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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

a total of 1200 metaphases for each dose) were analyzed for the following categories of CAs: gaps (G), chromatid breaks (B'), chromosome breaks (B"), dicentrics (DC), rings (R), tri- or tetra-radials (TR), acentric fragments (AF), rearrangements (Re) and numerical aberrations. Cells containing one of more types of CAs were scored as "aberrant cell" (Ab. C).

In order to determine cytotoxicity, the mitotic index (MI) was calculated from the number of metaphases in 2000 cells analyzed per subject per concentration (a total of 12000 cells per concentration).

2.6. Statistical analysis

Comparison of mean values of the percentage of cells with MNi, NPB, NBUD, CBPI, CAs and MI between exposure levels and their controls was assessed by the non-parametric Mann-Whitney test. Statistical calculations were carried out using the SPSS software package program (version 24.0, Inc., Chicago, IL, USA). All *P* values were two tailed, and *P* values of 5% or less were considered statistically significant for all tests carried out.

3. Results

3.1. Effect of CHT on CAs formation

Table 1 shows values of CAs found in the human peripheral lymphocytes cultured in the presence of different CHT concentrations.

CHT was found to induce seven types of structural CAs (gaps, chromatid and chromosome breaks, dicentric chromosomes, rings, acentric fragments and rearrangements). The most frequent observed aberrations were acentric fragments and chromatid breaks, respectively, whereas no numerical aberrations were found.

In Fig. 1 some examples of observed chromosomal aberrations were showed.

Because of the conflicting opinions about the possibility to consider gaps as indicators of genomic damage (Savage, 2004), we decided to exclude gaps from statistical analysis.

Data obtained indicated that human lymphocytes treated *in vitro* with CHT significantly ($P = 0.004$) increased the CAs and Ab.C frequencies at all tested concentrations when compared with the solvent control, including the concentration of 0.020 μg/mL ($P = 0.009$) that represents the ADI value established by FAO/WHO for this substance, but with exception of 0.015 μg/mL. Moreover, a dose-effect was observed since the regression analysis revealed a significant correlation between the CHT concentrations and the level of CAs ($P < 0.001$; β-coefficient = 0.870) and cells with CAs ($P < 0.001$; β-coefficient = 0.873). *Vice versa*, no significant differences were found between the DMSO solvent-control and the negative control, whereas the cultures treated with the known mutagen MMC showed a significant increase of CAs and Ab. C with respect to all CHT tested concentrations, including the negative and solvent control cultures.

No significant differences in the MI values were observed between DMSO and all tested concentrations of CHT, although at the highest concentration of 0.600 μg/mL, the reduction of the MI was very close to the significance values ($P = 0.051$).

Finally, no sexes differences were found in the levels of CAs induced by different CHT concentrations, with exception of MMC although with a borderline *P*-value of 0.046.

3.2. Effect of CHT on MNi formation

To verify both the aneugenic and clastogenic effects of CHT, the MNi test was assessed in parallel with CAs test (Table 2).

Similarly to what we already observed with the CAs assay, our results indicated that CHT significantly increased ($P = 0.004$) the MNi formation at all tested concentrations (including established ADI value of 0.020 μg/mL), with exception of 0.015 μg/mL. Moreover, a dose-effect was observed since the regression analysis revealed a significant correlation between the CHT concentrations and the frequencies of MNi and Cells with MNi ($P < 0.001$, β-coefficient = 0.863 and 0.879 for MNi and Cells with MNi, respectively). No significant differences were observed in the frequencies of NPB and NBUD between the different CHT concentrations and the DMSO solvent control, with exception of the highest concentration of 0.600 μg/mL ($P = 0.003$ and $P = 0.044$, for NPB and NBUD, respectively).

In Fig. 2 examples of observed bi-, tri- and tetra-nucleated cells with micronuclei, as well as of bi-nucleated cells with NBUD and NPB, were reported.

Also in this case, the DMSO solvent-control cultures did not show any difference with the negative controls ($P = 0.089$), further confirming that at this low concentration DMSO has no cytogenetic effects evaluable by MNi test. MMC showed a significant increase in the MNi formation compared with the negative control ($P = 0.004$), solvent controls ($P = 0.004$) and all tested concentrations of CHT ($P = 0.037$ for 0.600 μg/mL, $P = 0.006$ for 0.06 μg/mL and $P = 0.004$ for 0.030, 0.020 and 0.015 μg/mL).

After 48-h exposure, a significant reduction of the CBPI value in cultures treated with CHT was not observed, indicating that at the tested concentrations, CHT does not seem to produce effects on the proliferation index.

Similarly to what observed by CAs assay, females shows highest frequencies of MNi at all CHT concentrations tested, although these differences were not significant.

Table 1
Induction of chromosomal aberrations by Chlorothalonil in human lymphocytes *in vitro*. Number of scored metaphases for each concentration/subject: 200.

Test substance (μg/mL)	N	CAs								CAs	CAs + G	Ab. C	Ab. C + G	(%) CAs/N ± S.E.	(%) Ab. C/N ± S.E.	(%) MI ± S.E.
		G	B'	B''	DC	R	TR	AF	Re							
NC	1200	3	10	0	0	0	0	2	3	15	18	15	18	1.250 ± 0.214	1.250 ± 0.214	5.850 ± 0.097
0.1% DMSO	1200	5	13	2	0	0	0	3	2	20	25	20	25	1.6700 ± 0.211	1.6700 ± 0.211	5.775 ± 0.063
MMC (0.100)	1200	29	35	21	7	9	3	40	13	128	157	124	153	10.667 ± 1.388[a]	10.333 ± 1.160[a]	4.675 ± 0.084[c]
CHT (0.600)	1200	5	23	7	6	6	0	27	10	79	84	78	83	6.583 ± 0.352[a]	6.500 ± 0.288[a]	5.450 ± 0.124
CHT (0.060)	1200	3	20	7	3	1	0	18	10	59	62	59	62	4.917 ± 0.417[a]	4.917 ± 0.417[a]	5.558 ± 0.065
CHT (0.030)	1200	5	18	8	0	1	0	18	12	57	62	57	62	4.750 ± 0.382[a]	4.750 ± 0.382[a]	5.625 ± 0.038
CHT (0.020)	1200	4	10	1	3	1	0	19	2	36	40	36	40	3.000 ± 0. 423[b]	3.000 ± 0. 423[b]	5.767 ± 0.049
CHT (0.015)	1200	5	10	1	0	0	0	13	1	25	30	25	30	2.083 ± 0.327	2.083 ± 0.327	5.792 ± 0.040

N = total number of scored metaphases; G = gaps; CAs = chromosomal aberrations; Ab. C = aberrant cells (cells with 1 or more aberrations); MI = Mitotic Index; B': chromatid break; B": chromosome break; DC: dicentric; R: ring; TR = tri-tetraradials; AF = acentric fragments;Re = rearrangements; S.E. = standard error; NC = Negative Control; MMC = Mitomycin-C; CHT = chlorothalonil.

[a] $P = 0.004$.

[b] $P = 0.009$ and.

[c] $P = 0.004$ (significantly different from the DMSO solvent control, Mann-Whitney test).

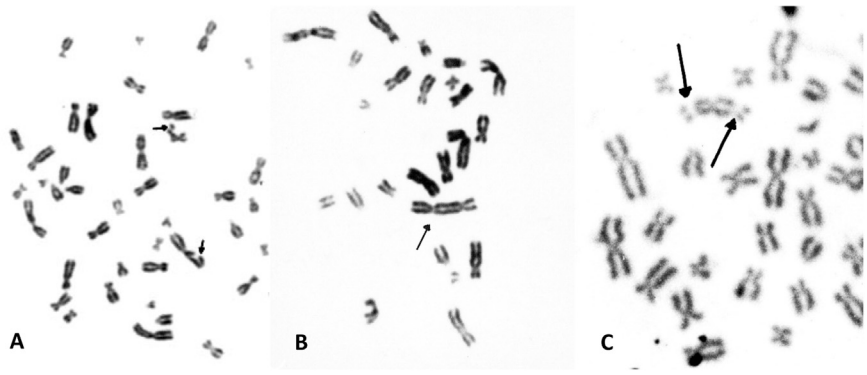

Fig. 1. Complete metaphase carrying chromosomal aberrations (A) and metaphase details (B and C) showing examples of observed chromosomal aberrations (1000 × magnification, Leitz Dialux 20, Germany). The arrows indicate, respectively: chromatid breaks (Figure A), dicentric chromosome (Figure B) and acentric fragments (Figure C).

Table 2
Induction of micronuclei produced by Chlorothalonil in human lymphocytes *in vitro*. Number of scored binucleated cells for each concentration/subject: 2000.

Test substance (μg/mL)	N	Distribution of BNCs according to the number of MNi				MNi	Ab. C	MNi/N ± S.E. (%)	Ab. C/N ± S.E. (%)	CBPI ± S.E	BNCs with NPB (‰)	BNCs with NBUD (‰)
		1	2	3	4							
NC	12000	21	1	0	0	23	22	0.192 ± 0.024	0.183 ± 0.021	1.680 ± 0.041	0.333 ± 0.105	0.750 ± 0.112
0.1% DMSO	12000	33	1	0	0	35	34	0.292 ± 0.040	0.283 ± 0.036	1.600 ± 0.056	0.417 ± 0.154	0.917 ± 0.154
MMC (0.100)	12000	235	14	3	1	276	253	2.300 ± 0.211[a]	2.108 ± 0.175[a]	1.344 ± 0.015[b]	2.500 ± 0.408[c]	2.833 ± 0.441[e]
CHT (0.600)	12000	174	8	0	2	198	188	1.650 ± 0.178[a]	1.567 ± 0.147[a]	1.499 ± 0.038	2.000 ± 0.183[d]	1.500 ± 0.183[f]
CHT (0.060)	12000	153	6	0	1	169	160	1.408 ± 0.137[a]	1.333 ± 0.103[a]	1.531 ± 0.027	0.917 ± 0.154	1.167 ± 0.105
CHT (0.030)	12000	119	4	1	0	130	124	1.083 ± 0.101[a]	1.033 ± 0.080[a]	1.543 ± 0.012	0.667 ± 0.105	1.083 ± 0.154
CHT (0.020)	12000	72	1	0	0	74	73	0.617 ± 0.061[a]	0.608 ± 0.061[a]	1.571 ± 0.063	0.500 ± 0.129	1.000 ± 0.129
CHT (0.015)	12000	41	0	0	0	41	41	0.342 ± 0.020	0.342 ± 0.020	1.586 ± 0.057	0.417 ± 0.154	1.000 ± 0.183

N = total number of scored binucleated cells; BNCs = Binucleated cells; MNi = micronuclei; Ab. C = cells with 1 or more micronuclei; CBPI = Cytokinesis-Block Proliferation Index; NPB = nucleoplasmic bridges; NBUD = nuclear buds; S.E. = Standard Error; NC = Negative Control; MMC = Mitomycin-C; CHT = Chlorothalonil.

[a] $P = 0.004$.
[b] $P = 0.016$.
[c] $P = 0.005$.
[d] $P = 0.003$.
[e] $P = 0.005$, and.
[f] $P = 0.044$ (significantly different from the DMSO solvent control, Mann-Whitney test).

4. Discussion

Exposure to pesticides is known to be an important environmental risk factor associated with the development of cancer (Alavanja et al., 2005). However, insufficient data are present in literature about the genotoxicity of many commercially available pesticides. In particular, the genotoxic potential of CHT was evaluated in different *in vitro* and *in vivo* studies but observed data were, in some cases, contradictory (Lebailly et al., 1997; Vigreux et al., 1998).

Results obtained in the present study evidenced a possible clastogenic and/or aneugenic effect of the CHT on human lymphocytes, also at the concentration of 0.020 μg/mL that represents the ADI value established for humans by FAO/WHO (2010). Our data seem to be concordant with results obtained by other authors with different cell lines and/or different assays. Lebailly et al. (1997), by a SCGE assay, observed a significant effect of CHT on human peripheral blood lymphocytes in terms of cell viability and DNA-damage. Similarly, CHT was found to induce SCEs and CAs *in vitro* in Chinese hamster ovary cells (Dearfield et al., 1993), whereas *in vivo* this increase of the DNA damage was observed in rat, mouse and Chinese hamster only after a chronic treatment for 5 successive days, but not after a single dose treatment (Dearfield et al., 1993). However, it should be emphasized that all data about CHT genotoxicity should be interpreted with particular attention also in view of the fact that, in pesticide formulations used by farmers, CHT is frequently associated to other pesticides, such as carbendazim, that are known to enhance the genotoxic effect of CHT on human PBL (Lebailly et al., 1997).

Chlorothalonil and other polychlorinated compounds like polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans, represent persistent organic contaminants of anthropic or natural origin, ubiquitously found in the environment and with the ability to bioaccumulate and biomagnify in food chains, causing the decline or disappearance of wild populations (Bruns-Weller et al., 2010). Like chlorothalonil, most of these polychlorinated compounds also showed genotoxic properties. For example, 1,2-dichloroethylene, 1,1,2-trichloroethane, 1,3-dichloropropane, and 1,1,3-trichloropropene, widely used as solvents and degreasing agents in industry, were found to induce MNi and DNA damage in human lymphocytes (Tafazoli and Kirsch-Volders, 1996). Similarly, 2,2′,4,4′,5,5′-hexachlorobiphenyl, 2,3′,4,4′,5- and 2,2′,4′,5,5′-pentachlorobiphenyls, were found to induce MNi and DNA breaks in fish RTG-2 cells (Marabini et al., 2011). The same polychlorinated biphenyls were found to affect the female reproductive system in humans, and some of these, like chlorinated bisphenol-A, were associated with metabolic conditions, such as obesity, lipid accumulation and type 2 diabetes mellitus (Andra et al., 2015). Moreover, dicloran (2,6-dichloro-4-nitroaniline), an aromatic amine commonly used as an agricultural fungicide throughout the Southern and Western United States, was found to be mutagen in Salmonella strains TA98 and TA100 (de Oliveira et al., 2009). Another aromatic fungicide, the *p*-cresol, at concentrations of 1.6 and 3.2 mM was found to be genotoxic toward HT-29 Glc–/+ and LS-174T human intestinal cells (Andriamihaja et al., 2015), whereas the fungicide hexachlorobenzene was found to be able to induce DNA damage and oxidative stress in human intestinal Caco-2 cells, also at the

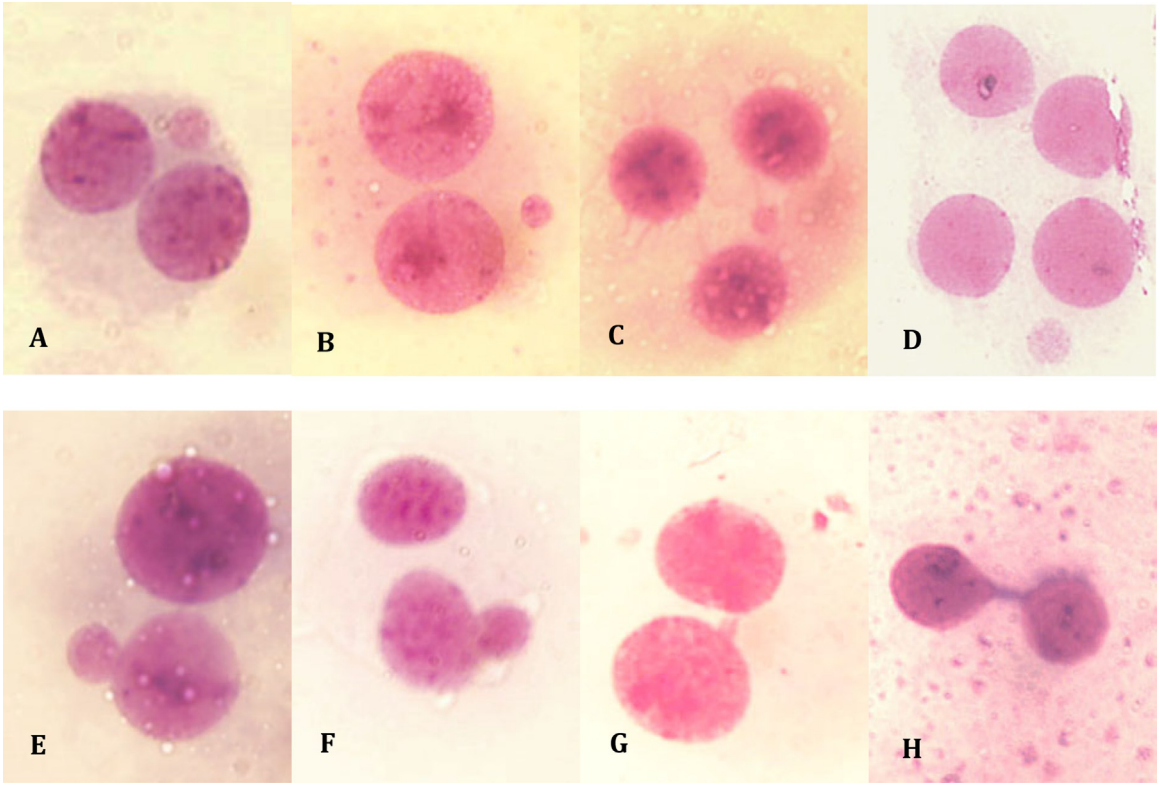

Fig. 2. Examples of micronuclei observed in binucleated cells (A-B), in trinucleated cell (C) and in tetranucleated cell (D) (1000 × magnification, Leitz Dialux 20, Germany). According to standardized procedures, micronuclei of tri- and tetra-nucleated cells were not scored in the evaluation of the total genomic damage. Examples of binucleated cells with nuclear buds (E-F) and bi-nucleated cells with nucleoplasmic bridges (G-H) are also reported.

low concentrations of 0.4 and 400 nM (Chalouati et al., 2015). Increased levels of MNi were observed also in *in vivo* studies, for example in several fish species after exposure to commercial polychlorinated biphenyls in contaminated areas, as well as abnormal karyotypes were observed in workers exposed to these compounds (Ludewig and Robertson, 2013).

The mechanisms underlying genotoxic potential of CHT alone or in complexation with other compounds are unknown, although it was found that exposure to CHT may trigger cytotoxic and inflammatory processes. These last were found able to induce DNA damage and the loss of cellular membrane integrity (Wilkinson and Killeen, 1996) as well as cytogenetic damage (Higashimoto et al., 2006; Santovito et al., 2016). In this sense, it is our opinion that the increase of cytogenetic damage observed by our group with both CAs and MNi assays, and by other groups with different assays and cell lines, requires further investigations and should pushes towards the adoption of lower reference limits. Indeed, increased CAs and MNi frequencies in peripheral blood lymphocytes have been positively associated with increased cancer risk and early events in carcinogenesis, respectively (Bonassi et al., 2004, 2011). Moreover, CHT was found to have *in vitro* tumor promoting effects in Syrian hamster embryo cells (Bessi et al., 1999), whereas, *in vivo,* chronic dietary treatment with CHT was found to causes in rodents (rats and mice) increased incidence of papillomas and carcinomas of the forestomach squamous epithelium and adenomas and carcinomas of the renal proximal tubule epithelium (Wilkinson and Killeen, 1996). All these considerations and the fact that also in our study we do not observed clastogenic/aneugenic effects at the CHT concentration of 0.015 µg/mL, seem to justify the Canadian health and welfare agency that, in contrast to FAO/WHO, established for CHT a lower ADI-value of 0–0.015 mg/kg/day (HWC, 1994).

The MI and the CBPI did not show any significant difference at all tested concentrations with respect to the solvent control, although at concentration of 0.600 µg/mL the *P*-values of both indices were borderline ($P = 0.051$ for MI and $P = 0.078$ for CBPI), indicating that the CHT cytotoxicity threshold could be close to this concentration value. These data differ from those found by other authors for other fungicides or insecticides (Kocaman et al., 2014; Yüzbaşioğlu et al., 2006), although it should be emphasized that these authors evaluated the MI at xenobiotic concentrations much higher than those tested in the present work.

Finally, as the role of sex, in the present study females showed higher levels of genomic damage than males, although with non-statistically significant values. The only exceptions were represented by MMC in the CAs assay, although with a borderline *P*-value of 0.046. However, it should be emphasized that the small sample size, typical of an *in vitro* study, does not allow to drawn definitive conclusions. In our previous *in vivo* studies involving a larger number of subjects, the results related to a possible role of sex in determining the level of genomic damage were conflicting. Indeed, a positive association between the frequencies of CAs, sister chromatid exchanges and sex was found by our group in two control populations (n = 101 and n = 230, respectively), with females showing highest levels of genomic damage (Santovito et al., 2017), whereas in another study this association was not observed (Santovito et al., 2016)

5. Conclusion

The results herein reported showed cytogenetic effects of CHT on cultured human lymphocytes in terms of increased CAs and MNi frequencies. Although simple experimental models like mammalian and bacterial cells cannot accurately mimic the complex *in vivo* kinetics of xenobiotic compounds, results we obtained with CHT point to the necessity of further investigations in order to establish the real genotoxic potential of this compound, alone and/or in association with other compounds, and, eventually, the adoption of more stringent measures able to reduce the presence of this compound in the environment and to

minimize the adverse effects of the CHT exposure on human health.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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