Title: Genomic damage induced by the widely used fungicide Chlorothalonil in peripheral human lymphocytes

Running Title: Genotoxic Effects of Chlorothalonil on 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 analysed the effects of chlorothalonil on human lymphocytes using *in vitro* chromosomal aberrations and micronuclei assays. Lymphocyte 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 MNs 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 chromosomal aberrations and micronuclei induced by different Chlorothalonil concentrations, as well as a significant reduction of the CBPI was not observed, indicating that Chlorothalonil does not seem to produce effects on the proliferation/mitotic index when its concentration is equal or less than 0.020 μg/mL.

Keywords: Chromosomal aberrations; Genotoxicology; Micronuclei; Pesticide

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 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, birds, amphibians and aquatic invertebrates (Gallo and Tosti, 2015; McMahon et al., 2011; Yu et al., 2013; Shelley et al., 2009; Guerreiro et al., 2017; Du Gas et al., 2017). 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 (Van Scoy and Tjeerdema, 2014; 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, 2002; 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 coll. (1998), using the alkaline comet assay, observed increased levels of DNA damage in mononuclear leukocytes of farmers exposed to selected pesticides, including CHT.

On the other hand, *in vitro* studies showed that CHT failed to induce CAs and micronuclei (MNs) 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 MNs in human peripheral lymphocytes after *in vitro* exposure to different concentrations of this pesticide, including those of 0.020 and 0.600 μg/mL that represent the ADI and Acceptable Reference Dose (ARfD), respectively, established by FAO/WHO for this compound, and the concentration of 0.015 μg/mL, that represents the ADI-value established by the Canadian health and welfare agency.

Among cytogenetic test systems, CAs and MNs 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, MNs assay allows

evaluation of both potential clastogenic and/or aneugenic effects of different xenobiotics. In particular, MNs 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 MNs 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 n. 1897-45-6). The CHT (obtained from Labservices, Bologna, Italy) was first dissolved in 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 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 venipuncture, 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, 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, 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 MNs assay, after 44 h of incubation, cytochalasin-B was added to the cultures at a concentration of 6 μg/mL to block cytokinesis.

Fixation of lymphocyte cultures was performed with a fresh mixture of methanol/acetic acid (3:1 v/v), using the procedure described in our previous published article (Santovito et al., 2018).

2.4. Cytokinesis-Block Micronucleus and Chromosomal Aberration Assays

Micronucleus and chromosomal aberration assays were performed according to previously described procedures (Santovito et al., 2018). MNs and CAs were scored in 1,000 binucleated lymphocytes and 200 well-spread complete metaphases per donor per concentration, respectively. 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. Statistical analysis

Comparison of mean values of the percentage of cells with MNs, CBPI and CAs 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 23.0, 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.

Insert Table 1

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 aberrations, respectively, whereas no numerical aberrations were found.

Because of the conflicting opinions about the possibility to consider gaps as indicators of genomic damage (Savage JR, 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 Aberrant Cells (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 ($P<0.001$) correlation between the CHT concentrations and the level of genomic damage (Table 2).

Insert Table 2

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. 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 (Table 3).

Insert Table 3

3.2. Effect of CHT on MNs formation

To verify both the aneugenic and clastogenic effects of CHT, the MNs test was assessed in parallel with CAs test (Table 4).

Insert Table 4

Similarly to what we already observed with the CAs assay, our results indicated that CHT significantly increased ($P = 0.004$) the MNs formation at all tested concentrations (including di 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 ($P<0.001$) correlation between the CHT concentrations and the frequencies of MNs and Cells with MNs (Table 2).

Also in this case, the DMSO solvent-control cultures did not show any difference with the negative controls ($P = 0.126$), further confirming that at this low concentration DMSO has no cytogenetic effects evaluable by MN test. MMC showed a significant increase in the MNs formation compared with the negative control ($P = 0.019$), solvent controls ($P = 0.020$) and all tested concentrations of CHT ($P = 0.029$ and for 0.06 μg/mL and $P = 0.021$ for both 0.030 and 0.020 μg/mL) with exception of 0.600 μg/mL ($P = 0.139$). 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/mitotic index.

Similarly to what observed by CAs assay, females shows higher frequencies of MNs at all CHT concentrations tested, although these differences were not significant. The only exception was

observed at the CHT concentration of 0.600 μg/ml, although, also in this case, with a borderline *P*-value of 0.046 (Table 5).

Insert Table 5

4. Discussion

Exposure to pesticides is known to be an important environmental risk factor associated with the development of cancer (Miligi et al., 2006; 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 (Vigreux et al., 1998; Lebailly et al., 1997).
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 coll. (1997), by a SCGE assay, observed a significant effects 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).

The mechanisms underlying genotoxic potential of CHT alone or in complexation with other

compounds are unknown, although it was known 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 MNs 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 MNs 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).

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 and the 0.6 μg/mL of CHT for the MNs, although both with a borderline *P*-value of 0.046 (Table 5). 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 higher levels of genomic damage (Santovito et al., 2017), whereas in another study this association was not observed (Santovito et al., 2016) Finally, the CBPI value was decreased with CHT treatments. However, this reduction was not statistically significant, indicating that CHT does not seem to produce significant effects on the proliferation/mitotic index when its concentration is equal or less than 0.020 μg/mL.

5. Conclusion

The results herein reported showed cytogenetic effects of CHT on cultured human lymphocytes in terms of increased CAs and MNs 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.

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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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Table 1 – Induction of chromosomal aberrations by Chlorothalonil in human lymphocytes *in vitro*.
Number of scored metaphases for each concentration of the test substance = 1200

Test substance (µg/ml)	CAs								CAs	CAs + Gaps	Ab.C	Ab.C + Gaps	(%) CAs/Cell ± S.E.	(%) Ab.C/Cell ± S.E.
	Gaps	B’	B’’	DC	R	TR	AF	Re						
NC	3	10	0	0	0	0	2	3	15	18	15	18	1.250±0.214	1.250±0.214
0.1% DMSO	5	13	2	1	0	0	3	2	20	25	20	25	1.6700±0.211	1.6700±0.211
MMC (0.100)	29	35	21	7	9	3	40	13	128	157	124	153	10.667±1.388 [a]	10.333±1.160 [a]
CHT (0.600)	5	23	7	6	6	0	27	10	79	84	78	83	6.583±0.352 [a]	6.500±0.288 [a]
CHT (0.060)	3	20	7	3	1	0	18	10	59	62	59	62	4.917±0.417 [a]	4.917±0.417 [a]
CHT (0.030)	5	18	8	0	1	0	18	12	57	62	57	62	4.750±0.382 [a]	4.750±0.382 [a]
CHT (0.020)	4	10	1	3	1	0	19	2	36	40	36	40	3.000±0. 423 [b]	3.000±0. 423 [b]
CHT (0.015)	5	10	1	0	0	0	13	1	25	30	25	30	2.083±0.327	2.083±0.327

CAs = chromosomal aberrations; Ab.C = aberrant cells (cells with 1 ore more aberrations);
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]$P = 0.009$ (significantly differs from DMSO solvent control)

Table 2 - Regression analysis evaluating the relationship between the different Chlorothalonil concentrations and the level of genomic damage

Biomarkers	**β-co**	***P*-value**	**95% CI** (Lower) – (Upper)
CAs	0.870	<0.001	(1.705) – (2.661)
Cells with CAs	0.873	<0.001	(1.686) - (2.614)
MNs	0.863	<0.001	(3.221) – (5.113)
Cells with MNs	0.879	<0.001	(2.949) – (4.517)
CBPI	0.199	0.292	(-0.042) – (0.013)

CAs = Chromosomal Aberrations; MNs = micronuclei;
CBPI = Cytokinesis-Block Proliferation Index; β-co = β-coefficient;
CI = Confidence Interval

Table 3 – Induction of chromosomal aberrations by Chlorothalonil in human lymphocytes *in vitro*, according to sex. Number of scored metaphases for each concentration of Chlorothalonil = 1200

Test substance (µg/ml)	**FEMALES**		**MALES**	
	CAs/Cell ± S.E. (%)	**Ab.C/Cell±S.E. (%)**	**CAs/Cell ± S.E. (%)**	**Ab.C/Cell±S.E. (%)**
NC	1.500±0.289	1.500±0.289	1.000±0.289	1.000±0.289
0.1% DMSO	1.833±0.333	1.833±0.333	1.500±0.289	1.500±0.289
MMC (0.100)	12.833±2.088*	12.166±1.667	8.500±0.763*	8.500±0.763
CHT (0.600)	7.167±0.441	7.167±0.441	6.000±0.289	6.000±0.289
CHT (0.060)	5.500±0.577	5.500±0.577	4.333±0.441	4.333±0.441
CHT (0.030)	5.167±0.333	5.167±0.333	4.333±0.667	4.333±0.667
CHT (0.020)	3.333±0.601	3.333±0.601	2.667±0.333	2.667±0.333
CHT (0.015)	2.333±0.333	2.333±0.333	1.8333±0.601	1.8333±0.601

CAs = chromosomal aberrations; Ab.C = aberrant cells (cells with 1 ore more aberrations);
S.E. = standard error; NC = Negative Control; MMC = Mitomycin-C; CHT = chlorothalonil;
$^{*}P = 0.046$

Table 4 - Induction of micronuclei produced by Chlorothalonil in human lymphocytes *in vitro*.
Number of scored binucleated cells for each concentration of the test substance = 6000

Test substance (µg/ml)	Distribution of BNCs according to the number of MNs				MNs	Ab.C	MN/cell ± S.E. (%)	Ab.C/cell ± S.E. (%)	CBPI ± S.E
	1	2	3	4					
NC	10	1	0	0	12	11	0.200±0.052	0.183±0.048	1.665±0.047
0.1% DMSO	23	0	0	0	23	23	0.383±0.087	0.425±0.025	1.593±0.059
MMC (0.100)	157	9	2	1	185	169	3.083±0.403 [a]	2.817±0.340 [a]	1.348±0.015 [d]
CHT (0.600)	101	5	0	2	119	108	1.983±0.271 [a]	1.817±0.227 [a]	1.523±0.041
CHT (0.060)	91	4	0	1	103	96	1.750±0.173 [a]	1.600±0.113 [a]	1.524±0.028
CHT (0.030)	65	3	1	0	74	69	1.233±0.099 [a]	1.150±0.089 [a]	1.536±0.011
CHT (0.020)	39	1	0	0	41	40	0.683±0.060 [b]	0.667±0.059 [c]	1.563±0.063
CHT (0.015)	25	0	0	0	25	25	0.417±0.048	0.417±0.048	1.575±0.058

BNCs = Binucleated cells; MNs = micronuclei; Ab.C = cells with 1 or more micronuclei;
CBPI = Cytokinesis-Block Proliferation Index = $[1 \times N_1] + [2 \times N_2] + [3 \times (N_3 + N_4)]/N$, where N_1–N_4 represents the number of cells with 1-4 nuclei, respectively, and N is the total number of cells scored; S.E. = Standard Error; NC = Negative Control; CHT = Chlorothalonil; MMC = Mitomycin-C.
[a]$P = 0.004$, [b]$P = 0.024$, [c]$P = 0.005$, [d]$P = 0.020$ (significantly differs from DMSO solvent control)

Table 5 - Induction of micronuclei produced by Chlorothalonil in human lymphocytes *in vitro,* according to sex.
Number of scored binucleated cells for each concentration of Chlorothalonil = 6000

	FEMALES			MALES		
Test substance (µg/ml)	**MN/cell±S.E. (%)**	**Ab.C/cell±S.E. (%)**	**CBPI±S.E.**	**MN/cell±S.E. (%)**	**Ab.C/cell±S.E. (%)**	**CBPI±S.E.**
NC	0.267±0.033	0.267±0.033	1.637±0.064	0.067±0.088	0.050±0.058	1.693±0.051
0.1% DMSO	0.233±0.088	0.233±0.088	1.653±0.040	0.267±0.088	0.267±0.088	1.532±0.109
MMC (0.100)	2.533±0.318	2.367±0.260	1.365±0.023	1.817±0.639	1.633±0.555	1.330±0.017
CHT (0.600)	1.400±0.153*	1.300±0.115	1.590±0.030	1.283±0.067*	1.150±0.058	1.457±0.054
CHT (0.060)	1.800±0.321	1.600±0.231	1.542±0.043	0.817±0.120	0.800±0.100	1.505±0.042
CHT (0.030)	1.100±0.153	1.000±0.116	1.521±0.019	0.683±0.088	0.650±0.058	1.551±0.077
CHT (0.020)	0.633±0.088	0.600±0.058	1.620±0.014	0.367±0.088	0.367±0.088	1.507±0.128
CHT (0.015)	0.333±0.033	0.333±0.033	1.626±0.009	0.250±0.058	0.250±0.058	1.524±0.119

BNCs = Binucleated cells; MNs = micronuclei; Ab.C = cells with 1 or more micronuclei;
CBPI = Cytokinesis-Block Proliferation Index = $[1 \times N_1] + [2 \times N_2] + [3 \times (N_3 + N_4)]/N$, where N_1–N_4 represents the number of cells with 1-4 nuclei, respectively, and N is the total number of cells scored; S.E. = Standard Error; NC = Negative Control; CHT = Chlorothalonil; MMC = Mitomycin-C.
$^{*}P = 0.046$.