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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. The co-exposure of OTA or DON with RES did not result in a decrease in cytotoxicity; on the contrary, it resulted in increased cytotoxicity not associated with an increase in 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).

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 and also in tea, coffee or

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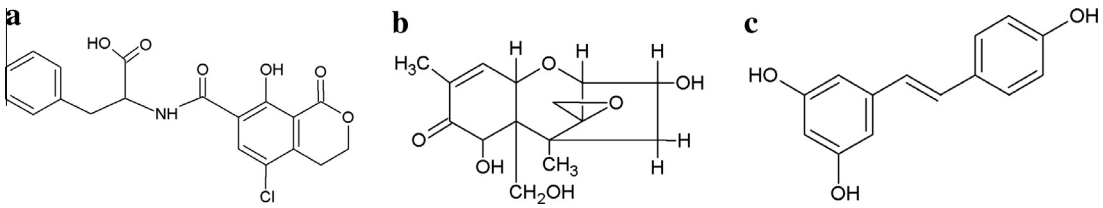

Fig. 1. Molecular structure of (a) ochratoxin A, (b) deoxynivalenol and (c) resveratrol.

chocolate (Burns et al., 2002; Fernández-Mar et al., 2012). 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, fibers 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). However little is known concerning the effect of a specific mycotoxin mixed with other mycotoxins and/or with bioactive compounds as RES, 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. 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-phenyl propanoic 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 °C 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 °C 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 × 250 mm) was used and column temperature was maintained at 30 °C 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.