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Deoxynivalenol induced oxidative stress and genotoxicity in human peripheral blood lymphocytes

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ABSTRACT

Deoxynivalenol (DON) is one of the most common mycotoxins. The aim of this study consists in using diverse cellular and molecular assays to evaluate cytotoxicity, genotoxicity as well as oxidative damage and to investigate their mechanisms in human peripheral blood lymphocytes. The human lymphocytes were cultured in eight different doses of DON (0, 6.25, 12.5, 25, 50, 100, 250 and 500 ng/mL) during 6, 12 and 24 h. DON was able to decrease cell viability and cause damage to the membrane, the chromosomes or the DNA at all times of culture. It was also able to induce lipid peroxidation and raise the levels of 8-OHdG and ROS in 6, 12 and 24 h. The results of the RT-PCR and the Western Blot indicated that DON is able to enhance mRNA or protein expressions of DNA repair genes and HO-1 in 6 h and to inhibit these expressions in 24 h. DON potentially triggers genotoxicity in human lymphocytes. This mechanism is probably related to depletion of antioxidase and oxidative damage to the DNA that reduced expression of HO-1, thereby inhibiting the ability of DNA repair.

1. Introduction

It has been demonstrated that trichothecenes, a group of *Fusarium* toxins, have diverse toxic effects on experimental animals and livestock, and that they may have adverse effects in the human body (Creppy, 2002; Gelderblom et al., 1988). Deoxynivalenol (DON, Fig. 1) is one of the most common mycotoxins that is produced by many *Fusarium* species and has been proven to be a major component of cereal grains. The main effects of trichothecene mycotoxins on animal species and human beings are reduced food intake, weight gain, gastrointestinal disturbances, neuroendocrine, hepatological changes, and the impairment of the immune system (Larsen et al., 2004; Pestka et al., 1994; Rotter et al., 1996; Tiemann et al., 2006).

During the last few years, the cytotoxicity of DON has been tested in individual cell lines or primary cell lines of laboratorial animals. For instance, DON was mutagenic to *Salmonella typhimurium* with and without metabolic activation (TA 98, 100, 1535, and 1537 of *S. typhimurium*) (Wehner et al., 1978). Furthermore, even at low concentrations (⩽1 μg/mL for 3 h), it induced an increase of the frequency of micronucleus cell (MN) and chromosomal aberration in cultures of rat primary cells (Knasmuller et al., 1997). Many studies have suggested that the DON-induced production of free radicals may be one of the mechanisms that cause damage to the membrane and to the DNA. Thus, oxidative stress is regarded to be an important factor in DON-induced toxicity (Rizzo et al., 1994). The latest research has demonstrated that DON-induced DNA fragmentation occurs in chicken spleen leukocytes, Caco-2 and Vero cell lines *in vivo* and *in vitro* (Bony et al., 2006; Frankic et al., 2006). It has also been proven that DON (1.7–20 μM) significantly increases the MDA level in Caco-2 cells (Kouadio et al., 2005). Moreover, many previous studies have presented the

Abbreviations: DCFH-DA, 2′, 7′-dichlorofluorescein; m5dC, 5-methylcytosine; 8-OHdG, 8-hydroxydeoxyguanosine; BER, base excision repair; Cyt B, cytochalasin B; DON, deoxynivalenol; Hb, hemoglobin; *h*OGG-1, human oxoguanine glycosylase-1; GSH, L-glutathione reduced; GSSG, L-glutathione oxidize; LC/MS, liquid chromatography/mass spectrometry; MN, micronucleus cell; Malonaldehyde, MDA, Lipid peroxidation; HO-1, heme oxygenase-1; HPLC, high-performance liquid chromatography; MMC, mitomycin C; OECD, Organization for Economic Cooperation and Development; PHA, phytohemagglutinin; ROS, reactive oxygen species; SCE, sister chromatid exchange; RT-PCR, reverse transcription-polymerase chain reaction; XRCC-1, X-ray repair cross-complementing protein-1.

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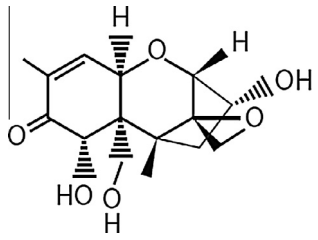

Fig. 1. Chemical structure of DON.

mechanism which triggers DON-induced DNA damage and cytotoxicity. For example, it has been discovered that DON-induced cytotoxicity down-regulates the expression of inflammatory genes (NF-kappaB and COX-2) in HT-29 cells (Krishnaswamy et al., 2010). In another study, it has been revealed that DON provokes DNA fragmentation followed by the activation of p53 and caspase-3 in human colon carcinoma cells and that it inhibits DNA synthesis, which is considered to be a toxic mechanism (Bensassi et al., 2009). Furthermore, it has been pointed out that, at a dose of 10 μM, DON causes a significant increase in the production of lipid peroxidation (MDA), a remarkable rise of the level of DNA fragmentation and an elevation of the portion of 5-methylcytosine (m5dC). The increased level of m5dC inhibits the DNA synthesis in Caco-2 cells (Kouadio et al., 2007). No data on the effect of DON on human primary cells have been published so far, because of the the activation of oxidative damage and the formation of DNA adducts which induce double- and single-strand DNA breaks and reduce DNA repair gene expression.

Furthermore, the degradation of heme to biliverdin, CO, and iron is catalyzed by one of two isoforms of heme oxygenase (HO). HO-1 is an inducible isoform that is thought to be a homeostatic and protective gene against various stress-related conditions. The diversity of HO-1 inducers supports the speculation that HO-1 may also play a pivotal role in maintaining cellular homeostasis. Low or inappropriate HO-1 expression or activity in a prooxidant environment leads to genomic instability, accelerated DNA damage, poor DNA repair, and potentially to the development of disease pathologies (Leo et al., 2011). Furthermore, no data concerning the effect of DON on the expression of HO-1 and DNA repair in human primary cells are available. Therefore, our aim also consists in observing the heme oxygenase-1 (HO-1) gene expression in order to assess the regulation of the oxidative stress response system with DON in human peripheral blood lymphocytes.

In the framework of this study, various cellular and molecular assays were used to evaluate cytotoxicity, genotoxicity as well as oxidative damage, and to investigate toxic mechanisms that are induced by DON in human peripheral blood lymphocytes. The cell viability, the extent of DNA damage, and the chromosomal breaks were measured by the CCK-8 assay, the comet assay, the cytokinesis-block micronucleus assay and the sister chromatid exchange assay. Glutathione reduced/glutathione oxidized (GSH/GSSG), lipid peroxidation, reactive oxygen species (ROS), and phospholipids membrane symmetry were measured with the liquid chromatography–mass spectrography technique (LC/MS), the high performance liquid chromatography technique (HPLC), the fluorescent probe (DCFH-DA, 2′, 7′-dichlorofluorescein), and with the flow cytometry technique. The DNA adducts (8-OHdG) was measured by enzyme linked immunosorbent assay (ELISA). The mRNA expression levels of *h*OGG-1, XRCC-1 and HO-1 were measured by reverse transcription-polymerase chain reaction (RT-PCR) and the protein expression levels of *h*OGG-1, XRCC-1, and HO-1 were measured by Western blot (WB) assay.

2. Materials and methods

2.1. Materials

DON (12, 13-epoxy-3, 4, 15- trihydroxytrichotec-9-en-8-one, $C_{15}H_{20}O_6$, MW: 296.32, CAS No.: 51481-10-8, ⩾99%), GSH (⩾99%), DMSO, normal melting point agarose (NMP), low melting point agarose (LMP), cytochalasin B, phytohemagglutinin (PHA) and GSSG (⩾98%) were purchased from Sigma (St. Louis, MO, USA). Annexin V-FITC and DCFH-DA were purchased from the KeyGen biochemistry company (Nanjing, China). The ELISA kit (8-OHdG) was acquired from the Cayman chemical company (Ann Arbor, MI, USA). RPMI 1640 culture medium and fetal calf serum (FCS) were purchased from Gibco/Invitrogen (Grand Island, NY, USA). The CCK-8 kit was acquired from the Dojindo chemical company (Kyoto, Japan).

2.2. Cell culture

The heparinized blood samples were gathered from 172 healthy and non-smoking volunteers at the clinical department of the Hubei Provincial Centre for Disease Control and Prevention (Wuhan, China). This group consisted of 86 males with a mean age of 27.34 ± 2.04 years and 86 females with a mean age of 26.02 ± 1.47 years (The volunteers were recruited for different experiments). Written informed consent was obtained from all participants and the study was approved by the Medical Ethics Committee of the Tongji Medical College. The lymphocyte cultures were prepared from heparinized blood as had already been described in a previous report (Thomas and Fenech, 2011). In addition, DON powder was dissolved in ultra-pure water at eight different doses (0, 6.25, 12.5, 25, 50, 100, 250, and 500 ng/mL) (Ouyang et al., 1996). In the 0 ng/mL samples, no DON powder was added. These samples were used as negative controls.

2.3. Cell viability test

Cell viability was measured by a CCK-8 assay [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl) -2H-tetrazolium, monosodium salt]. The lymphocyte samples were seeded on 96-well cell culture plates (1.0×10^6 cells/well) with RPMI 1640 culture media. After overnight incubation, these samples were treated with various concentrations of DON (0, 6.25, 12.5, 25, 50, 100, 250 and 500 ng/mL) for 6, 12 and 24 h. Next, the cells were exposed to CCK-8 at 37 °C for 1 h. The tests were performed in 96-well cell culture plates in triplicate. The cell viability was evaluated at an absorbance of 450 nm (Synergy 2 Multi-Mode Microplate Reader, Bio-Tek Instruments Inc., Winooski, VT, USA). The effects of DON on the cell viability were assessed as the percentage of cell viability compared to untreated control cells.

2.4. Comet assay

The concentrations of DON were adjusted to the guidelines of the Organization for Economic Cooperation and Development (OECD), and the Comet assay was performed under alkaline conditions that had already been described in a previous report with some minor modifications (Singh et al., 1991). In culture, the different lymphocyte samples were exposed to five different concentrations of DON (0, 6.25, 12.5, 25 and 50 ng/mL) for 6, 12 and 24 h (the samples with a dose of 0 ng/mL were used as negative controls). The samples with H_2O_2 (20 μM) were positive for 30 min. The five concentrations of DON and mitomycin C (1 μg/mL) were both positive for 6, 12 and 24 h. After the incubation, each frosted microscope slide was covered with 70 μl NMP agarose (0.8%) in Ca^{2+}- and Mg^{2+}-free PBS. Next, a coverslip was placed on top of each slide and the slides were allowed to solidify for 10 min at 4 °C. Then, the LMP (0.8%) was melted in PBS, 10 μl cell suspension were mixed with 130 μl LMP agarose in Eppendorf tubes. After removing the coverslip, the suspension was put onto two slides coated with NMP agarose. Next, the mixture was spread on a microscope slide and allowed to solidify for 10 min at 4 °C. Then, the slides were immersed in a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO freshly added prior to use) in the refrigerator for 4 h. After the completion of the lysis, the slides were rinsed with distilled water and placed in a horizontal gel electrophoresis box containing fresh, chilled electrophoresis buffer up to a level of 0.25 cm above the slides. Electrophoresis was performed in an alkaline solution (1 mM Na_2EDTA and 300 mM NaOH, pH = 13) for 30 min at 25 V and 300 mA at 4 °C. After the completion of your electrophoresis, the slides were neutralized twice with 0.4 mM Tris (pH = 7.5) for 15 min, rinsed with water, and dehydrated in 95% ethanol for 5 min at room temperature. Subsequently, the slides were stained with ethidium bromide and analyzed under the Olympus IX-71 fluorescence microscope (Olympus, Tokyo, Japan). The tail DNA percentage, the tail length and the tail moment were used as indexes of the DNA damage in lymphocytes. At least 150 randomly selected cells per sample (50 cells from each one of the three replicate slides) were analyzed with the Comet Assay Software Project (CASP) 1.2.2 software (University of Wroclaw, Poland).