminated by this toxin

(JECFA, 2001). Acute effects of food poisoning by DON in humans are abdominal pains, dizziness, headache, throat irritation, nausea, vomiting, diarrhoea, and blood in the stool. It has also been shown that DON can act severely against the intestinal cells causing inflammation and increasing the permeability of the monolayers, resulting in a reduction of viability and immune function (Pinton et al., 2009; Van de Walle et al., 2010). DON also increases cytokine levels, a fact that is closely related with immune function reduction and increases caspase-3 levels, a well-known apoptosis mediator (Pestka et al., 2004; Pestka, 2008). Additionally, DON induced oxidative stress has been proposed as the mechanism of DNA damage in hepatic cells HepG2 (Pestka et al., 2004).

Red wine is one of the most important beverages in the Mediterranean diet, and it is considered to have a more protective effect than white wine due to its greater content in antioxidant substances released from the grape's skin and seeds, mainly polyphenols. Red wine contains a total of 1.8 g/L of polyphenols, whereas white wine contains only 0.2-0.3 g/L of polyphenols (Bertelli and Das, 2009). Resveratrol (RES; Fig. 1c), a polyphenolic product synthesized by a wide variety of plant fruits, including grapes, is naturally present in red wine. RES has gained considerable attention because of its potential as a chemopreventive and its anticancer properties, as well as for the evidence of decreasing heart disease and neural degeneration in animal studies (Vang et al., 2011). Among its various biological actions, RES was demonstrated to inhibit cellular survival signaling, and to interfere with apoptosis pathways, both by directly triggering apoptosis-promoting signaling cascades and by blocking antiapoptotic mechanisms (Fulda and Debatin, 2006).

Human dietary intake of food and its relationships with the digestive tract make up a very complex system in the framework of exposure to food contaminants. Most toxicity studies commonly evaluate the effect of individual mycotoxins on the target cell

biological functions. However human exposure to mycotoxins is well-known to be far off this simplified paradigm, involving sophisticated interactions with a lot of different matrices and different chemical species, including other mycotoxins, but also major nutrients, fibres and natural bioactive compounds. Co-contamination of different mycotoxins in food and feed has been frequently demonstrated, being a more common event than single contamination (Streit et al., 2013). Most of the published studies are reporting the toxicological properties of mycotoxins alone, and little is known concerning the effect of a specific mycotoxin mixed with other mycotoxins and/or with bioactive compounds, which may lead to unexpected subsequent effects. DON and OTA have shown to be among the most ubiquitous mycotoxins in foods of Mediterranean countries, and thus, continuously consumed in moderate levels through dietary intake. Moreover, the mycotoxin mixtures can interact with complex food matrices containing bioactive food compounds, as RES. Polyphenols such as RES are also widely present in the diet with the main dietary sources being fruits, wine, tea, coffee or chocolate (Burns et al., 2002; Fernández-Mar et al., 2012). These compounds are known for their beneficial effects on human health due to their antioxidants properties. Thus, the main goal of the present work was to assess the effect of DON and OTA on the viability of Caco-2 cells when these mycotoxins are combined with RES. Additionally, to try to understand the underlying mechanism of cytotoxicity, production of reactive oxygen species (ROS), as a first indicator of oxidative stress status, has been measured.

2. Materials and methods

2.1 Chemicals

DON (3α, 7α, 15-trihydroxy-12,13-epoxytrichotech-9-en-8-one, purity ≥ 98 %), OTA ((2S)-2-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-2-benzopyran-7-yl]formamido}-3-phenylpropanoic acid, purity ≥ 98%), RES (3,40,5-trihydroxystilbene, purity ≥ 99%), trypsine, dimethylsulfoxide (DMSO), L-glutamine (200 mM), penicillin-streptomycin (10,000 UI/mL-10,000 µg/mL), Minimum Essential Medium (MEM, ref 56416C, dry powder), MEM non-essential amino acids 100x (NEAA), Hepes (Bio-performance grade), chloramine-T and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (Sigma, Madrid, Spain). Dulbecco's Modified Eagle Medium (DMEM, ref. SH30022) was supplied by Thermo Scientific HyClone (Barcelona, Spain) and fetal bovine serum (FBS) by Biosera (Santa Coloma de Gramenet, Spain). CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) kit was purchased from Promega (Charbonnières, France). Solid phase extraction cartridge BondElut C18 was purchased from Varian (California, USA).

2.2 Cell culture and treatment

The Caco-2 cell line (ATCC HTB-37) derived from a human colorectal adenocarcinoma was purchased from LGC Standards, S.a.r.l. (Molsheim Cedex, France). Caco-2 cells were cultured at 37 ºC and 5 % CO_2 in DMEM medium supplemented with penicillin (100 UI/mL), streptomycin (100 µg/mL), 15 % fetal bovine serum, 2 mM L-glutamine, and 1% NEAA. DON, OTA and RES were dissolved in DMSO and stored at -20 °C before dilution in cell culture media. Solvent control cells received the maximal DMSO concentrations used in the treated cells (0.1%, v/v). Caco-2 cells were seeded at a concentration of 10^5 cells/well in 100 µL of culture medium in flat-bottomed 96-well plates. After culturing for 48 h, different concentrations of OTA, DON and RES or their

combinations (OTA-RES, DON-RES, OTA-DON) were added to the cells. Cell lines were exposed for 6, 24 and/or 48 h, depending on the assay.

2.3 Stability of resveratrol in the culture medium

Yang et al. (2010) reported the oxidation of resveratrol in culture medium due to bicarbonate ions. We checked the stability of resveratrol (160 µM) in two culture media, MEM (medium without bicarbonate) and DMEM (a medium that contains 3.7 g/L of bicarbonate). Hepes (25 mM) was added to MEM to maintain the pH in a range of 7.2-7.5.

Stability assays were carried out with 3 mL of culture medium (spiked at 160 µM RES). Samples were incubated at 37 °C for 12, 24 and 48 h in the same conditions described for cell culture. Three culture medium samples were incubated for each time. Samples were collected at the end of the incubation period, and RES was extracted immediately.

2.4 Solid-phase extraction of resveratrol and high performance liquid chromatography analysis (HPLC)

RES from culture medium samples was extracted by solid-phase extraction using a Bond Elut C18 cartridge and ethanol as eluting solvent. Samples were dried under a nitrogen stream and stored at 4 ºC until analysis.

RES was quantified by HPLC, using a Waters 2695 Separations Module coupled to a Waters 2475 Multi λ fluorescence detector (Waters, Milford, MA, USA). HPLC conditions were a modification of those described by Serra et al. (2009). Mobile phase

was: A) acetic acid 0.2% and B) acetonitrile, according to the following gradient: 0-10 min 95% A, 10-20 min 75% A, 20-25 min 95% A, with a flow-rate of 1 mL/min. A Waters Spherisorb ODS2 C18 column (3 μm, 4.6 x 250 mm) was used and column temperature was maintained at 30 ºC during analysis. Detection was performed at 280 nm, and retention time was 14.66 min. Quantification was achieved with a software integrator (Empower 2, Milford, MA, USA).

RES detection (LOD) and quantification (LOQ) limits were experimentally determined from the calibration curve of a set of seven standards (from 1 to 320 μM RES), which was linear in the range of 10-320 μM RES ($r^2 = 0.989$). The LOD and LOQ determined was 10 and 25 μM, respectively.

2.5 MTS assay

The effects on the mitochondrial activity of Caco-2 cells exposed to OTA and RES at concentrations ranging from 0.5 to 160 μM and DON (0.25-30 μM), and the combinations of OTA (20, 40 and 80 μM) or DON (0.5, 1 and 5 μM) with RES (1-100 μM), and OTA (20, 40 and 80 μM) with DON (0.5, 1 and 5 μM), after a 24 and 48 h exposure period, were studied using the MTS assay kit. The kit is composed of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) and phenazinemethosulfate (PMS), an electron coupling reagent. MTS is reduced by dehydrogenase enzymes in metabolically active cells into a formazan product that is soluble in tissue culture medium. After the 24 h or 48 h exposure period, the cells were washed with PBS, and 20 μL of a freshly prepared MTS/PMS solution in fresh medium was added to the wells. The cells were further incubated for 2-4 h. The amount of soluble formazan produced by cellular reduction of

the MTS was measured by the absorbance at 490 nm on an ELISA plate reader (Genios, TECAN, Männedorf, Switzerland). Results were expressed as percentage of the solvent control.

2.6 Reactive oxygen species (ROS) assay

To study the ROS production, cells were exposed for 6, 24 and 48 h to OTA and RES at concentrations ranging from 0.5 to 160 µM, and DON at 0.25-30 µM. For the combination effects study, concentrations of OTA (5 and 80 µM) or DON (1 and 30 µM) with RES (10, 40 and 160 µM), and OTA (5 and 80 µM) with DON (1, 10 and 30 µM) were tested. Intracellular ROS production was determined by using the dichlorofluorescein (DCF) assay (Wang and Joseph, 1999). DCFH-DA (100 mM) in phenol red and serum-free MEM medium was added after the incubation period and maintained during 30 min at 37 ºC in the dark. Chloramine-T, an effective inducer of oxidative stress, was used as a positive control, with cells being exposed to concentrations in the range of 0.3-10 mM. Fluorescence was measured at 485 nm excitation and 535 nm emission at 37 ºC, using a microplate reader. Fluorescence readings were taken every 15 min for 60 min, with the plates being incubated at 37 ºC with 5 % CO_2 between measurements. Oxidative stress was calculated as the percentage of increase in fluorescence (Ft) per well over a 60-min period by the formula [(Ft60-Ft0)/Ft0 x 100]. This result was expressed as percentage of the solvent control.

2.7 Statistical analysis

The sodium bicarbonate effect on stability of RES in culture medium was determined by the analysis of variance ANOVA and the Bonferroni post-test. Cytotoxicity data are represented as mean ± standard deviation (SD) of three to five independent experiments (in each experiment, each concentration was applied by triplicate in the culture plates). Statistical analysis was performed using Sigma Plot version 12.0 (Jandel Scientific, San Rafael, CA, USA). Significant differences among treatments were determined by one-way repeated measures analysis of variance (RMANOVA, $p < 0.05$). Previously, normality (Shapiro-Wilk test, $p < 0.05$) and equal variance ($p < 0.05$) of the distribution was tested. Means of treatments were contrasted with respect to the control group using Dunnett's test.

3. Results

3.1. Stability of resveratrol in culture media.

Table 1 reports the decrease of RES (160 µM) over time (12-48 h) on a culture medium with or without bicarbonate. Results showed that RES concentration decreased with time in both culture media used, regardless the presence of sodium bicarbonate in its composition. The decrease in RES level was moderate (10.5% loss) in samples incubated for 12 h in culture media with sodium bicarbonate ($p < 0.05$), and were increasing with time (19 to 41% after 24 and 48 h, respectively). Our results also show a statistical difference in RES levels due to the presence or absence of bicarbonate in the media .RES showed a moderate decrease after 24 h incubation in DMEM (19%), lower than in MEM (32.3%). However, at 48 h a minor decrease was observed in MEM (30.6%) than in DMEM (41.4%).

Table 1. Decrease of RES (160 µM) in cell culture medium with or without sodium bicarbonate

Incubation time (h)	**Resveratrol stability**			
	Medium with bicarbonate (DMEM)		**Medium without bicarbonate (MEM)**	
	RES (µM)	**(% RES loss)**	**RES (µM)**	**(% RES loss)**
12	143.18 ± 7.05*	10.51	n.d.	n.d.
24	129.70 ± 5.29$^{**, A}$	18.94	108.33 ± 9.22$^{**, B}$	32.29
48	93.84 ± 7.53$^{**, A}$	41.35	111.09 ± 3.15 $^{**, B}$	30.57

Data are represented as mean ± SD of three analyses. n.d.: not determined.
Stars indicate statistical differences regarding to RES control in DMEM medium (0 h) (*$p < 0.05$, **$p < 0.0005$).
Different capital letters next to DMEM/MEM treatments, at the same time, mean significant differences ($p < 0.005$) due to bicarbonate.

3.2 MTS assay

The effect of OTA, DON and RES treatment on cell viability was tested by the MTS assay over 24 h and 48 h. Similar responses were observed between both times of exposure and only results after 48 h exposure are presented in Figure 2. A dose-response curve was obtained after OTA and DON treatment. A significant reduction in cell viability was observed with OTA at 40 µM ($p < 0.01$) (Fig. 2a) and DON 1 µM ($p < 0.001$) (Fig. 2b). The non observed effect concentration (NOEC) for OTA and DON were 20 and 0.5 µM, respectively. However, Caco-2 cells did not show any response after treatment with RES in the range 0-160 µM (Fig. 2c).

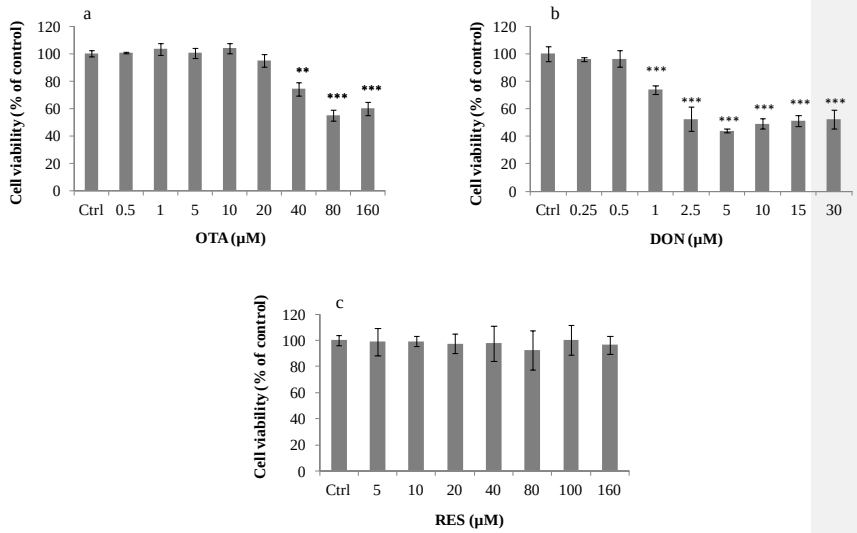

Figure 2. Effect of OTA (a), DON (b) and RES (c) on Caco-2 cell viability after 48 h exposure measured with the MTS assay. Results are expressed as percentage of viability compared with the solvent control (0.1% DMSO). Data are represented as mean ± SD of 3-5 independent experiments. Statistical differences with respect to the controls are indicated (**$p < 0.01$, ***$p < 0.001$).

The 48 h co-exposure of DON at non-toxic concentrations (0.5 µM) with increasing concentrations (1-100 µM) of RES induced an increase in cytotoxicity at a concentration of RES of 100 µM ($p < 0.001$) (Fig. 3a). However the cytotoxic effect at concentrations of DON of 1 and 5 µM was not significantly affected by the presence of RES. On the other hand, the cytotoxicity of OTA at 80 µM increased significantly ($p < 0.05$) when co-exposed with 100 µM of RES (Fig. 3b). An increase in toxicity could also be observed when cells were co-exposed to a non toxic concentration of OTA (20 µM) and non toxic

or low concentrations (0.5 µM and 1 µM) of DON (Fig. 3c).

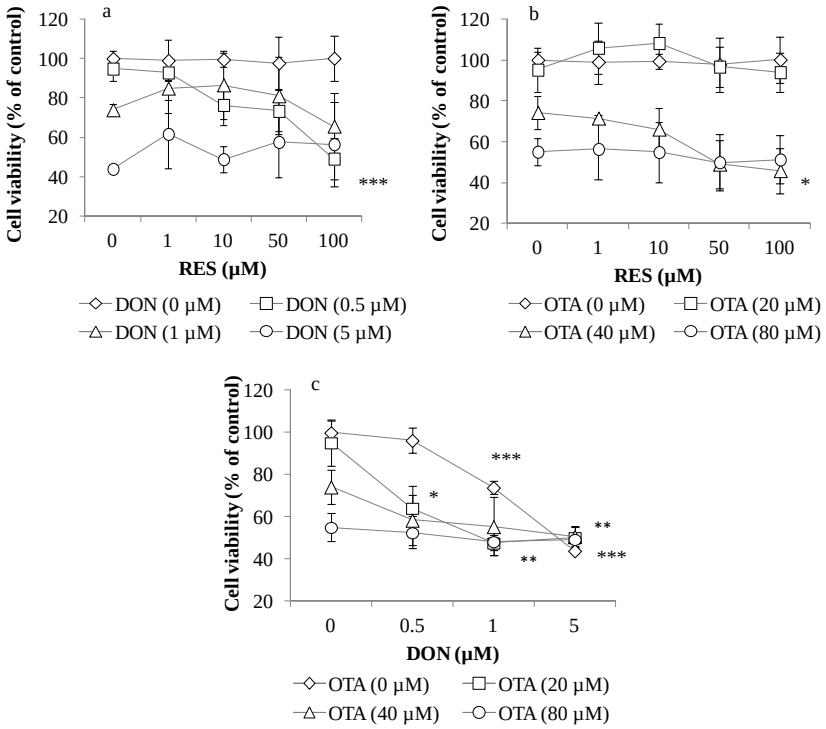

Figure 3. Combined effect of DON, OTA and RES on Caco-2 cell viability after 48 h of treatment. Results are expressed as percentage of viability compared with the solvent control (0.1% DMSO). Data are represented as mean ± SD of 3-5 independent experiments. Statistical differences with respect to the control of RES for each concentration level of DON (3a) or OTA (3b) and with respect to the control of DON for each concentration level of OTA (3c) are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.3 Reactive oxygen species (ROS) production

ROS produced by OTA, DON and RES was measured by comparison of fluorescence emitted by treated cells with the control using a dichlorofluorescein (DCF) assay (Fig. 4). Significant dose-dependent production of ROS was obtained when the cells

were treated with OTA and RES for 24 h (data not shown) and 48 h (Fig. 4a and 4b), but not when treated for only 6 h (data not shown). The best response was obtained after 48 h. OTA and RES induced production of ROS at 80 and 20 µM ($p < 0.01$), respectively. However, there was not an increase in ROS production when Caco-2 cells were treated with DON during 6 and 24 h (data not shown) or 48 h (Fig. 4c).

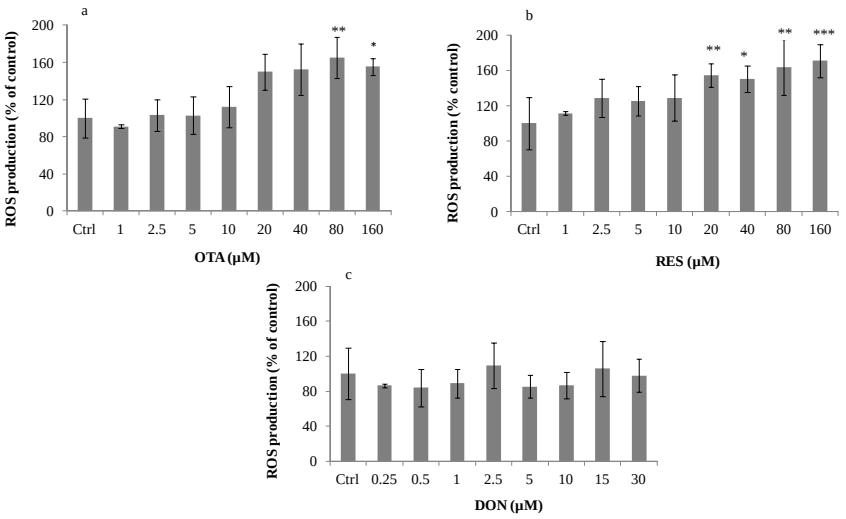

Figure 4. Individual effect of DON, OTA and RES on ROS production by Caco-2 cells after 48 h of treatment. Results are expressed as percentage of ROS production compared with the solvent control (0.1% DMSO). Data are represented as mean ± SD of 3-5 independent experiments. Statistical differences with respect to the controls are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Binary mixtures of DON and OTA with different concentrations of RES (10, 40 and 160 µM) were tested (Fig. 5). To study the effects of DON on the ROS production, a low and a high cytotoxic concentration of DON (1 µM and 30 µM) were selected for these co-exposure studies. RES produced a notable increase of ROS levels when it was treated individually, however this ROS production decreased completely when co-

exposed with DON at both concentration levels (Fig. 5a). To study the effects of the co-exposure of OTA with RES a non ROS-producing and non-cytotoxic concentration (5 µM), and a high cytotoxic and high ROS-producing concentration (80 µM) of OTA were selected. Combination of OTA at both doses with RES blocked the production of ROS by RES (Fig. 5b). Mixtures of OTA-DON didn't increase the ROS production observed for the highest concentration of OTA (80 µM) (Fig. 5c).

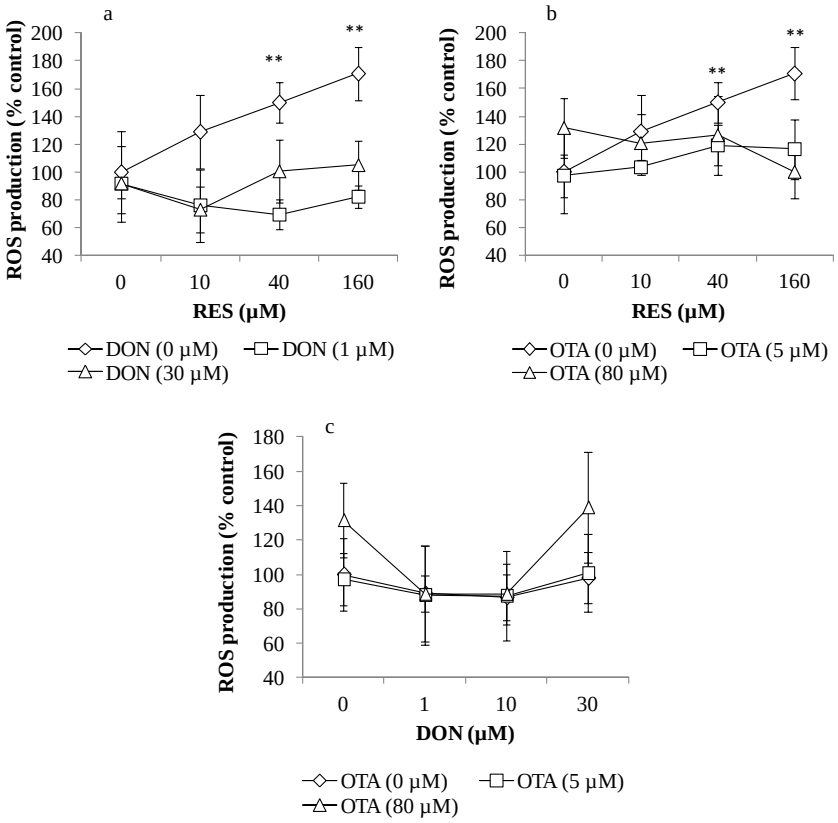

Figure 5. Combined effect of DON, OTA and RES on ROS production by Caco-2 cells after 48 h of treatment. Results are expressed as percentage of ROS production compared with the solvent control (0.1% DMSO). Data are represented as mean ± SD of 3-5 independent experiments. Statistical differences with respect to the control of RES for

each concentration level of DON (5a) or OTA (5b) and with respect to the control of DON for each concentration level of OTA (5c) are indicated (**$p < 0.01$).

4. Discussion

In this study we have used the human cell line Caco-2 to study the possible toxic effects of the presence in foods of two different mycotoxins when they are present alone or together as well as when the antioxidant RES is also present in food. Caco-2 cell model is an accepted intestinal model widely implemented to assess the effect of chemical compounds on the intestinal function. Hence, undifferentiated and proliferating Caco-2 cells share some characteristics with crypt enterocytes. Studies focusing to assess the trans-cellular transport, barrier function and permeability properties are performed with differentiated cells, which exhibit structural and molecular characteristics similar to villous enterocytes. Cytotoxicity, viability and proliferation assays may be carried out also with un-differentiated (proliferating) Caco-2 phenotypes, which share some characteristics with crypt enterocytes (Manda et al. 2015; Sambruy et al 2001). Bony et al (2006) reported that proliferating cells were found to be more sensitive to DON than differentiated cells, therefore this model can be considered as the most conservative approach for hazard characterization.

In the present work we have shown that the degradation of RES in DMEM, a medium with sodium bicarbonate, is lower than 20 % after 24 h of incubation and around 40% after 48 h. These results do not agree with those found by Yang et al. (2010) that reported a 96 % of RES degradation after 24 h of incubation at 37 ºC when it was added at 200 µM to the Base Modified Eagle (BME) medium, a medium with bicarbonate (2.2 g/L) in its composition. Our results also show that, independently of the presence or

absence of bicarbonate, the decrease in RES levels after 24 h (18.94% and 32.29%) and 48 h (41.35% and 30.57%) of incubation is relatively similar.

Regarding ROS production, Yang et al. (2010) reported a production of high levels (90 µM) of H_2O_2 after incubating RES for 24 h in a medium with bicarbonate. Nakamura et al. (2003) demonstrated a significant toxicity in HeLa cells after a 15 min exposure to H_2O_2 at concentrations as low as 0.06 mM (2 µg/mL). However, our results show that after 24 h and 48 h of exposure, RES is capable to increase ROS levels but not after 6 h, and no induction of cytotoxicity was observed with RES. This suggests that the enhanced production of ROS by RES in our study is mainly due to RES itself.

Both assayed mycotoxins are cytotoxic for the Caco-2 cell line. The NOEC and the lowest observed effect concentration (LOEC) were 0.5 and 1 µM for DON and 20 and 40 µM for OTA, respectively, indicating a highly toxic effect, especially for DON. Contrary to our results, Calvert et al. (2005) didn't find cytotoxicity according to the MTT assay for the Caco-2 cell line after 48 h exposure to 0.34-1.7 µM of DON, but HeLa and Hep-2 cells were very sensitive to DON $\geq$ 0.34 µM. Also in the mammalian kidney epithelial (Vero) cells the cytotoxicity of DON, evaluated by the Neutral Red (NR) and MTT assays over 24, 48 and 72 h, showed IC_{50} values ranging from 3.30 to 10 µM (Ruiz et al., 2011). Berger et al. (2003) reported the toxic effect of OTA on Caco-2 cells with the MTT assay. They determined a low IC_{50} (0.4 µM) after a 48 h exposure period. However, similar results are reported in the HepG2 cell line. The IC_{50} of OTA, as measured by the NR assay and the MTT assay, was 35 µM after 24 and 48 h exposure (Hundhausen et al., 2005; Zheng et al., 2013). As expected, RES didn't show cytotoxic effects up to concentrations of 160 µM. However, Sergent et al. (2005) found a slight cytotoxic effect of RES on Caco-2 cells after 48 h of treatment at 100 µM.

To investigate the capacity of these compounds and their combinations to induce oxidative stress, production of ROS was also measured. Studies carried out with individual compounds showed that only OTA and RES increased ROS levels with NOECs and LOECs of 40 and 80 µM for OTA and 10 and 20 µM for RES, respectively. The implication of oxidative stress in the cytotoxicity induced by OTA has been previously suggested (Schaaf et al., 2002; Petrik et al., 2003; Kamp et al., 2005; Mally et al., 2005; Hsuuw et al., 2013). In our study, the production of ROS is associated with a parallel reduction in viability, suggesting the same conclusion of other authors. Interestingly, although RES is known for its anti-oxidative properties, it was able to produce high levels of ROS at concentrations as low as 20 µM in the Caco-2 cell line. Juan et al. (2008) have also reported the generation of mitochondrial ROS in the human colon cancer cell line (HT-29) exposed to RES (150 µM) for 4 h. Despite this ROS production, the basal redox status of the cells was not disturbed and no cytotoxicity was recorded with this compound in the cell line studied. From our results, the cytotoxicity induced by DON might not be explained by an oxidative stress mechanism. Previous results points to an apoptotic mechanism (Petska et al., 2004; Pestka, 2008). Recently, Ma et al. (2012) demonstrated the capacity of DON to induce apoptosis in the HT-29 cell line at very low concentrations ranging from 0.8 to 3.4 µM. They showed that the mechanism of apoptosis was caused by mitochondrial dysfunction and subsequent release of cytochrome c into the cytoplasm and successive activation of caspase-9 and caspase-3.

There have been few works published to date attempting to contribute on the knowledge of mycotoxins interactions with bioactive compounds such as dietary polyphenols. The most common approach followed involves pre-treatments with the bioactive compound and a subsequent treatment with the mycotoxin (Hundhausen et al.,

2005; Bosch-Saadatmandi et al., 2006; Costa et al., 2007). In real exposure scenarios, interactions between the bioactive compounds and mycotoxins will occur simultaneously, which suggest that pre-treatment may not be the best approach.

In this study using a co-exposure approach, we didn't found a protective effect when OTA was co-exposed with RES. In fact, co-exposure of OTA at concentrations of 40 µM (moderately toxic dose) with the highest concentration of RES (100 µM) resulted in an increase in cytotoxicity. Sergent et al. (2005) showed that the Caco-2 absorption of OTA, at concentrations that should be easily encountered in the gut, is increased in the presence of RES. This would imply a greater bioavailability of the mycotoxin which could increase the toxicity of this compound. It could be that this also happens in the Caco-2 cell line, although this possibility has not been checked in this study. Co-exposure of DON with RES did neither result in a protective effect of the polyphenols to the toxic effects of this mycotoxin. Moreover, in the case of DON, the presence of RES at high doses (100 µM) increases the toxicity of a non-cytotoxic dose of DON (0.5 µM). In contrast, Kolesarova et al. (2012) showed in porcine ovarian granulosa cells that RES protected cells from the reproductive toxicity induced by DON. Co-exposure of OTA and DON with RES resulted in the complete abrogation of ROS levels induced by RES. This finding is difficult to explain especially in the case of the co-exposure of RES with the non-toxic and non-ROS producing dose of OTA (5 µM). The decrease in ROS production of RES observed when this compound is co-exposed with toxic doses of DON (1 µM and 30 µM) or OTA (80 µM) could be the result of loss in cell viability. The mechanism triggering the cytotoxic effect of DON and OTA enhanced by RES at a non- toxic and a sub-toxic concentration respectively cannot be completely elucidated with the present data. Several studies have reported that RES modulates multiple vias that elicite to apoptosis and cell damage. Fouad et al. (2013) and Pasciu et al. (2010) have related a

response mediated by the caspase-3 and the cells damage observed. Zhai et al. (2015) reported that RES inhibits proliferation and induces apoptosis of fibroblasts through the mechanism involving transforming growth factor and Smad proteins (TGF-b1/Smads) signaling pathway. On the one hand, TGF-b1 is a multifunctional cytokine that controls a diverse set of cellular processes (cell proliferation, differentiation, and apoptosis). On the other hand, Smad proteins are downstream messengers of TGF-b signaling pathways, conveying the TGF-b-mediated extracellular signals to the nucleus (Zhai et al., 2015).

When both mycotoxins were co-exposed at non-toxic concentrations of DON and OTA (0.5 and 20 µM, respectively) a significant increase in cytotoxicity was observed. Also the toxic response induced by DON (1 µM) increased significantly with increasing doses of OTA (40 and 80 µM), indicating that cocktails of these mycotoxins can suppose a risk for consumers. This increase in cytotoxicity could not be explained by an increase in ROS production. To our knowledge this is the first study where the *in vitro* toxicity of the co-exposure of DON and OTA has been studied. Other authors have reported different interactions between binary and tertiary combinations of DON or OTA with other mycotoxins (T-2 toxin, zearalenone, beauvericin, fumonisin B1, nivalenol, fusarenon-X) resulting in antagonism, synergism or additive effects depending on the combinations, the cell lines, the concentrations and the assays used (Ruiz et al., 2011; Alassane-Kpembi et al., 2013).

Our results elucidated the complex behavior of mixtures of chemicals and bioactive compounds and the need of further research to better understand the biological responses and potential effects on the human health. Although the extrapolation of *in vitro* models to humans is very complex and requires an accurate approach, the concentration doses used in the present study are expected in the range of those levels expected from a

common European diet. For example, the mean and highest (percentile 95) exposure levels of DON in Spain were estimated to be 0.37 and 1.9 mg/kg bw/day (Cano-Sancho et al., 2011). Thus, a mean intestinal dose of 0.5 μM can be expected in a relevant group of population. RES can be detected in Spanish wine at mean concentrations of 4.6 mg/L (Martelo-Vidal and Vázquez, 2014), leading to estimate an intestinal dose of 20 μM. While the risk assessment frameworks are developed on the basis of animal or human dose-response studies with isolated mycotoxins, potential interactions with other mycotoxins and bioactive compounds are missed. We focused the study to assess the simultaneous exposure of DON and OTA, the most concerning mycotoxins in Spain (Cano-Sancho et al., 2011: Coronel et al. 2012) with RES, a polyphenol widely consumed on the Spanish households. Despite the risk characterization did not reveal a concern for the human health, in the present study we elucidated toxicological issues commonly misplaced on risk assessment, such as the potential interaction of bioactive compounds at sub-toxic doses of mycotoxins. Future studies should confirm these results on animal models and assess the interactions with other bioactive compounds, as well as to translate the effect of these interactions on the risk characterization frameworks.

5. Conclusion

The results from our study showed that the co-exposure of both mycotoxins increased significantly the cytotoxicity of these compounds in Caco-2 cells without increasing the ROS production. Opposite to our expectations, the co-exposure with RES didn't result in a protection for the cell line from the mycotoxins, resulting in an increase in cytotoxicity at sub-toxic doses of mycotoxins, unrelated with an increase in ROS production. Further studies should be conducted to elucidate the mechanisms underlying the toxic effects

observed in the co-exposures and confirm these findings in animals. These results also elucidated a need to better understand the interactions with other bioactive compounds present in the diet, and evaluate the impact on risk assessment.

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