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Ochratoxin A activates neutrophils and kills these cells through necrosis, an effect eliminated through its conversion into ochratoxin α

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by several species of fungi from the *Aspergillus* and *Penicillium* genera that frequently grow in improperly stored food products. OTA has carcinogenic, teratogenic and nephrotoxic potential and sustains a high half-life in human blood. Despite the recently efforts to decontaminate OTA through its conversion into its metabolite ochratoxin alpha (OTα), there are just a few reports in literature comparing the toxic effects of these toxins. Thus, herein we studied and compared the proinflammatory and toxicological effects of OTA and its metabolite OTα in human neutrophils *in vitro*. The effect of OTA and OTα on human neutrophils viability was evaluated by trypan blue, annexin-V and propidium iodide methods as well as by the analysis of cytomorphological alterations. The ATP levels were also evaluated using the luciferin-luciferase bioluminescence assay. The alteration on mitochondrial potential was assessed by a mitoscreen flow cytometry mitochondrial membrane potential detection kit and the intracellular calcium levels through the probe FLUO-4/AM. To study the human neutrophils' oxidative burst, the fluorescent probe dichlorodihydrofluorescein diacetate was used. OTA induced an increase on the intracellular calcium, human neutrophils' oxidative burst followed by depletion of ATP levels and alterations on mitochondrial potential leading to cell death by necrosis, while OTα did not induce significant toxic effects. Our results strongly suggest that the toxicity in human neutrophils induced by OTA started with the release of calcium from internal stores triggering several neutrophils' activities that culminate in cell death by necrosis.
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1. Introduction

Ochratoxins are mycotoxins produced by several species of *Aspergillus* and *Penicillium* genera. Ochratoxin A (OTA) (Fig. 1), a phenylalanyl derivative of a substituted isocoumarin (*R*)-*N*-[5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-*oxo*-1H-2-benzo-pyran-7-y1)-carbonyl]-L phenylalanine), is one of the most common mycotoxin (Vanderme et al., 1965).

OTA is commonly found in cereals and their derivates (Zinedine et al., 2010), grapes (Mitchell et al., 2004), coffee (Vecchio et al., 2012), cocoa (Copetti et al., 2013), beer (Reinsch et al., 2007), meat (Dall'Asta et al., 2010) and spices (Ozbey and Kabak, 2012). The human exposure to OTA can occur by two routes: direct exposure, due to the consumption of moldy plant products, or indirect exposure through the consumption of contaminated animal products (Duarte et al., 2009). OTA is a stable compound that is not destroyed by common food preparation procedures and is rather persistent in the human organism (Reddy and Bhoola 2010; Vidal et al., 2014).

Toxic effects of OTA have already been described, namely the inhibition of protein synthesis, oxidative damage, enhancement of lipid peroxidation, binding to proteins and DNA, disturbance of calcium homeostasis, impairment of mitochondrial oxidation and induction of apoptosis in several cell types (Bouaziz et al., 2008; Ringot et al., 2006). Human epidemiological data are scarce and the food safety data of OTA is essentially limited to animal studies, where the renal toxicity and carcinogenicity are considered the pivotal effects (Marin-Kuan et al., 2008).

Considering the harmful effects caused by the ingestion of this toxin, maximum tolerable levels were established for different

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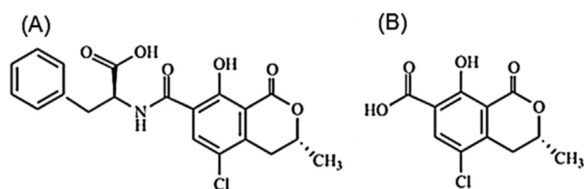

Fig. 1. Chemical structure of OTA (A) and its principal metabolite OTα (B).

food products, varying from 0.5 to 10 μg/kg and the reduction of this mycotoxin in food and feed to levels as low as technologically possible is highly recommended (Abrunhosa et al., 2011; Ridgway et al., 2007). There are several reports in the literature concerning the biotransformation of OTA into several metabolites (Wu et al., 2011; Xiao et al., 1996). Among them, ochratoxin alpha (OTα) is the main metabolite obtained through the biodegradation of OTA in the gastro-intestinal tract by microorganisms, through hydrolysis by carboxipetidase A, which consists in a specific, environmentally friendly and a very promising strategy for the control of OTA levels in foods and feeds (Abrunhosa et al., 2011). Despite the recent efforts to decontaminate OTA (Fig. 1A) into its metabolite OTα (Fig. 1B), there are just a few reports in literature comparing the toxicity of these toxins. Xiao et al. (1996) evaluated the toxicity of OTA and its metabolite in 6-week-old mice and observed a death rate ranging between 30 and 90% after 72 h exposure to OTA, while no death was observed for OTα. The lower toxic effect of OTα was also observed by Muller et al. (2003) in THP-1 monocyte cell line.

OTA is frequently found in human blood, due to its unfavorable kinetics of renal elimination (Sorrenti et al., 2013), highlighting the importance of studying the toxicological effect of OTA and its metabolite in human blood cells. Human neutrophils are an interesting *in vitro* model to study the OTA and OTα behavior, since they are the most common type of white blood cells and represent an important first-line defense against invading pathogens. In this work we aimed to evaluate and compare the proinflammatory and toxicological effects of OTA and OTα in human neutrophils *in vitro*.

2. Material and methods

2.1. Reagents

The following reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, USA): RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin, streptomycin, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), diphenyleneiodonium chloride (DPI), Dulbecco's phosphate buffer saline, without calcium chloride and magnesium chloride (PBS), 4-methyl-4′-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-carboxanilide (BTP2), 2-Aminoethyl diphenylborinate (2-APB), trypan blue solution, luciferin, luciferase. Annexin-V-FLUOS Staining Kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany) and BD™ MitoScreen Flow Cytometry Mitochondrial Membrane Potential Detection Kit were purchased from BD Biosciences (San Diego, USA). *DC* Protein Assay was purchased from Bio-Rad (California, USA). FLUO-4/AM was purchased from Life Technologies (California, USA). Vacuum tubes with K_3EDTA were purchased from Vacutainer Systems (U.K.). The compounds employed on this study, OTA and OTα, were purchased from Sigma Aldrich Co. LLC (St. Louis, USA) and KareBay™ Biochem, Inc. (New Jersey, USA), respectively.

2.2. Methods

2.2.1. Isolation of human neutrophils

In accordance with Helsinki Declaration, the design and execution of the experiment were thoroughly explained to the participants, and informed consent was obtained. Venous blood was collected by antecubital venipuncture, from each human healthy volunteer, into vacuum tubes with K_3EDTA. The isolation of human neutrophils was performed by the density gradient centrifugation method as previously reported (Freitas et al., 2008). Cells were resuspended in RPMI 1640 incubation medium [(pH 7.4) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin]. Incubations with OTA and OTα were always performed in 5% CO_2 humid atmosphere at 37 °C.

2.2.2. Cell viability

Isolated neutrophils (4×10^6 cells/mL) were incubated with OTA and OTα (0–300 μM) during 8–24 h. Neutrophils suspension was added to an equal volume of trypan blue solution 0.4% in a microtube and gently mixed. After 2 min on ice, neutrophil number and viability (viable cells excluding trypan blue) were counted.

2.2.3. Assessment of neutrophils' apoptosis and necrosis

Annexin-V and propidium iodide (PI) binding assay. Isolated neutrophils (4×10^6 cells/mL) were incubated with OTA and OTα (0–300 μM) during 4–20 h. After incubation with OTA and OTα, the apoptotic and necrotic neutrophils were analysed by flow cytometry after simultaneous staining with annexin-V labelled with fluorescein and PI, according to Freitas et al. (2014). Cells were analysed by flow cytometry, using the commercial Annexin-V-FLUOS Staining Kit, according to the manufacturer's instructions. The fluorescence signals were collected using a BD Accuri™ C6 flow cytometer. A polygon gate was set according to the neutrophils' light scattering properties (in a forward vs. side scatter plot) excluding cell debris and other blood cells, restricting the analysis to neutrophils only. At least 10,000 cells were collected and their fluorescence signal detected, in logarithmic mode, and the data were analysed using the BD Accuri™ C6 software. The green fluorescence due to Annexin-V was followed in channel 1 (FL1) and plotted as a histogram of FL1 staining. Fluorescence due to the PI incorporation was followed in channel 3 (FL3).

2.2.4. Cytomorphological alterations

Isolated neutrophils (4×10^6 cells/mL) were incubated with OTA and OTα (0–300 μM) during 2–20 h. Subsequently, samples were cytocentrifuged (28g, 5 min), stained with Hemacolor®, and counted under light microscopy (100×) to determine the proportion of cells with cytomorphological alterations (Freitas et al., 2014).

2.2.5. ATP measurement

Isolated neutrophils (4×10^6 cells/mL) were incubated with OTA and OTα (0–300 μM) during 4–20 h. Subsequently, cell samples were acidified to a final concentration of 5% $HClO_4$ and centrifuged at 1600g, 4 °C, for 1 min. ATP levels were quantified after the reaction of 100 μL of neutralized supernatant with luciferin/luciferase solution. ATP standards (0–10 μM) were prepared in 5% $HClO_4$ (Costa et al., 2007). The pellet was resuspended in 0.4 M NaOH and protein quantification was performed in a microplate reader using the commercial *DC* Protein Assay according to the manufacturer's instructions.

2.2.6. Assessment of neutrophils' mitochondrial membrane potential (Ψm)

Isolated neutrophils (4×10^6 cells/mL) were incubated with OTA and OTα (0–300 μM) during 2–20 h. After this period, alterations of the mitochondrial potential were analysed by flow cytometry, using the commercial BD™ MitoScreen Flow Cytometry Mitochondrial Membrane Potential Detection Kit, according to

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