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In vitro genotoxicity of mycotoxins ochratoxin A and fumonisin B_1 could be prevented by sodium copper chlorophyllin – Implication to their genotoxic mechanism

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ABSTRACT

The aim of this study was to investigate the possible protective effect of sodium copper chlorophyllin (CHL) against cytotoxicity and DNA damage induced by mycotoxins ochratoxin A (OTA) and fumonisin B_1 (FB_1). CHL (0.1–100 μg/ml) alone had no impact on cell viability and genome damage in the primary human peripheral blood lymphocytes (HPBLs) and exhibited free radical scavenging activity in the DPPH assay. Both mycotoxins, OTA (4 μmol/l) and FB_1 (20 μg/ml), induced DNA damage in HPBLs already after 1 h exposure. When the HPBLs were co-exposed to CHL (10 and 100 μg/ml) and OTA (4 μmol/l) or FB_1 (20 μg/ml) for 1 h, CHL protected against cell and DNA damage induced by both mycotoxins, implying that OTA and FB_1 cytogenotoxicity mechanisms function at least partially through oxidative stress. Therefore, CHL could be a perfect candidate for possible use as an antioxidant.

1. Introduction

Sodium copper chlorophyllin (CHL) is a semi-synthetic mixture of water-soluble sodium copper salts derived from chlorophyll, widely used as food dye (Chernomorsky, Rancourt, Virdi, Segelman, & Poretz, 1997; Tumolo & Lanfer-Marquez, 2012). Its anti-mutagenic and anti-carcinogenic activities against numerous dietary and environmental agents, including the mycotoxin aflatoxin B_1, heterocyclic amines, and polycyclic aromatic hydrocarbons have been recorded (reviewed in Tumolo & Lanfer-Marquez, 2012). Mechanistic studies have revealed that the anti-mutagenic/anti-carcinogenic properties of CHL may be attributed to its ability to bind carcinogens by forming tight complexes, thus diminishing its bioavailability (Egner, Muñoz, & Kensler, 2003; Tumolo & Lanfer-Marquez, 2012). However, according to some authors, the anti-carcinogenic properties of CHL may be due to its antioxidant properties (Ferruzzi, Böhm, Courtney, & Schwartz, 2002; Tumolo & Lanfer-Marquez, 2012). For example, CHL was found to inhibit the formation of DNA strand breaks caused by reactive oxygen species (ROS) derived from the H_2O_2/Cu(II) system (Park, Park, Jung, & Chung, 2003). Moreover, CHL was able to reduce the formation of the major oxidative DNA damage product 8-hydroxydeoxyguanosine (8-OH-dG) induced by hydrogen peroxide (H_2O_2) and UV or by cyclophosphamide or benzo[a]pyrene (Ibrahim, Elbehairy, Ghoneim, & Amer, 2007; Park et al., 2003). It has also been reported that CHL effectively suppresses chemically-, radiation-, and photosensitization-induced lipid peroxidation (LPO) (Boloor, Kamat, & Devasagayam, 2000; Ibrahim et al., 2007; Kamat, Boloor, & Devasagayam, 2000). It is important to emphasise that the antioxidant activity of CHL was found to be much higher than that of natural chlorophylls, including chlorophylls *a* and *b*, pheophytins and pheophorbids (Ferruzzi et al., 2002; Lanfer-Marquez, Barros, & Sinnecker, 2005).

Ochratoxin A (OTA) and fumonisin B_1 (FB_1) are mycotoxins found as feed and food contaminants world-wide (IPCS, 2001). The nephrotoxicity, hepatotoxicity, genotoxicity and carcinogenicity of OTA and FB_1 have already been established in experimental animals (Domijan & Peraica, 2010). Human exposure to OTA is connected to the development of Balkan endemic nephropathy and urinary bladder tumours (Miletić-Medved, Domijan, & Peraica, 2005), while human exposure to FB_1 is associated with a higher incidence of oesophageal cancer, primary liver tumours and neural tube defects (Voss, Smith, & Haschek, 2007). Due to a lack of epidemiological data, it is not possible to establish a direct connection between exposure to OTA or FB_1 and the development of human

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diseases. Therefore, the International Agency for Research on Cancer (IARC) classified both OTA and FB_1 as Group 2B (possible human carcinogen) (IARC, 1993, 2002).

Although experimental data confirm that both mycotoxins OTA and FB_1 are genotoxic, the mechanism of their genotoxicity and carcinogenicity is still a matter of debate. Based on findings of OTA-related DNA adducts in cell cultures as well as in animal and human kidneys, some authors support the notion of direct OTA genotoxic action after metabolic activation (Pfohl-Leskowicz & Manderville, 2007). However, some studies indicate that OTA is not able to form reactive intermediates capable of interacting with DNA (Mally et al., 2005), and it has been suggested that OTA can induce DNA damage through oxidative stress (Arbillaga, Azqueta, Ezpeleta, & Lopez de Cerain, 2007; Domijan, Želježić, Kopjar, & Peraica, 2006; Kamp, Eisenbrand, Schlatter, Würth, & Janzowski, 2005; Liu et al., 2012). Similarly, some authors have proposed that oxidative stress may be the underlying mechanism of FB_1 genotoxicity (Domijan et al., 2006; Mary, Theumer, Arias, & Rubinstein, 2012; Mobio et al., 2000, 2003), while others have failed to find an increase in ROS production together with DNA damage after FB_1 exposure, suggesting oxidative stress is not involved in FB_1-induced DNA damage (Galvano et al., 2002).

Since the mechanism of OTA and FB_1 genotoxicity as well as cytotoxicity is still controversial, the present study aimed to investigate if CHL, as an antioxidant, has the potential to prevent cytogenotoxicity induced by these mycotoxins and thus clarify the role of oxidative stress in cell death and DNA damage induced by these naturally occurring toxins. First, we evaluated the toxicological profile of CHL by measuring the cytotoxic and genotoxic potential of CHL in primary human peripheral blood lymphocytes (HPBLs). DNA damage was measured with the alkaline comet assay, while further CHL genotoxic activity was assessed with the cytokinesis-block micronucleus (CBMN) assay. Also, CHL free radical scavenging capacity/antioxidant activity was determined with the DPPH assay. Subsequently, the genotoxic potential of OTA and FB_1 in HPBLs was established. The possible protective role of CHL against OTA and FB_1 cytotoxicity and genotoxicity was followed by co-exposing HPBLs to CHL and OTA or FB_1. In these experiments, H_2O_2 was used as positive control.

2. Materials and methods

2.1. Chemicals

CHL, acridine orange (AO), cytochalasin-B, disodium EDTA, ethidium bromide (EtBr), histopaque, low melting point (LMP) and normal melting point (NMP) agaroses, RPMI 1640 medium, Triton X-100, L-ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and butylated hydroxytoluene (BHT) were purchased from Sigma (St Louis, MO, USA). Giemsa solution was acquired from Merck (Darmstadt, Germany) and Chromosome kit P from Euroclone (Milano, Italy). All other chemicals used were laboratory-grade and were purchased from Kemika (Zagreb, Croatia).

2.2. Blood sampling and treatment

Whole blood samples were taken from a healthy female non-smoking donor (age 32). The donor had not been exposed to ionizing radiation for diagnostic or therapeutic purposes, or vaccinated and treated with drugs that might have interfered with the results of testing for a year before blood sampling. The subject gave informed consent to participate in this study. The study was part of the project approved by the institutional ethics committee and observed the ethical principles of the Declaration of Helsinki. Whole venous blood was collected under sterile conditions in heparinised vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing lithium heparin as anticoagulant.

The comet assay and CBMN assay were conducted on whole blood, while cytotoxicity was performed on isolated HPBLs. CHL stock solution (1 mg/ml), and CHL dilutions used for treatments (in concentrations 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml) were prepared in sterile redistilled water. Three OTA concentrations (1, 2 and 4 μmol/l; dissolved in ethanol) and three concentrations of FB_1 (5, 10 and 20 μg/ml; dissolved in sterile redistilled water) were tested. Also, for each experiment and each exposure time, control samples (solvent control) were included. After treatments, individual experiments were conducted according to the standard protocols listed below.

2.3. Cell viability (cytotoxicity) test

Cell viability was determined by differential staining with AO and EtBr using a fluorescence microscope (Duke & Cohen, 1992). HPBLs were isolated by histopaque density centrifugation method (Singh, 2000). The slides were prepared using 200 μl of HPBLs and 2 μl of stain (AO (100 μg/ml) and EtBr (100 μg/ml, 1:1; v/v), both diluted in phosphate-buffered saline, PBS). A total of 100 cells per repetition were examined with an Olympus BX-51 microscope (Tokyo, Japan) at 400× magnification. The cells were classified as follows: live cells with functional membrane with uniform green staining of the nucleus, and necrotic cells with uniform red staining of the nucleus.

2.4. Alkaline comet (SCGE) assay

The alkaline comet assay was carried out as described by Singh, McCoy, Tice, and Schneider (1988) with minor modifications (Gajski, Garaj-Vrhovac, & Oreščanin, 2008). Briefly, after the exposure, 5 μl of whole blood was mixed with 100 μl of 0.5% LMP agarose and added to fully frosted slides pre-coated with 0.6% NMP agarose. Once the NMP agarose was solid, the slides were covered with 0.5% LMP agarose, and cells lysed (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide; pH 10) over night at 4 °C. After the lysis, the slides were placed in alkaline solution (300 mmol/l NaOH, 1 mmol/l Na_2EDTA; pH 13) for 20 min at 4 °C to allow DNA unwinding and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in 0.4 mol/l Tris buffer (pH 7.5) for 5 min 3 times, stained with EtBr (20 μg/ml) and analysed at 250× magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK). The level of DNA damage was expressed as percentage of DNA in the tail, and a total of 100 randomly captured comets were examined from each slide. The results are shown as box plots.

2.5. Cytokinesis-block micronucleus (CBMN) assay

The CBMN assay was performed according to the guidelines of Fenech and Morley (1985) with minor modifications (Gajski et al., 2008). After the exposure (4 and 24 h) to CHL the whole blood (500 μl) was incubated in a Euroclone medium (Chromosome kit P) at 37 °C in an atmosphere of 5% CO_2. Cytochalasin-B was added at a final concentration of 3 μg/ml 44 h after the culture was started. The cultures were harvested after 72 h. The lymphocytes were fixed in methanol–acetic acid (3:1), air-dried and stained with 5% Giemsa solution. All slides were randomised and coded prior to analysis. The binuclear lymphocytes were analysed under a light microscope (Olympus CX41, Tokyo, Japan) at 400×