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# *In vitro* effect of karathane LC (dinocap) on human lymphocytes

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**Karathane LC (active ingredient dinocap), a contact fungicide and a non-systemic acaricide was investigated for its ability to induce chromosome aberrations (CAs) and sister chromatid exchanges (SCEs) in cultured human lymphocytes of peripheral blood. In addition to the cytogenetic analysis, the effect of karathane LC on the cell proliferation kinetics (CPK) by the replication index (RI) was studied. The mitotic index (MI) was also determined to detect the cytotoxic effect. Lymphocytes were treated with four different concentrations (5, 10, 15 and 20 μg/ml) of karathane LC for 24 and 48 h. Significant differences between exposed and non-exposed groups found in CAs, SCEs and MI demonstrate the mutagenic, clastogenic and also the cytotoxic effect of karathane LC.**

## Introduction

Despite the valuable contributions associated with the use of pesticides, many of these biologically active chemicals represent a potential hazard to humans and to nature. Many of these chemicals are carcinogenic (1–3) and mutagenic, inducing gene mutations, chromosomal alterations and DNA damage (4,5). In epidemiological studies, a significant increase was found in the risk of leukemia and multiple myeloma and also stomach, liver, pancreatic and bladder cancer associated with pesticide exposure (3,6–8). Owing to increasing evidence of carcinogenic, mutagenic and teratogenic effects in exposed humans and experimental animals, interest in pesticide toxicity is increasing every day.

A number of *in vitro* and *in vivo* test systems have been developed to study the effects of chemicals on cellular DNA and chromosomes. The analysis of chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) in peripheral blood lymphocytes are the widely used biomarkers of genotoxic, carcinogenic and/or mutagenic effects (9–12). CAs can trigger the development of cancer (13–15) and an increased frequency of SCEs could be an indicator of persistent DNA damage (11,16).

In this study, cultured human peripheral lymphocytes were used for the evaluation of genotoxic and mutagenic activity of the commercial fungicide karathane LC with the active ingredient dinocap. Dinocap is a contact fungicide and a non-systemic acaricide. It is registered for use on powdery mildews in pome fruit, stone fruit, citrus fruit, soft fruit, vines, cucurbits, ornamentals, tobacco, hops and some vegetables. It is also used in the control of mites (*Panonycus*, *Tetranycus* and *Aculus* sp.) in fruit trees and vines. EPA has classified dinocap as a Group E 'not likely' carcinogen (17). The results on the genotoxicity of dinocap covering a large number of systems and organisms *in vivo* and *in vitro* have taken into account several different genetic endpoints, including DNA damage, gene mutations and chromosomal damage, and are negative (18). However, contrasting results have been reported using the Ames test and the Allium test, wherein positive results were obtained by Moriya *et al*. (19) and Çelik (20). The present study was undertaken to examine the ability of karathane LC (containing 475 g/l dinocap) to induce CAs and SCEs in cultured human peripheral lymphocytes. Changes in cell proliferation kinetics (CPK) by the replication index (RI) and cytotoxic effect by the mitotic index (MI) were also evaluated.

## Materials and methods

Human peripheral blood cells were used as the test system, and the commercial fungicide karathane LC as the test substance. The chemical structure of dinocap, the active component of karathane LC, is shown in Figure 1.

Peripheral blood was obtained from healthy non-smoking male (aged 25 years) and female (aged 25 years) donors, free of any known exposure to genotoxic agents. A whole blood sample of 0.2 ml was added to 2.5 ml Chromosome Medium B (Biochrom) supplemented with 10 μg/ml bromodeoxyuridine. The cultures were incubated at 37°C for 72 h. The cells were treated with 5, 10, 15 and 20 μg/ml concentrations of karathane LC (obtained from Agro-San, Turkey) on the basis of active ingredient dinocap for 24 and 48 h. In addition, a negative and a positive control (mitomycin-C (MMC), 0.10 μg/ml) were also used for each experiment to ensure the validity of the assay. The test substance karathane LC and positive control MMC were dissolved in distilled water. Colchicine (0.06 μg/ml) was added during the last 2 h of culture. To collect the cells, the cultures were centrifuged (1200 r.p.m., 10 min) and treated with hypotonic solution (0.075 M KCl) for 30 min at 37°C. Then the cells were fixed in cold methanol:acetic acid (3:1) for 20 min at room temperature. The treatment with the fixative was repeated three times. The cells were spread on glass slides and air dried.

For chromosomal aberrations, 1-day-old slides were stained with 5% Giemsa (pH 6.8) prepared in Sorensen buffer solution, for 15–20 min, washed in distilled water, dried at room temperature and mounted with depex. For the SCEs, slides were stained with Giemsa according to the Speit and Houpter's method (21) with some modifications.

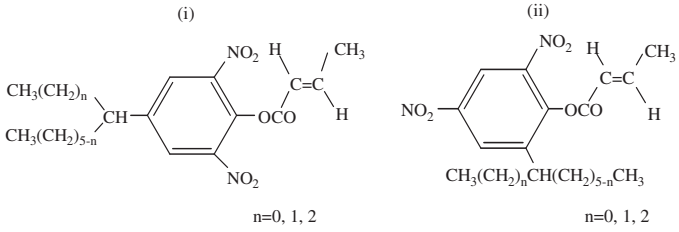


**Fig. 1.** Chemical structure of dinocap, the active component of karathane LC. Compounds of type (i) are more effective as a fungicide, and those of type (ii) as an acaricide.

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A total of 200 well spread metaphases/concentration (100 metaphase/donor) were analysed for the CA assays. Numerical and structural chromosomal abnormalities within each metaphase were recorded. A total of 50 second mitosis was analysed in each treatment for the SCE assays. The results were recorded as the frequency of SCEs/metaphase. The MI was determined by scoring 1000 cells from each donor.

In the SCE study, a total of 200 cells (100 cells from each donor) were also scored to obtain the Proliferative Rate Index (PRI), calculated according to the formula M1 + 2M2 + 3M3/N, where M1, M2 and M3 represent the number of cells undergoing first, second and third mitosis and N the number of observed cells (22,23).

The significance between the percentage of abnormal cells, CA/cell, PRI and MI in treated cultures and their controls were determined using the *z*-test. The significance between mean SCE in treated cultures and their controls were determined using the *t*-test. Regression analysis was carried out to find out the dose–response relationship.

## Results

Analysis of the cultured human lymphocytes revealed that karathane LC induced various types of chromosomal aberrations compared with the control (Table I). The data obtained from two donors are equivalent and are pooled together. Chromatid breaks, fragments, sister chromatid union (except 5 μg/ml), dicentric chromosomes and gaps were observed with all concentrations of karathane LC at both treatment times. Ring chromosomes, chromatid exchanges and polyploidies were also observed at some treatments. In addition, contraction and morphological defect of chromosomes were also determined. Concentrations of 10, 15 and 20 μg/ml of karathane LC induced a significant amount of abnormal cells (%) over negative control at both 24 and 48 h when gaps were included. Concentrations of 5 μg/ml also induced higher aberrations at both the 24- and 48-h durations; however, they are not significant over the control. When gaps were excluded, 15 and 20 μg/ml concentrations at 24 h and 10, 15 and 20 μg/ml concentrations at 48 h showed significant increase in the percentage of abnormal cells. Similarly, chromosomal aberrations including gaps/cell are significantly increased at 10, 15 and 20 μg/ml concentrations over the negative control at both 24- and 48-h treatments. CA/cell values without a gap at 10, 15 and 20 μg/ml concentrations were also significantly different from the negative control at both 24- and 48-h periods. The regression analyses revealed that there is a significant correlation between the dose and the percentage of abnormal cells at 24 h ($r = 0.98$, +G/−G) and 48 h ($r = 0.97$, +G and $r = 0.99$, −G). The regression analyses between the concentration and CA/cell was also correlated significantly at 24 h ($r = 0.99$, +G and $r = 0.98$, −G) and 48 h ($r = 0.99$, +G/−G). The 48-h treatment period induced more abnormalities and higher frequency of CA/cell than the 24-h treatment in all concentrations. The potency of karathane LC for the induction of abnormal cells (%) and CA/cell (+G/−G) was lower than those of the positive control, MMC, at both the 24-and 48-h treatments.

It is apparent from Table II that karathane LC also induced SCEs. SCE frequency was significantly higher than in the negative control with all concentrations at 48 h, but only with 20 μg/ml at the 24-h treatment. The 48-h period induced more SCE/cell than the 24-h treatment at all concentrations. Minimum and maximum numbers of SCEs are also given in Table II. The increase of SCEs was dose-dependent at both the 24-h ($r = 0.90$) and 48-h ($r = 0.98$) treatment periods. The potency of karathane LC for the induction of SCEs was lower than those of the positive control, MMC at both the 24- and 48-h treatments.

Karathane LC, in general, decreased the RI at both treatment periods with increasing concentrations ($r = -0.95$ at both 24 and 48 h) but this decrease was not significantly different from the negative control value (Table II). RI was lower than those of the positive control at the 20 μg/ml concentration at both the 24- and 48-h treatments. Karathane LC also decreased MI significantly in a progressive dose-related manner at both 24- ($r = -0.99$) and 48-h ($r = -0.98$) treatment groups compared with the negative control. Reduction of the MI value was more with the test chemical than with the positive control MMC at 20 μg/ml for 24 h and at 15 and 20 μg/ml concentrations for 48 h. Decrease in MI was not significant only at 5 μg/ml for the 24-h treatment.

## Discussion

The results obtained in this *in vitro* study demonstrate that karathane LC induced CAs as well as SCEs in cultured

**Table I.** Induction of chromosomal aberrations in cultured human lymphocytes treated with karathane LC

| Treatment | Dose (μg/ml) | Aberrations | | | | | | | | Abnormal cell (%) ± SE (+G) | Abnormal cell (%) ± SE (−G) | CA/cell ± SE (+G) | CA/cell ± SE (−G) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | B | F | SU | DC | G | R | CE | P | | | | |
| 24 h | | | | | | | | | | | | | |
| Negative control | | 2 | 1 | 1 | 1 | — | — | — | — | 2.50 ± 1.00 | 2.50 ± 1.00 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| MMC | 0.1 | 17 | 9 | 3 | 2 | 1 | 2 | 1 | — | 15.00 ± 2.50 | 14.50 ± 2.48 | 0.18 ± 0.03 | 0.17 ± 0.03 |
| Karathane LC | 5 | 3 | 1 | — | 1 | 1 | 1 | — | — | 3.50 ± 1.30 | 3.00 ± 1.20 | 0.04 ± 0.01 | 0.03 ± 0.01 |
| | 10 | 4 | 3 | 2 | 2 | 1 | 1 | 1 | — | 7.00 ± 1.80[a] | 6.50 ± 1.74 | 0.07 ± 0.02[a] | 0.07 ± 0.02[a] |
| | 15 | 5 | 6 | 2 | 4 | 2 | — | 1 | 1 | 9.50 ± 2.00[b] | 8.50 ± 1.97[b] | 0.11 ± 0.02[b] | 0.10 ± 0.02[b] |
| | 20 | 7 | 5 | 4 | 3 | 3 | 1 | — | 1 | 11.00 ± 2.20[c] | 9.50 ± 2.00[b] | 0.12 ± 0.02[c] | 0.11 ± 0.02[b] |
| 48 h | | | | | | | | | | | | | |
| Negative control | | 3 | 2 | 2 | — | — | — | — | — | 3.50 ± 1.30 | 3.50 ± 1.30 | 0.04 ± 0.01 | 0.04 ± 0.01 |
| MMC | 0.1 | 23 | 12 | 4 | 2 | 1 | 1 | 1 | 1 | 19.50 ± 2.80 | 19.00 ± 2.77 | 0.23 ± 0.03 | 0.23 ± 0.03 |
| Karathane LC | 5 | 5 | 3 | 3 | 1 | 1 | — | — | — | 6.50 ± 1.74 | 6.00 ± 1.68 | 0.07 ± 0.02 | 0.07 ± 0.02 |
| | 10 | 8 | 4 | 4 | 2 | 3 | — | — | — | 10.00 ± 2.12[b] | 8.50 ± 1.97[a] | 0.11 ± 0.02[b] | 0.11 ± 0.02[b] |
| | 15 | 13 | 4 | 2 | 2 | 4 | — | 1 | 1 | 11.50 ± 2.25[b] | 9.50 ± 2.00[b] | 0.14 ± 0.02[c] | 0.14 ± 0.02[c] |
| | 20 | 14 | 5 | 5 | 1 | 3 | 1 | 2 | 1 | 13.00 ± 2.37[c] | 11.50 ± 2.25[b] | 0.16 ± 0.02[c] | 0.16 ± 0.02[c] |

B, chromatid break; F, fragment; SU, sister chromatid union; DC, dicentric; G, gap; R, ring chromosome; CE, chromatid exchange; P, polyploidy.
[a]Significantly different from the control $P < 0.05$ (*z*-test).
[b]Significantly different from the control $P < 0.01$ (*z*-test).
[c]Significantly different from the control $P < 0.001$ (*z*-test).

**Table II.** The frequency of the SCE, RI and the MI in cultured human lymphocytes treated with karathane LC

| Treatment | Dose (μg/ml) | Minimum–maximum SCE | SCE/cell ± SE | M1 | M2 | M3 | RI ± SE | MI ± SE |
|---|---|---|---|---|---|---|---|---|
| 24 h | | | | | | | | |
| Negative control | — | 2–8 | 5.12 ± 0.286 | 44 | 70 | 86 | 2.21 ± 0.06 | 6.25 ± 0.54 |
| MMC | 0.1 | 10–33 | 20.74 ± 0.882 | 76 | 64 | 60 | 1.92 ± 0.05 | 3.70 ± 0.42 |
| Karathane LC | 5 | 1–10 | 5.18 ± 0.380 | 46 | 94 | 60 | 2.07 ± 0.05 | 5.70 ± 0.51 |
| | 10 | 1–12 | 5.38 ± 0.316 | 64 | 82 | 54 | 1.96 ± 0.05 | 4.80 ± 0.47[a] |
| | 15 | 1–10 | 5.46 ± 0.283 | 70 | 74 | 56 | 1.93 ± 0.06 | 4.15 ± 0.44[b] |
| | 20 | 2–11 | 6.32 ± 0.314** | 72 | 78 | 50 | 1.89 ± 0.05 | 3.60 ± 0.41[c] |
| 48 h | | | | | | | | |
| Negative control | — | 1–11 | 5.16 ± 0.297 | 45 | 72 | 83 | 2.19 ± 0.06 | 5.95 ± 0.52 |
| MMC | 0.1 | 11–38 | 24.26 ± 0.945 | 93 | 72 | 35 | 1.71 ± 0.07 | 3.20 ± 0.39 |
| Karathane LC | 5 | 1–14 | 6.68 ± 0.368** | 58 | 96 | 46 | 1.94 ± 0.05 | 4.55 ± 0.46[a] |
| | 10 | 2–16 | 7.32 ± 0.450*** | 74 | 72 | 54 | 1.90 ± 0.06 | 3.55 ± 0.41[c] |
| | 15 | 3–15 | 7.76 ± 0.402*** | 78 | 74 | 48 | 1.85 ± 0.06 | 2.85 ± 0.37[c] |
| | 20 | 3–16 | 8.96 ± 0.430*** | 110 | 56 | 34 | 1.62 ± 0.07 | 2.40 ± 0.34[c] |

[a]Significantly different from the control $P < 0.05$ (*z*-test).
[b]Significantly different from the control $P < 0.01$ (*z*-test).
[c]Significantly different from the control $P < 0.001$ (*z*-test).
**Significantly different from the control $P < 0.01$ (*t*-test).
***Significantly different from the control $P < 0.001$ (*t*-test).

human peripheral lymphocytes in a dose-dependent manner. The seven types of aberrations recorded were chromatid break, fragment, sister chromatid union, dicentric chromosome, chromatid exchange, ring chromosome and polyploidy as observed in other studies (4,5,9,10,24). This fungicide has also induced chromosome breaks, sister chromatid union, bridges, laggards, c-mitosis, stickiness, multipolarity and polyploidy in *Allium cepa* (20). In this study, karathane LC significantly increased CAs and CA/cell frequency both with and without a gap. Several studies on the clastogenic action of different compounds have suggested that gaps may indeed be associated with a mutagenic action and may be considered as aberrations (11,25,26). However, the gaps were not elevated as CA by some authors (9,27). Chromosomal aberrations are very important biomarkers for DNA damage induced by chemical agents with or without gaps (26).

In this study, the most pronounced aberration was the chromatid break, suggesting that the chemical acts mostly in the late S or $G_2$ phase of the cell cycle (28). Fragments were the second common aberrations in the karathane LC-treated lymphocytes. Presence of fragments reveals the increased mutagenic potential of the test compound (29). Fragments and sister chromatid union observed in this study may result from the terminal deletion (30). In this study, quadriradial and triradial type of chromatid exchanges were also observed. Quadriradial chromosomes can be formed from homologous or non-homologous chromosomes (31).

Karathane LC has also caused polyploidy in some cells, indicating that the spindle apparatus made of proteins seems to be affected (9,27,32). Dicentric and ring chromosomes resulting from breakage–fusion–bridge cycle (28) were also induced by karathane. In this investigation, chromosome contractions were also evident in the cultures treated with the highest concentration of karathane LC. The contraction may have arisen from the effects of the chemical on the histone proteins (9).

Karathane LC has not induced a significant effect on the PRI, indicating that it does not influence the kinetics of cell growth. Similar results were obtained by some other pesticides (33–35). However, karathane LC significantly decreased the MI in a dose-related manner. Karathane LC may possibly decrease the MI by affecting $G_2$ and prophase or synthesis and assembly and/or formation of the mitotic spindle (24). Karathane LC has also decreased MI in *Allium cepa* (20).

Karathane LC induced the formation of SCEs in 20 μg/ml concentration at 24 h and all concentrations at 48-h treatments compared with the control. SCE analysis is one of the most sensitive genotoxic assays and has been widely used to detect the mutagenic and carcinogenic potential of chemicals (26,36). SCEs arise from the reciprocal exchange of DNA at apparently identical loci of the sister chromatids of a duplicated chromosome in response to a damaged DNA template (36–38). The SCEs were induced by substances, which form covalent adducts with DNA or by those that interfere in the metabolism or in the DNA repair reactions (39). Our results are in agreement with those obtained after treating the human peripheral lymphocytes with different pesticides (4,9,24).

In conclusion, the results obtained in this research suggest that karathane LC should be considered as cytotoxic, clastogenic and mutagenic *in vitro* and as posing a genotoxic hazard to humans in all probability. Pesticides that are genotoxic–mutagenic to somatic cells could affect germinal cells as well (40,41). Hence, human exposure to karathane LC should be restricted.

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