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Evaluation of Potential Genotoxic Effects Induced by Chlorothalonil in Human Lymphocytes in Vitro

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Abstract

Chlorothalonil is a widespread non-systemic fungicide used for the control of fungal infections in agriculture of vegetables and fruits. Due to the widespread use of chlorothalonil, chlorothalonil has been detected in all environmental systems, which poses potential risks to human health. The genotoxic effects of the fungicide Chlorothalonil were analyzed in cultured human peripheral lymphocytes by use of cytogenetic parameters including chromosomal aberrations, sister chromatid exchange and mitotic index. Blood samples were obtained from two healthy Non-smoker volunteers (aged 25 to 26 year old). Human lymphocytes were treated with four different concentrations of Chlorothalonil (1, 5, 20, and 50 µg/mL). The results of this study showed that Chlorothalonil significantly increased the frequency of chromosomal aberrations and sister chromatid exchange compared to the negative control. Moreover, chlorothalonil significantly decreased the mitotic value at all concentrations. Current findings show that Chlorothalonil is clastogenic and induces in vitro DNA damage in human lymphocytes.

Keywords: Chlorothalonil; Genotoxicity; Chromosome Aberrations; Mitotic Index; sister chromatid exchange; human peripheral lymphocytes.

1. Introduction

The rapid expansion of agriculture and the increasing demand for food has led to extensive use of pesticides. Fungicides, which protect plants from fungal diseases, are one of the most effective and extensively used pesticide groups (Petit *et al.* 2012; Ijaz *et al.* 2015). Chlorothalonil (CHT) is a highly reactive, widely used, broad-spectrum non-systemic chlorinated fungicide that is used on vegetables, fruit trees, and agricultural crops. CHT controls fungal and bacterial infestations by inhibiting cellular respiration and metabolism (Carlo-Rojas *et al.,* 2004; Liang *et al.,* 2010). Several studies confirmed CHT toxicity to fish, crustaceans, amphibian, and aquatic invertebrates; however, toxic effects were not found for small mammal avian species or honeybees (Du Gas *et al.,* 2017; Guerreiro *et al.,* 2017; Yu *et al.,* 2013). Chlorothalonil shows toxicity towards humans, with effects including contact dermatitis, severe eye and skin irritation and gastrointestinal problems (Penagos *et al.,* 2004).Moreover, several rodent studies have revealed that long-term treatment with CHT is associated carcinoma and increased rates of adenomas (Wilkinson and Killeen, 1996). According to the "International Agency for Research on Cancers (IARC)", there was inadequate evidence of CHT carcinogenicity in humans; however, CHT was classified as possibly carcinogenic to humans (Group 2B) (IARC, 1999). Genotoxic effects of CHT have been reported as absent in chromosome aberration assays in rats, mice, and Chinese hamsters (Mizens *et al.,* 1998; Dearfield *et al.,* 1993). Chlorothalonil also failed to increase DNA damage to bone marrow, thymus, liver, kidneys and leukocytes in rats after intraperitoneal injection at 200 and 2000 mg/kg (Godard *et al.,* 1999).

By contrast, recent studies demonstrated that CHT induced DNA damage in both in vivo and in vitro studies (Santovito *et al.,* 2018; Knopper *et al.,* 2005). These contradictory results as well as the extensive use of CHT led us to investigate the genetic effects of CHT in human lymphocytes in vitro using three genetic endpoints: CAs, SCEs, and MI.

2. Materials and Methods

2.1 Chemicals

All the chemicals used, including CHT, were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2 Lymphocyte cultures

Peripheral venous blood was obtained from two male donors in good health (both non-smokers, 24-26 years old); neither subject had exposure to any known mutagenic agents or drug therapies in the previous 24 months and had not had an x-ray for at least six months. They had no recent viral infections or a history of chromosome fragility. Both blood donors provided written informed consent, with the study gaining approval from al-Baha university ethics committee and carried out in line with ethical standards described in the 1964 Helsinki Declaration.

2.3 Chromosomal aberrations, sister-chromatid exchange assay and mitotic index

Cytogenetic analysis was conducted according to Zengin *et al.* (2011), with slight modifications. Heparinized whole-blood sample (0.2 mL) was added to 2.5 mL Chromosome Medium B (containing fetal bovine serum, heparin, antibiotics, and phytohaemagglutinin) supplemented with 10 µg/mL bromodeoxyuridine. Cultures were incubated in the dark, at 37 °C for 72 h and the cells were treated with 1, 5, 20 and 50 µg/mL of CHT dissolved in dimethyl sulfoxide (DMSO). A negative (DMSO) control, which did not exceed 0.3% (v/v) of the culture medium and a positive control (mitomycin-C (MMC), 0.20 mM) were included for each experiment to ensure validity of the assay. Colchicine (0.06 µg/mL) was added to the culture at the last 2 h of incubation time for arrest cell cycle at metaphase. The cultured peripheral blood lymphocytes were harvested by centrifugation (2000 rpm, 5 min) and treated with KCl (75 mM) for 30 min at 37 °C and fixed with freshly prepared cold methanol:glacial acetic acid (3:1 v/v) for 20 min at room temperature. Fixation process was repeated three times. At last, metaphase spreads were prepared by dropping the concentrated cell suspension onto slides. Slides for CAs were conventionally stained for 15 – 20 min with 5% Giemsa stain (pH = 6.8) prepared in Sorensen buffer. For the SCE assay, slides were stained by use of the FPG (fluorescence plus Giemsa) technique according to the method described by Speit and Houpter (1985), with some modifications. The slides were dried at room temperature. One hundred well spread metaphases by donor have been evaluated for CA (total of 200 metaphases per concentration), 50 metaphases have been recorded for each concentration in the SCE assy. In addition, MI was determined by scoring 1,000 cells from each donor.

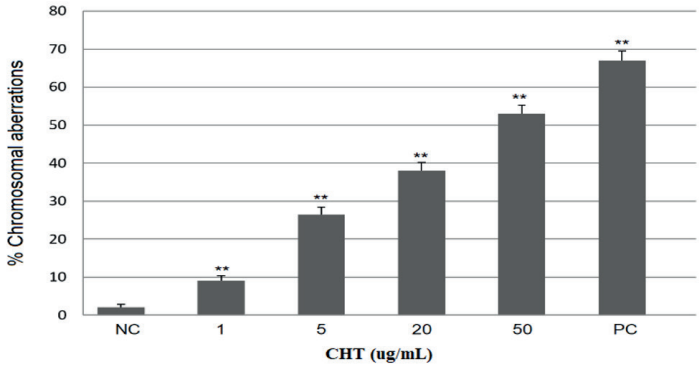
2.4 Statistical Analysis

All of the results were expressed as mean ± standard deviation. The differences among the groups were evaluated with Student's t-test. The mean difference was considered significant at the 0.05 level.

3. Results and Discussion

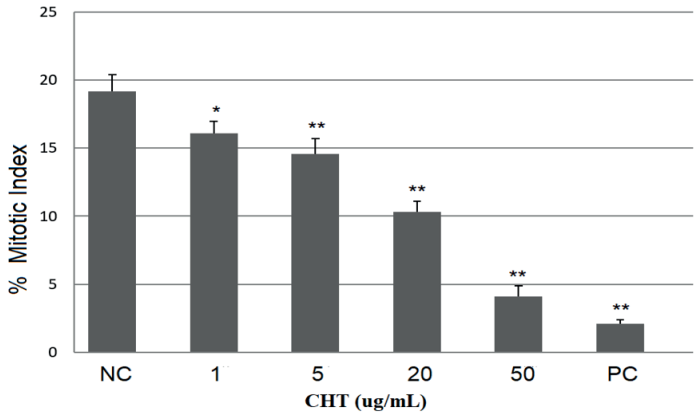
The effect of CHT on human lymphocytes was assessed by three endpoints. Figure 1 presents CAs induced in human lymphocytes culture after treatment with different concentrations of CHT. The results showed that the CHT significantly increased the frequency of CAs compared to the negative control. The frequency of chromosomal aberrations generally increased with increasing concentrations of CHT. The mean percentages of induced CAs were 9 ± 1.4, 26 ± 1.8, 38 ± 2.1 and 53 ± 2.2 % at CHT doses of 1, 5, 20 and 50 µg/mL, respectively. The potency of CHT for CAs induction was lower than that caused by the positive control.

A total of five types of chromosomal aberrations were observed, such as gaps, fragments, RCF, stickiness and polyploidy. Stickiness (33.6%) and gaps (19.1%) were the most common types of chromosomal abnormalities (Fig.3). Furthermore, compared with the negative control, CHT significantly decreased MI at all concentrations. Mitotic indexes of 16.25 ± 1.7, 14.65 ± 1.7, 10.25 ± 1.9, and 4 ± 0.8% were observed for CHT doses of 1, 5, 20 and 50 µg/mL, respectively, and these findings are summarized in Figure 2. However, these decrements were not as great as in the positive control. The results in Table (1) indicated that CHT significantly increased the frequency of SCE in all concentrations in comparison to the control. The mean values of the induced SCE were 4.2 ± 0.62, 6.8 ± 0.7, 10.1 ± 0.8 and 16.2 ± 1.2 at CHT doses of 1, 5, 20 and 50 µg/mL, respectively (Figure 4).



NC= Negative Control
PC= Positive Control
** Significant from the control P<0.01 (T- test).

Figure 1. Percentage of CAs induced in cultured human lymphocytes treated with different doses of CHT



NC= Negative Control
PC= Positive Control
* Significant from the negative control P<0.05 (T- test).
** Significant from the negative control P<0.01 (T- test).

Figure 2. Percentage of MI induced in cultured human lymphocytes treated with different doses of CHT

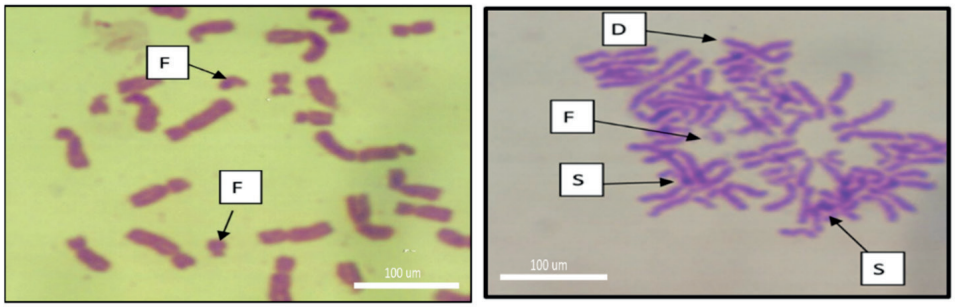


Figure 3. Different types of aberrations induced by CHT in human peripheral lymphocytes. (S) Sticky chromosomes; (F) Chromosomal fragments; (D) Chromatid deletion

Table 1. Frequency of SCEs in cultured human lymphocytes exposed to CHT

Dose (ug/ml)	No. of metaphases	mean ± S.D	Min-max SCE
1	100	4.2 ± 0.62 *	0-6
5	100	6.8 ± 0.7**	1-8
20	100	10.1 ± 0.8 **	2-12
50	100	16.2 ± 1.2 **	3-18
NC	100	1.4 ± 0.3	0-3
PC	100	24.5 ± 1.5 **	22-27

NC= Negative Control
PC= Positive Control
* Significant from the negative control P<0.05 (t test)
** Significant from the negative control P<0.01 (t test.

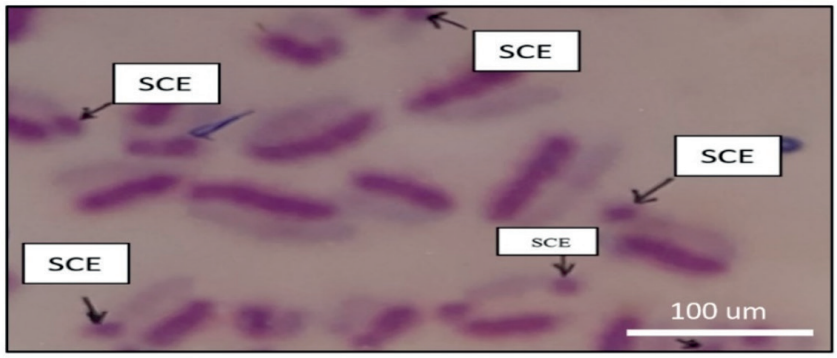


Figure 4. Sister chromatid exchange (SCE) in human peripheral lymphocytes after treated with CHT

Contact with pesticides has been associated with numerous human disorders, such as various types of cancer (Silva *et al.,* 2016; Falzone *et al.,* 2016). Moreover, there is a lack of published data regarding the genotoxicity of several commercial pesticides. Specifically, both in vitro and in vivo studies have examined the genotoxic potential of CHT, but the results have sometimes been conflicting (Lebailly *et al.,* 1997; Vigreux *et al.,* 1998). This study assessed the genetic impact of CHT on cultured human lymphocytes using three endpoints: CAs, SCEs and MI. The analyses of CAs and SCEs in human peripheral blood lymphocytes as well as the MI are sensitive indicators for genotoxic effects (Mamur *et al.,* 2010). Peripheral lymphocytes have been used extensively for the detection of the genotoxic effects of various compounds in many studies, as they are considered suitable for the detection of genome damage

(Azab *et al.,* 2017). According to the findings of this study, CHT significantly increased the number of CAs. The most common types of CA found were chromatid gap, polyploidy, fragment, RCF, and stickiness. When chromosome material is broken or exchanged, the changes in chromosome structure can cause chromosome aberrations. Most CAs will be fatal, but there are numerous corresponding aberrations that have vitality and may be responsible for genetic effects, both inherited and somatic (Swierenga *et al.,* 1991). Such results are consistent with previous studies that suggested CHT's ability to increase the frequency of CAs. It was reported that chronic treatment with CHT led to a significant increase in CAs for Chinese hamsters, mice, and rats (Dearfield *et al.,* 1993). Santovito *et al.* (2018) reported that CHT caused a significant increase in CA and micronucleus (MNi) frequencies in human lymphocytes. Vigreux *et al.* (1998) reported that CHT also yielded positive results, inducing CAs in Chinese Hamster Ovary cells (CHOK1). In human toxicology, SCE assay has proven to be a reliable and sensitive method due to its ability to provide biomarkers for the effects of human exposure to toxic agents (Wilson and Thompson, 2007; Bonassi *et al.,* 1999). The occurrence of SCEs is particularly likely when two sister chromatids of a duplicating chromosome engage in a symmetrical exchange of DNA segments during the S phase of the cell cycle. Sister chromatid exchange (SCE) can be increase by the effect of S phase-dependent DNA - damaging agents (Johnson *et al.,* 2009). The outcomes of this study demonstrated that, in comparison to a control, there were statistically significant variations in SCE induction frequencies in human lymphocyte cultures that had undergone exposure to CHT. These findings concur with those of Dearfield *et al.* (1993), who found that the treatment of mice, rats, and Chinese hamsters with high doses of CHT for five days in succession resulted in a rise in SCE frequencies, in contrast to treatment with one dose. Moreover, the aforementioned study also found that SCE frequencies increased in Chinese hamster ovary cells exposed to CHT. The mitotic index (MI) is a further parameter that was employed in the current study as a means of evaluating the cytotoxic potential of CHT. As such, MI plays a fundamental role in determining cell division rate (Moore *et al.,* 2011) and has been considered to represent an indication of the cytotoxicity of several compounds. A low MI is indicative of an inhibition of the cell cycle, which has a negative impact on cell division (do Carmo *et al.,* 2012). A significant reduction in MI was observed in response to CHT at all concentrations in comparison to the control. The reduction in MI could be attributed to an obstruction at G2, which could impede the cell from undergoing mitosis or reduce the ATP level and the pressure from the energy production center (Jain and Andsorbhoy, 1988; Yılmaz *et al.,* 2008; Zengin *et al.,* 2011). The MI outcome accords with the results in this study for CA and SEC assays. Several studies indicated that CHT increased the frequency of chromosomal damage measured with the SCGE assay (comet assay) in human peripheral blood lymphocytes (Lebailly *et al.,* 1997), Chinese hamster ovary cells (Vigreux *et al.,* 1998), and leukocytes of meadow voles (Knopper *et al.,* 2005). It is not known what mechanisms underpin CHT's genotoxic potential, either alone or in combination with additional compounds, though it has been demonstrated that inflammatory and cytotoxic processes can be triggered by CHT exposure. These processes have been proven to cause cytogenetic damage, DNA damage, and losses in the integrity of the cellular membrane (Santovito *et al.,* 2016). In conclusion, this study demonstrates that DNA damage and chromosomal alterations can be induced in human lymphocytes by CHT due to increases in SCE and CA frequencies. The repression of the MI demonstrates that CHT could potentially be responsible for the induction of the inhibition of cell growth or for arresting growth. These outcomes show that CHT has genotoxic and /or clastogenic potential.

Conflict of Interest

The author declares that no conflict of interest of any type exists in the current research.

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