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Genomic damage induced by the widely used fungicide chlorothalonil in peripheral human lymphocytes

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ABSTRACT

Chlorothalonil is an important broad spectrum fungicide widely used in agriculture, silviculture, and urban settings. As a result of its massive use, chlorothalonil was found in all environmental matrices, with consequent risks to the health of terrestrial and aquatic organisms, as well as for humans.

We analyzed the effects of chlorothalonil on human lymphocytes using *in vitro* chromosomal aberrations (CAs) and micronuclei (MNi) assays. Lymphocytes were exposed to five concentrations of chlorothalonil: 0.600 μg/mL, 0.060 μg/mL, 0.030 μg/mL, 0.020 μg/mL, and 0.015 μg/mL, where 0.020 and 0.600 μg/mL represent the ADI and the ARfD concentration values, respectively, established by FAO/WHO for this compound; 0.030 and 0.060 μg/mL represent intermediate values of these concentrations and 0.015 μg/mL represents the ADI value established by the Canadian health and welfare agency.

We observed cytogenetic effects of chlorothalonil on cultured human lymphocytes in terms of increased CAs and MNi frequencies at all tested concentrations, including the FAO/WHO ADI and ARfD values of 0.020 and 0.600 μg/mL, respectively, but with exception of the Canadian ADI value of 0.015 μg/mL.

Finally, no sexes differences were found in the levels of CAs and MNi induced by different chlorothalonil concentrations. Similarly, the mitotic index and the cytokinesis-block proliferation index did not show any significant effect on the proliferative capacity of the cells, although at the chlorothalonil concentration of 0.600 μg/mL the *P*-values of both indices were borderline.

1. Introduction

Chlorothalonil (CHT) is a broad spectrum, non-systemic chlorinated isophtalonitrile fungicide widely used in agriculture, silviculture, and urban settings. It reacts with functional cellular thiols and inhibits fungal respiration and energy metabolism. For this reason, it was used to control fungal and bacterial infestations in many fruit, vegetable and agricultural crops including peanuts, tomatoes, potatoes, onions and celery (FAO/WHO, 2010).

As a result of its massive use, CHT was found in all environmental matrices (Arinaitwe et al., 2016; Wu et al., 2014) and its possible genotoxicity has been investigated by many authors. CHT was found to be relatively non-toxic for avian species, small mammals and honeybees, but highly toxic for fish, crustaceans, amphibians and aquatic invertebrates (Du Gas et al., 2017; Gallo and Tosti, 2015; Guerreiro et al., 2017; Yu et al., 2013). Moreover, in rodents, chronic dietary exposure to CHT was found to cause an increased incidence of papillomas and carcinomas of the stomach squamous epithelium as well as of adenomas and carcinomas of the renal proximal tubule epithelium (FAO/WHO, 1992).

In humans, CHT exposure was associated with contact dermatitis, severe eye and skin irritation and gastrointestinal problems. In particular, allergic contact dermatitis, conjunctivitis and upper airway complaints were described in fruit and vegetable growers (Penagos et al., 2004), in floriculturists and in trailer tent factory workers (Lensen et al., 2007, 2011). *Vice versa*, epidemiological evidences for an association between CHT and different type of cancers, such as colon, lung, and prostate cancers among humans were not found (Mozzachio et al., 2008).

Form genotoxic point of view, *in vivo* results showed that, mice and Chinese hamsters chronically treated with CHT revealed increased levels of DNA damage in terms of chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) (Dearfield et al., 1993). Moreover, Lebailly et al. (1998), using the alkaline comet assay, observed increased levels of DNA damage in mononuclear leukocytes of farmers exposed to selected pesticides, including CHT.

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On the other hand, *in vitro* studies showed that CHT failed to induce CAs and micronuclei (MNi) in mammalian cell lines (Vigreux et al., 1998), whereas positive results in terms of loss of cell viability and increased frequencies of damaged cells were found in human peripheral blood lymphocytes analyzed with the SCGE assay (Lebailly et al., 1997).

Based on evidences of carcinogenicity from animal studies but no from human epidemiologic data, CHT was classified by U.S. Environmental Protection Agency (EPA) as a Group B2 (probable human carcinogen) (EPA, 1999). Similarly, the International Agency for Research on Cancers (IARC), despite the lack of available data about human carcinogenicity, classified CHT as a possible carcinogen (2B) (IARC, 1999). Different FAO/WHO reviews confirmed that CHT did not show a genotoxic hazard for humans and, on the basis of the available information, estimated the Acceptable Daily Intake (ADI) value to 0–0.02 mg/kg/ bw and the Acceptable Reference Dose value to 0.6 mg/kg/ bw (FAO/WHO, 2010). However, it should be emphasized that, in a previous published report and partially in contrast to FAO/WHO, the Canadian health and welfare agency established for CHT the more stringent ADI concentration value of 0–0.015 mg/kg/ bw (HWC, 1994).

The widespread use of CHT in agriculture and the limited data about its genotoxicity in humans lymphocytes, prompted us to investigate the frequency of CAs and MNi in human peripheral lymphocytes after *in vitro* exposure to different concentrations of this pesticide, including the ADI-value and the Acceptable Reference Dose (ARfD) established by FAO/WHO, as well as the ADI-value established by the Canadian health and welfare agency.

Among cytogenetic test systems, CAs and MNi assays are important tools in the measurement of the genotoxic potential of many chemicals. The CAs assay allows the detection of cells carrying unstable aberrations (*i.e.* chromosome/chromatid breaks, fragments, rings and dicentrics) that will lead to cell death during proliferation (Garcia-Sagredo, 2008). On the other hand, MNi assay allows evaluation of both potential clastogenic and/or aneugenic effects of different xenobiotics. In particular, MNi originates from acentric chromosome fragments or whole chromosomes that fail to segregate properly during mitotic division and appear in the cytoplasm of interphase cells as small additional nuclei (Fenech, 2016). Interestingly, previous published studies provided evidences for a relationship between high levels of CAs and MNi in peripheral blood lymphocytes and increase of cancer risk (Bonassi et al., 2004, 2011).

2. Materials and methods

2.1. Chemicals and reagents

The IUPAC name of CHT is: Tetrachloroisophthalonitrile (CAS no. 1897-45-6). The CHT (obtained from Labservices, Bologna, Italy) was first dissolved in dimethyl sulfoxide (DMSO) (CAS no. 67-68-5) at a final concentration of 0.6 mg/mL (stock solution) and was kept at 4 °C until prepared for the final exposure solutions in culture medium. Gibco RPMI 1640 cell culture media supplemented with L-glutamine, foetal calf serum, phytohemagglutinin (PHA), and antibiotics were purchased from Invitrogen-Life Technologies, Milan, Italy. Cytochalasin-B, colchicine and Mitomycin-C (MMC) were obtained from Sigma-Aldrich, Milan, Italy. Methanol, Acetic acid, Giemsa stain solution, and conventional microscope slides were purchased from Carlo Erba Reagenti, Milan, Italy. Potassium chloride (KCl) and Sörensen buffer were obtained from Merck S.p.A., Milan, Italy. Vacutainer blood collection tubes were from Terumo Europe, Rome, Italy. Distilled water was used throughout the experiments.

2.2. Subjects

Peripheral venous blood was collected from 6 healthy subjects (3 males and 3 females, mean age ± S.E., 32.90 ± 1.84, range 23–40 years), non-smoking, not alcoholics, not under drug therapy, and with no recent history of exposure to mutagens. Informed consent was obtained from all blood donors. The study was approved by the local ethics committee and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

2.3. Blood sample collection and lymphocyte cultures

Blood samples were obtained by venepuncture (about 10 mL of blood per subject), collected in heparinised tubes, cooled (4 °C) and processed within 2 h after collection. Heparinised venous blood (0.3 mL) were cultured in 25 cm^2 flasks containing 6 mL of RPMI-1640 medium, 2 mL of foetal calf serum (FCS), 200 µL of the mitogenic agent Phytohemagglutinin-L (2.3% v/v), and 100 µL of antibiotics solution (100 IU/mL penicillin, and 100 µg/mL streptomycin), for a total of 8.6 mL for each lymphocyte culture. The cultures were successively incubated at 37 °C and under 5% of CO_2 in the air in a humidified atmosphere. After 24 h of incubation, 8.6 µL of CHT stock solution at concentration of 0.6 mg/mL were added to the lymphocyte culture in order to reach a final CHT concentration of 0.600 µg/mL. Similarly, 8.6 µL of CHT stock solution diluted 10, 20, 30 and 40 times with DMSO were added to the lymphocyte cultures in order to reach the final CHT concentrations of 0.060 µg/mL, 0.030 µg/mL, 0.020 µg/mL and 0.015 µg/mL, respectively. In particular, 0.020 and 0.600 µg/mL represent the ADI and the ARfD concentrations, respectively, established by FAO/WHO for this compound, 0.030 and 0.060 µg/mL intermediate values of these concentrations and 0.015 µg/mL represent the ADI concentration established by Canadian health and welfare agency. Three control cultures were assessed: 1) positive control, by adding only MMC (final concentration 0.1 µg/mL culture); 2) 0.1% DMSO solvent control, obtained by adding 8.6 µL of DMSO to the lymphocyte culture; 3) negative control culture without both CHT and DMSO, obtained adding 8.6 µL of RPMI medium to the lymphocyte culture. Only for MNi assay, after 44 h of incubation, cytochalasin-B was added to the cultures at a concentration of 6 µg/mL to block cytokinesis. Similarly, only for CAs assay, to arrest cells in mitosis, colchicine was added at a concentration of 0.06 µg/mL during the last 2 h of culture.

After 48 h (for CAs assay) and 72 h (for MNi assay) of incubation at 37°, the cells were collected by centrifugation and treated for 10 min with a pre-warmed mild hypotonic solution (75 mM KCl). After centrifugation and removal of the supernatant, the cells were fixed with a fresh mixture of methanol/acetic acid (3:1 v/v). The treatment with the fixative was repeated three times. Finally, the supernatant was discarded and the pellet, dissolved in a minimal volume of fixative, was seeded on the slides to detect CAs and MNi by conventional staining with 5% Giemsa (pH 6.8) prepared in Sörensen buffer.

2.4. Cytokinesis-block micronucleus assay

Microscope analysis was performed at 400 × magnification on a light microscope (Dialux 20, Leitz, Germany). MNi, nucleoplasmic bridges (NPB) and nuclear buds (NBUD) were scored in 2000 binucleated lymphocytes with well-preserved cytoplasm per subject (total 12000 binucleated cells per concentration). A total of 2000 lymphocytes per donor per concentration were scored to evaluate the percentage of cells with 1–4 nuclei. The cytokinesis-block proliferation index (CBPI) was calculated, according to the following formula: $[1 \times N1] + [2 \times N2] + [3 \times (N3 + N4)]/N$, where N1-N4 represents the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored.

2.5. Chromosomal aberration assays

Microscope analysis was performed at 1000 × magnification on a light microscope (Dialux 20, Leitz, Germany). For each subject and CHT concentration, 200 well-spread first-division complete metaphases (for