

## *In vitro* CYTOGENETIC EFFECTS OF CANNABIDIOL ON HUMAN LYMPHOCYTE CULTURES\*

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### ABSTRACT

The *in vitro* effect of cannabidiol (CBD) on mitotic index and frequency of cells with numerical and/or structural aberrations was studied in human lymphocyte cultures. In the first phase of the study, CBD was dissolved in absolute ethyl alcohol (0.01 ml/ml medium) at the concentrations of 0.001, 0.01, 0.1, 1.0 and 10.0  $\mu\text{g}$  CBD/ml medium, and in the second phase ethanol was evaporated before the culture medium was added. The clastogenic effect of CBD was stronger when the drug was combined with alcohol. The action of ethanol was predominantly antimitogenic, whereas CBD had a sharper effect on the production of cells with chromosome aberrations. After ethanol evaporation, the proportion of cells with structural chromosome aberrations maintained an approximately linear increasing relationship with the CBD concentration.

### INTRODUCTION

Cannabidiol (CBD) is a natural product found in recently picked *Cannabis sativa* plants from which it can be extracted in the crystalline form (Salemink, 1976). In contrast to other cannabinoids\*\* present in the plant, CBD has no hallucinogenic action on man (Dalton *et al.*, 1976; Turner *et al.*, 1980) but has an anticonvulsant effect both on laboratory animals (Turkanis, 1974; Carlini *et al.*, 1975; Karler *et al.*, 1982) and on man (Cunha *et al.*, 1980; Carlini and Cunha, 1981), as well as a hypnotic and ansiolytic effect (Carlini *et al.*, 1979; Zwicker *et al.*, 1982).

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\* Part of a thesis submitted by G.J.F.G. to the Instituto de Biociências, Universidade de São Paulo, in partial fulfillment of the requirements for the Masters degree.

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\*\*Group of substances with a cyclic carbon chain without nitrogen.

The literature concerning the effect of CBD on genetic material is still very limited. *In vivo* studies on marihuana users who received oral doses of CBD combined or not with other cannabinoids did not indicate an increase in the frequency of cells with chromosome aberrations (Nichols *et al.*, 1974; Matsuyama and Fu, 1981). *In vitro* administration of marihuana preparations containing CBD did not increase the proportion of cells with chromosome aberrations but altered normal chromosome segregation (Martin *et al.*, 1974; Stenchever *et al.*, 1976; Henrich *et al.*, 1980). In a study on mice treated with CBD, Zimmerman and Raj (1980) observed an increased frequency of micronucleoli in polychromatic bone marrow, red blood cells and of cells with chromosome aberrations in blood.

The objective of the present study was to evaluate the possible *in vitro* cytogenetic effects of CBD on human lymphocyte short-term cultures by karyotyping and mitotic index analysis. CBD, a component of marihuana, whose isolated effect has been little investigated, in addition to being consumed by marihuana smokers, has been indicated as a drug with possible therapeutic uses for man.

## MATERIAL AND METHODS

Lymphocytes from 20 ml heparinized peripheral blood were cultured by the method of Moorhead *et al.* (1960), modified. Blood was obtained from a single individual with no history of viral infection or of exposure to drugs and radiation. After red blood cell sedimentation, 0.4 ml of the supernatant was added to flasks containing 5 ml culture medium (RPMI-1640 Gibco, supplemented with fetal calf serum, penicillin and streptomycin). The study consisted of two phases:

**Phase 1** – three cultures were prepared for each of the five dilutions of the drug in absolute ethyl alcohol (0.001, 0.01, 0.1, 1.0 and 10.0  $\mu\text{g}$  CBD/ml medium), as well as drug-free control cultures and control cultures containing 0.01 ml alcohol/ml medium. All cultures were incubated at 37°C for 72 hours. Two hours before fixation, 0.1 ml colchicine ( $4.5 \times 10^{-5}$  M) was added. Slides were prepared, stained with Giemsa and coded for use in a blind test.

**Phase 2** – using blood from the same individual, the previous test was repeated after alcohol evaporation. The same dilutions of CBD in alcohol were used and the flasks were incubated at 37°C for 24 hours before starting the culture, a time sufficient for alcohol evaporation as determined by thin-layer chromatography.

One hundred mitoses from each culture were analyzed in the two experiments, for a total of 1400 cells. Cytogenetic analysis was based on the detection of numerical chromosome aberrations (cells with a chromosome number of 44, 45 or  $\geq 47$ ) and/or structural chromosome aberrations such as breaks, deletions and chromatid and chromosome rearrangements. Using a blind test, the mitotic index was also calculated as percentage of metaphases in 2000 nuclei from each culture.

*Statistical analysis*

The data were submitted to analysis of variance and when significant results were obtained they were further analyzed by the multiple comparison test of Student-Newman-Keuls. When heterogeneity among variances was detected, the data were submitted to analysis by the nonparametric Kruskal-Wallis test. The critical level of rejection of the null hypothesis was considered to be 5% of probability.

**RESULTS***Mitotic index*

Tables I and II show the mitotic indices obtained in phase 1 and phase 2, respectively. Analysis by the nonparametric Kruskal-Wallis test showed that the data did not reach statistical significance at the critical level of probability.

Table I - Mitotic index (%) detected in CBD-treated and control cultures in phase 1 of the experiment.

Cultures	No. of metaphases	Total No. of nuclei	%
Control	85	2,000	4.25
Control with ethanol	23	2,000	1.15
0.001 $\mu\text{g}$ CBD	17	2,000	0.85
0.01 $\mu\text{g}$ CBD	26	2,000	1.30
0.1 $\mu\text{g}$ CBD	25	2,000	1.25
1.0 $\mu\text{g}$ CBD	20	2,000	1.00
10.0 $\mu\text{g}$ CBD	23	2,000	1.15

$$\chi^2 = 11.72; \text{d.f.} = 6; 0.05 < P < 0.10.$$

CBD present in combination with alcohol in 72-hour lymphocyte cultures caused significant aberrations in mitotic index only at the 6% level of probability. The frequency of metaphase cells observed in the control culture was on average four times higher than in the control culture containing ethanol and did not differ from the remaining cultures treated with alcohol (Table I).

After ethanol evaporation, there was no decrease in mitotic index in control cultures or in cultures containing CBD (Table II). Even though the mean mitotic index of cultures treated with CBD was lower than that detected in control cultures, the difference was not statistically significant ( $P = 0.35$ ).

Table II - Mitotic index (%) detected in CBD-treated and control cultures in phase 2 of the experiment.

Cultures	No. of metaphases	Total No. of nuclei	%
Control	43	2,000	2.15
Control with evaporated ethanol	39	2,000	1.95
0.001 $\mu\text{g}$ CBD	28	2,000	1.40
0.01 $\mu\text{g}$ CBD	27	2,000	1.35
0.1 $\mu\text{g}$ CBD	37	2,000	1.85
1.0 $\mu\text{g}$ CBD	28	2,000	1.40
10.0 $\mu\text{g}$ CBD	33	2,000	1.65

$$\chi^2 = 6.91; \text{d.f.} = 6; 0.30 < P < 0.50.$$

### *Chromosome aberrations*

Figure 1 presents some of the types of chromosome aberrations observed in phase 1 and phase 2 of the experiment. Some cells presented more than one aberration and the frequency of chromosomal alterations per cell was considered in the analysis of the results.

Chromosome aberrations such as chromatid and chromosome breaks and, less frequently, deletions and rearrangements were detected. The frequency of cells with numerical aberrations was low in both experimental phases. The proportion between chromosome breaks and chromatid breaks ranged from three times in phase 1 to nine times in phase 2.

Tables III and IV present the total frequency of cells with chromosome aberrations in control cultures and in cultures with increasing CBD dilutions. Analysis of variance of the results was significant at the 5% level of probability in both experimental phases.

In phase 1, the total frequency of cells with structural chromosome aberrations in the control culture was lower than in the remaining cultures, which did not differ amongst themselves (Table III). The frequency of cells with structural chromosome alterations was sharply lower in the control culture (3%) compared to that of the control culture containing ethanol (16%); in turn, the frequency of the latter did not differ statistically from that observed in cannabidiol-treated cultures, except for the culture treated with 0.01  $\mu\text{g}$  CBD/ml medium, which reached 31% (Table III).

In phase 2 (alcohol evaporation), the control culture presented a statistically

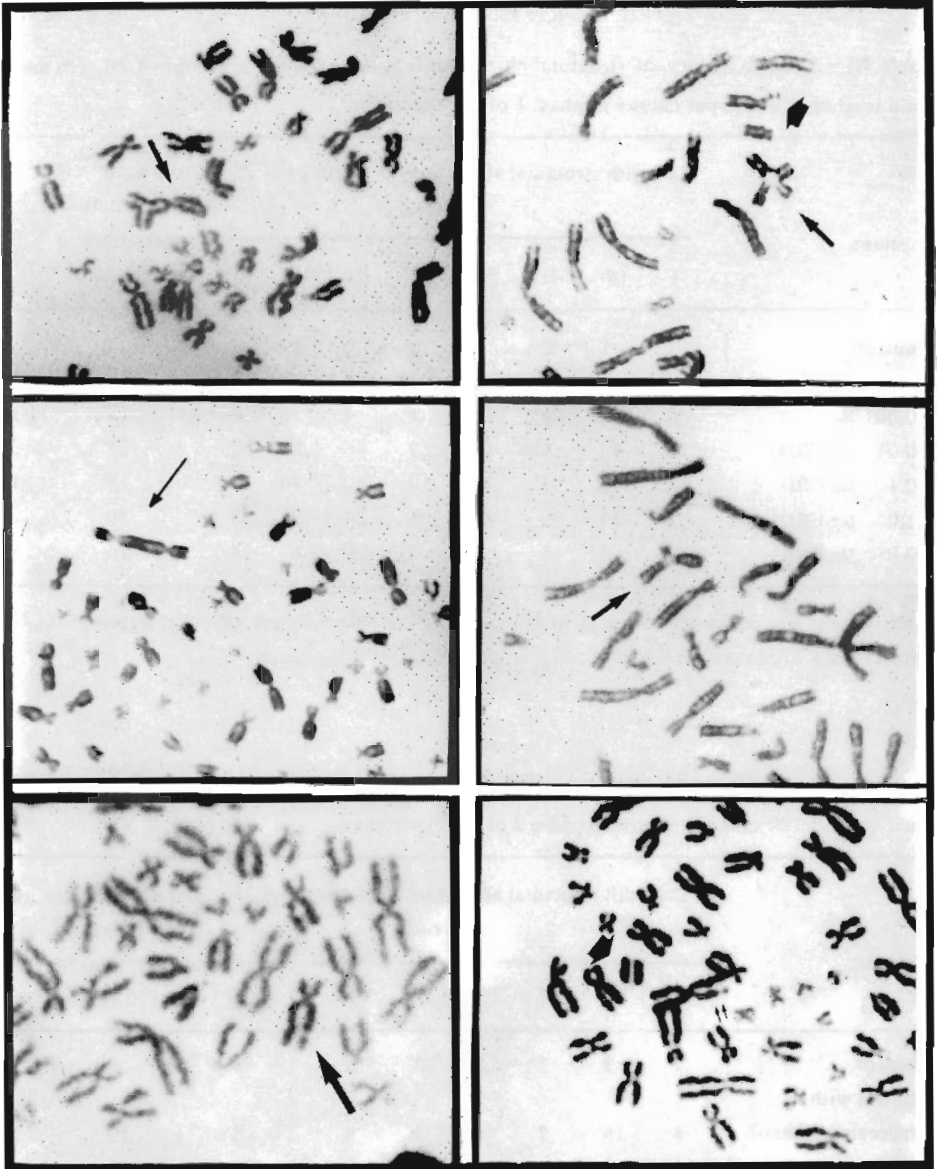


Figure 1 - Metaphase chromosomes with chromatid deletions (  $\leftarrow$  ), chromatid breaks ( = ), chromosome breaks (  $\blacklozenge$  ), chromatid rearrangements (  $\rightarrow$  ), and chromosome rearrangements (  $\leftarrow$  ).

different frequency of chromosome aberrations when compared with the culture treated with 1.0  $\mu\text{g}$  CBD/ml medium, while the remaining comparisons did not reveal any statistical differences (Table IV).

Table III - Total frequency of structural chromosome aberrations per 4 groups of 25 cells each, for a total of 100 cells per culture in phase 1 of the experiment.

Cultures	Cell with structural aberrations in 4 groups of 25 cells each								Total no. of cells with structural aberrations in 100 cells	f%
	I	f%	II	f%	III	f%	IV	f%		
Control	1	4	1	4	0	0	1	4	3	3
Control with ethanol	7	28	4	16	2	8	3	12	16	16
0.001 $\mu\text{g}$ CBD	3	12	4	16	1	4	3	12	11	11
0.01 $\mu\text{g}$ CBD	10	40	4	16	9	36	8	32	31	31
0.1 $\mu\text{g}$ CBD	2	8	3	12	10	40	4	16	19	19
1.0 $\mu\text{g}$ CBD	6	24	6	24	6	24	2	8	20	20
10.0 $\mu\text{g}$ CBD	2	8	3	12	5	20	6	24	16	16

Data were analyzed by the Student-Newman-Keuls test. Mean square between groups = 0.0230; mean square within groups = 0.0047;  $F_{(6; 21)} = 4,92$ ;  $P < 0.01$ .

Table IV - Total frequency of cells with chromosome aberrations in per 4 groups of 25 cells each, for a total of 100 cells per culture in phase 2 of the experiment.

Cultures	Cell with structural aberrations in 4 groups of 25 cells each								Total no. of cells with structural aberrations in 100 cells	f%
	I	f%	II	f%	III	f%	IV	f%		
Control	2	8	2	8	0	0	1	4	5	5
Control with evaporated ethanol	4	16	2	8	2	8	2	8	10	10
0.001 $\mu\text{g}$ CBD	3	12	4	16	3	12	8	32	18	18
0.01 $\mu\text{g}$ CBD	9	36	7	28	6	24	1	4	23	23
0.1 $\mu\text{g}$ CBD	4	16	8	32	2	8	3	12	17	17
1.0 $\mu\text{g}$ CBD	9	36	11	44	5	20	5	20	30	30
10.0 $\mu\text{g}$ CBD	6	24	5	20	8	32	3	12	22	22

Data were analyzed by the Student-Newman-Keuls test. Mean square between groups = 0.0202; mean square within groups = 0.0056;  $F_{(6; 21)} = 3.62$ ;  $0.05 < P < 0.01$ .

## DISCUSSION

CBD maintained in culture together with ethanol induced a drastic decrease in mitotic index and an increase in the total frequency of cells with structural chromosome aberrations. A similar effect was also observed in the control culture containing ethanol only, and for this reason a second experimental phase was planned in an attempt to eliminate the effect of ethanol, which was used as the CBD vehicle in the cultures.

In phase 2, after a chromatographic test it was possible to demonstrate that CBD was dissolved in the culture medium after alcohol evaporation. Even though a quantitative analysis of CBD was not done, concentrations close to those planned in the investigation were obtained. However, comparison of the cytogenetic results between the control cultures indicated that alcohol evaporation was not complete.

After alcohol evaporation, the mitotic index was not significantly changed when CBD-treated cultures were compared with control cultures. However, the frequency of metaphase nuclei in the control culture of phase 1 was approximately twice that observed in phase 2. This difference was probably due to several factors not subjected to systematic control. When the two experimental phases are compared, the predominant effect of alcohol on the reduction of mitotic index becomes clear, as does the absence of an antimutagenic effect of CBD after alcohol evaporation. Similar results were obtained by Neu *et al.* (1970) using ethyl alcohol added to lymphocyte cultures at the same concentration 24 hours before the addition of colchicine.

As to the effect of alcohol on chromosome aberrations, the control culture containing ethanol analyzed in phase 1 presented a mean frequency five-fold higher than observed in the alcohol-free control culture. However, after alcohol evaporation this value fell to twice the control value, suggesting a residual effect of alcohol in the evaporated cultures. However, the literature on the cytogenetic effects of ethanol *in vitro* are quite contradictory in terms of the amount of drug that would cause chromosome damage (Neu *et al.*, 1970; Bregman *et al.*, 1971; Cadotte *et al.*, 1973; Badr *et al.*, 1977; Obe and Ristow, 1979).

The results obtained here in the two experimental phases show that CBD had a greater clastogenic effect when combined with alcohol. This fact may be due to a potentiating action of alcohol or even to greater cell penetration by CBD in the presence of alcohol since CBD is a liposoluble substance (Nahas *et al.*, 1976). After alcohol evaporation there was a practically increasing effect of CBD in the production of cells with structural chromosome aberrations even though the effect was statistically significant at the 5% level only at the 1.0  $\mu\text{g}$  CBD/ml dilution. This action was not clearly linear, probably because of CBD concentration, differential alcohol evaporation, and random drug precipitation especially at concentrations higher than 1.0  $\mu\text{g}$  CBD/ml medium.

Figure 2 permits a comparative analysis of the effect of alcohol and of CBD on mitotic index and chromosome aberration rate. The figure indicates a synergistic action between CBD and alcohol. Alcohol appears to have a predominantly antimitotic effect resulting in reduced availability of cells for the detection of the action of CBD. In the absence or in the presence of reduced amounts of alcohol, this antimitogenic action practically disappears (A and B in Figure 2) and the almost linearly increasing effect of CBD on the frequency of chromosome aberrations becomes evident (C and D in Figure 2).

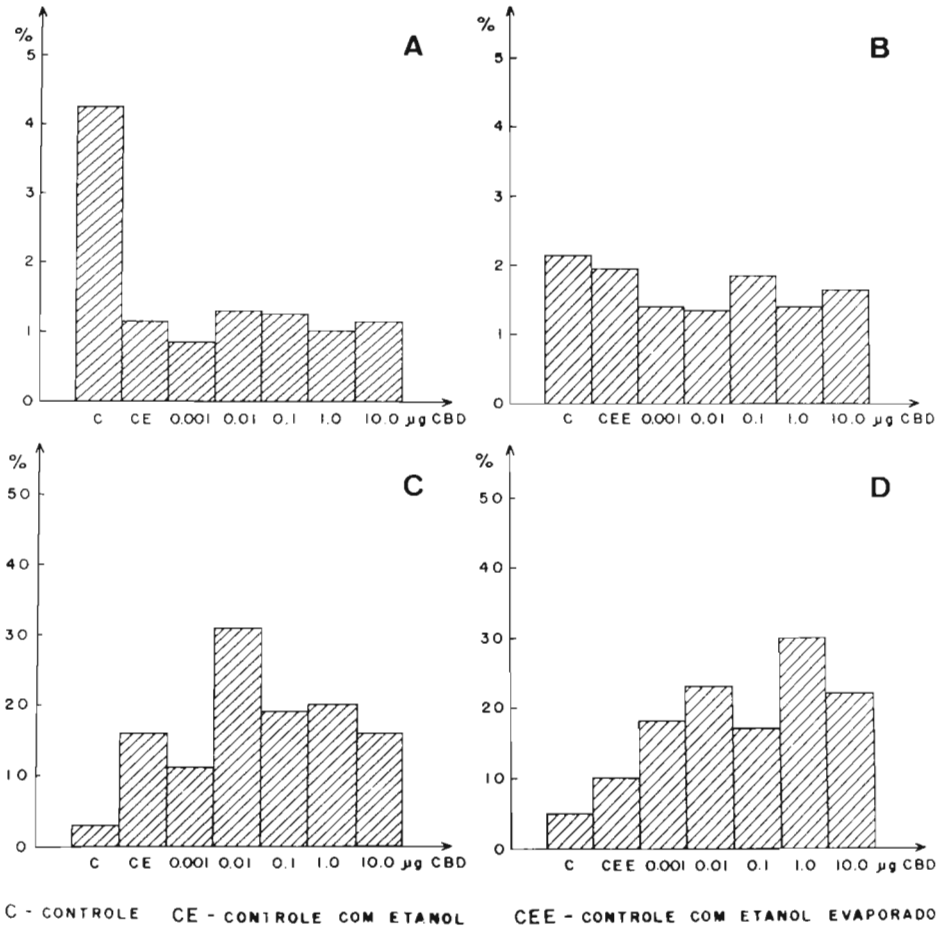


Figure 2 - Comparative frequency of metaphase nuclei (A and B) and of cells with structural chromosome aberrations (C and D) observed in control cultures and in cultures with different CBD dilutions in phase 1 (A and C) and phase 2 (B and D) of the experiment.

The perceptible reduction of mitotic index caused by alcohol may also explain the reduced fertility or even sterility of alcoholics reported in the literature (Lester and Van Thiel, 1977).

The clastogenic effects observed in the presence of CBD may also have been affected by a particular response of the donor's cells, since all experiments were carried out with blood from a single individual in order to standardize all of the experiments.

These results obtained *in vitro* cannot be easily extrapolated to *in vivo* situation because of the homeostatic mechanisms of metabolism and biological repair. However, the data agree with those obtained by Zimmerman and Raj (1980). Further studies are needed to evaluate the mutagenic potential of CBD, not only because it is a cannabinoid present in large amounts in the *Cannabis sativa*, but also because of its possible therapeutic uses as an anticonvulsivant drug (Carlini and Cunha, 1981).

### ACKNOWLEDGMENTS

We are grateful to Prof. P.A. Otto for assistance with the statistical analyses and to Prof. E.A. Carlini for providing the drug. This study was partially financed by CNPq and Laboratórios de Investigação Médica – HC – FMUSP, Brazil. Publication supported by FAPESP.

### RESUMO

Investigou-se o efeito *in vitro* do canabidiol (CBD) sobre o índice mitótico e a frequência de células com aberrações cromossômicas numéricas e/ou estruturais, em culturas de linfócitos humanos. Em uma primeira fase o CBD foi dissolvido em álcool etílico absoluto (0,01 ml/ml de meio) nas concentrações de 0,001, 0,01, 0,1, 1,0 e 10,0  $\mu\text{g}$  de CBD/ml de meio e, na segunda fase, o etanol foi evaporado antes de ser adicionado o meio de cultura. O efeito clastogênico do CBD foi maior quando associado ao álcool. A ação do etanol foi predominantemente anti-mitogênica enquanto o CBD teve efeito mais nítido sobre a produção de células com aberrações cromossômicas. Após a evaporação do etanol, a proporção de células com aberrações cromossômicas estruturais manteve uma relação aproximadamente crescente com o aumento da taxa de CBD.

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(Received April 4, 1988)