Clastogenicity of Beta-Caryophyllene in Mouse

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Beta-caryophyllene (BC) is a chemical found in the essential oil of numerous plants which have shown various biomedical properties. In the present investigation we examined for the first time its genotoxic capacity *in vivo* by evaluating its potential to induce micronuclei in mouse. In a first assay we administered three doses of the compound once, and in a second assay, we administered three doses of BC in three consecutive days. In each assay, the results were compared with those obtained in a group of mice administered corn oil and in another treated with adriamycin. Our results revealed no genotoxic effect by BC in the two assays. Moreover, no bone marrow cytotoxicity was observed when the proportion of polychromatic erythrocytes was determined with respect to the number of normochromatic erythrocytes. Therefore, our study showed the relevance of extending research on BC regarding its beneficial properties.

Key words beta-caryophyllene; micronucleus; mouse

Plants are used for various purposes including their cosmetic, nutritive, and biomedical properties. In this context, a number of therapeutic effects have been determined for different plant extracts or specific compounds. Beta-caryophyllene (BC) in particular, is a volatile sesquiterpene which has been detected in the essential oil of plants such as *Salvia*, *Artemisia* and *Eugenia*, as well as in various spices which include clove, origanum, thyme, pepper and cinnamon.^{1–3)} The compound participates in the odor and flavor of the plants, besides, due to its fragrance, BC is used in creams, lotions, and soaps.⁴⁾ On the other hand, an anti-anemic effect was suggested for BC in the middle of the last century, and more recently, studies in mouse, rat, rabbit, bacteria, and mammalian cell lines have shown its effect as an anti-inflammatory agent and antimutagen, a protector of gastric damage and oxidative stress, a local anesthetic, a potential medication against acne, and as an anticarcinogen due to its capacity to detoxify xenobiotics or to attack cancerous cell lines.^{1,2,5)}

However, in spite of the potentially useful biomedical properties mentioned above, only one report concerning the effect of BC on DNA has been published to date. The authors evaluated its mutagenic potential by using the bacterial reverse mutation assay and obtained negative results²⁾; therefore, the study of its genotoxic potential in an *in vivo* assay was necessary. In this investigation we determined the effect of BC on the induction of micronucleated polychromatic erythrocytes (MNPE) in mouse, as well as its cytotoxicity by detecting its capacity to modify the bone marrow polychromatic erythrocyte rate in the same model.

MATERIAL AND METHODS

Chemicals and Animals Beta-caryophyllene (97% pure), acridine orange corn oil and adriamycin (AD) (98% pure) were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.), and dibasic sodium phosphate, monobasic sodium phosphate, methanol and ethanol were acquired from J. T. Baker (Mexico City).

For the experiment we used six-week old male mice (strain NIH) weighing 25 g obtained from the National Institute of Hygiene (Mexico City). The animals were kept in polypropy-

lene cages at 23 °C in a 12 h dark–light schedule, and allowed to freely consume food (Rodent Laboratory chow 5001, Purina) (Mexico City) and water. The experimental protocol was approved by the Committee of Ethics and Biosecurity of the National School of Biological Sciences.

Determination of LD₅₀ The acute toxicity of BC was determined according to the method described by Lorke.⁶⁾ The compound was dissolved in corn oil and administered orally to mice. In a first step, the animals received doses of 10, 100, and 1000 mg/kg. We detected no mortality or other signs of toxicity along 7 d post-administration. Therefore, we administered up to 5000 mg/kg of BC and again, no sign of toxicity was observed in the tested animals.

Genotoxicity Protocols In the first assay we organized five groups with five individuals each as follows: a control group administered orally with 0.1 ml/g of corn oil; three groups treated orally with BC in doses of 20, 200, and 2000 mg/kg, respectively; and a positive control group injected intraperitoneally with AD (2.5 mg/kg). Blood samples from the tail of each mouse were obtained before the chemical administration and at 24, 48, 72, and 96 h post-administration.

A second experimental protocol was performed to confirm the results obtained in the first assay. The mice were organized and treated as indicated above except for the dose of AD (1.0 mg/kg). As for the other groups we used 0.1 ml/g of corn oil, and 20, 200, and 2000 mg/kg of the tested chemical administered to three groups. The main difference with the previous protocol was that the administration of chemicals to the corresponding groups was made in three consecutive days. Blood samples from the tail of each mouse were also taken before the chemical administration and at 24, 48, 72, and 96 h post-administration.

Slide Preparation, Staining, and Micronuclei Determination In both protocols, two blood smears per mouse were made, fixed in methanol for 5 min and stained for 1 min with a 0.1% solution of acridine orange made in phosphate buffered saline (PBS), pH 6.8. We then rinsed the slides three times with PBS and observed them in a fluorescence microscope (Carl Zeiss, Axioskop) with a 420 nm filter. Under these conditions, micronuclei show green-yellowish fluores-

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cence, polychromatic erythrocytes (PE) have an orange color, and normochromatic erythrocytes (NE) show very low fluorescence.⁷⁾ The capacity of BC to induce micronuclei was determined in 1000 PE per mouse in each of the five time points of the assays. Besides, we took advantage of our test model to determine the effect of the chemical on bone marrow cytotoxicity, by quantifying (in 1000 erythrocytes per mouse) the proportion of PE with respect to the number of normochromatic erythrocytes (PE/NE percent). The obtained results were statistically evaluated with the ANOVA and the Student *t* tests.

RESULTS AND DISCUSSION

The results on the rate of MNPE obtained in the first assay are shown in Table 1. We found the number of micronuclei induced by BC to be in the same range determined for the control group. The variability in the number of MNPE induced by BC in the evaluated schedule was small and non-significant (between 0.6 and 1.6). On the contrary, the administration of AD revealed a significant increase in all four post-administration points, with the high effects presented at 48 and 72 h. The PE/NE index determined in this protocol is shown in Table 2. No significant modification by BC was observed on the production of PE; however AD significantly reduced the index starting at 72 and 96 h, where a 53% and 36% suppression of the erythrocyte production was registered, respectively. AD is a widely used topoisomerase inhibitor antineoplastic agent that is known to cause reactive oxygen species, DNA damage, and apoptosis.⁸⁾ Since the chemical has also been reported to induce severe chromosome damage in mice by measuring sister chromatid exchanges (SCE) and micronuclei (MN), it is used as a positive agent in experimental studies.^{9,10)}

The results obtained in the assay with three administrations of BC are presented in Tables 3 and 4. The first table shows that the level of micronuclei induced by BC with the two low doses tested along the study was similar to the one detected in the control animals; in contrast, as regards the high dose we found an increase in the rate of MNPE from 48 to 96 h of the experiment, although without statistical significance. Considering the high level of such a dose, we feel that the obtained result does not imply a genotoxic risk by BC in our model. AD induced a significant increase in the level of micronuclei with respect to the control value during the four evaluation days. Table 4 shows that, once again, BC was un-

able to produce a cytotoxic effect in the bone marrow of the treated animals while AD did have this effect at 96 h of its administration.

Terpenes, and BC in particular, are promising agents with various useful therapeutic properties; moreover, BC is a constituent in about 39% of cosmetic products.⁴⁾ Therefore, they deserve to be solidly scrutinized as regards their toxic and genotoxic effects to assure their safe use. Studies on the group have given varying results: for example, in a bacterial assay linalool and BC showed no mutagenicity but linalyl acetate did; on the other hand, linalool was not antimutagenic whereas BC gave positive results; and finally, the toxicity of linalool and linalyl acetate proved to be stronger than BC.²⁾ Cytotoxic data about sesquiterpenes in mammalian cells have

Table 2. Effect of a Single Administration of Beta-Caryophyllene (BC) on the Relationship Polychromatic Erythrocytes/Normochromatic Erythrocytes (PE/NE Percent) in Mouse

Time (h)	PE/NE percent ($\bar{X} \pm \text{S.E.M.}$)				
	Corn oil	BC 20 mg/kg	BC 200 mg/kg	BC 2000 mg/kg	Adryamicin 2.5 mg/kg
0	2.88±0.26	2.90±0.07	2.88±0.07	2.64±0.07	3.12±0.13
24	2.76±0.06	2.58±0.08	3.06±0.22	3.08±0.09	3.12±0.11
48	2.83±0.10	2.60±0.13	2.74±0.17	3.22±0.13	2.50±0.10
72	2.77±0.02	2.60±0.15	2.80±0.11	3.02±0.21	0.28±0.18*
96	2.94±0.06	2.68±0.13	2.94±0.13	3.66±0.15	0.82±0.08*

The indicated results correspond to the mean $\pm$ S.E.M. of 5 mice per dose. 1000 erythrocytes per mouse scored to determine the PE/NE percent. * Statistically significant difference with respect to the control value ANOVA and Student *t* tests, $p=0.001$.

Table 3. Effect of Three Administrations of Beta-Caryophyllene (BC) on the Rate of Micronucleated Polychromatic Erythrocytes (MNPE) in Mouse

Time (h)	MNPE percent ($\bar{X} \pm \text{S.E.M.}$)				
	Corn oil	BC 20 mg/kg	BC 200 mg/kg	BC 2000 mg/kg	Adryamicin 2.5 mg/kg
0	2.4±0.24	2.4±0.68	2.2±0.37	2.2±0.58	2.5±0.25
24	2.2±0.48	2.2±0.37	1.4±0.39	2.2±0.56	18.0±0.81
48	2.4±0.39	0.8±0.48	1.8±0.37	4.2±0.42	24.0±0.96*
72	2.2±0.48	2.6±0.74	1.6±0.51	4.5±0.77	23.0±1.15*
96	3.0±0.54	2.2±0.37	1.8±0.37	4.7±0.31	11.2±1.05*

The indicated results correspond to the mean $\pm$ S.E.M. of 5 mice per dose. 1000 polychromatic erythrocytes scored per mouse. * Statistically significant difference with respect to the control value ANOVA and Student *t* tests, $p=0.01$.

Table 4. Effect of Three Administrations of Beta-Caryophyllene (BC) on the Relationship Polychromatic Erythrocytes/Normochromatic Erythrocytes (PE/NE Percent) in Mouse

Time (h)	PE/NE percent ($\bar{X} \pm \text{S.E.M.}$)				
	Corn oil	BC 20 mg/kg	BC 200 mg/kg	BC 2000 mg/kg	Adryamicin 2.5 mg/kg
0	2.46±0.18	2.80±0.15	3.14±0.10	2.78±0.11	2.80±0.09
24	2.58±0.11	2.84±0.18	3.26±0.17	2.32±0.20	2.50±0.13
48	2.78±0.13	2.92±0.16	3.02±0.19	2.80±0.20	2.67±0.08
72	2.76±0.13	3.08±0.20	3.04±0.13	2.85±0.18	2.35±0.05
96	2.66±0.06	3.16±0.18	2.88±0.09	3.12±0.22	1.77±0.11*

The indicated results correspond to the mean $\pm$ S.E.M. of 5 mice per dose. 1000 erythrocytes per mouse scored to determine the PE/NE percent. * Statistically significant difference with respect to the control value ANOVA and Student *t* tests, $p=0.01$.

Table 1. Effect of a Single Administration of Beta-Caryophyllene (BC) on the Rate of Micronucleated Polychromatic Erythrocytes (MNPE) in Mouse

Time (h)	MNPE ($\bar{X} \pm \text{S.E.M.}$)				
	Corn oil 0.4 ml	BC 20 mg/kg	BC 200 mg/kg	BC 2000 mg/kg	Adryamicin 2.5 mg/kg
0	1.7±0.22	1.6±0.39	1.8±0.37	1.8±0.66	2.8±0.37
24	2.0±0.31	1.4±0.39	1.2±0.48	3.0±0.31	8.6±1.02*
48	1.6±0.26	1.6±0.24	1.4±0.51	2.0±0.70	40.4±1.77*
72	2.3±0.26	1.4±0.6	2.6±0.68	2.2±0.58	50.0±3.18*
96	2.2±0.37	2.4±0.68	1.8±0.48	1.4±0.39	30.6±1.86*

The indicated results correspond to the mean $\pm$ S.E.M. of 5 mice per dose. 1000 polychromatic erythrocytes scored per mouse. * Statistically significant difference with respect to the control value ANOVA and Student *t* tests, $p=0.001$.

also varied; for example, in cultivated cells a stronger effect of alpha humulene is generally shown, in comparison with that of alpha or gamma caryophyllene, BC being the less toxic of these agents.¹⁾ In regard to genotoxic studies in mammalian cells, no previous studies on the BC potential have been made. However, contrary to our negative results and those of Di Soto *et al.* in bacteria,²⁾ other sesquiterpenes have given positive results: this is the case of the compound lactone lynchnopholide that was reported as a chromosome damaging agent in *in vitro* and *in vivo* assays, and was shown to be cytotoxic *in vitro*¹¹⁾; another such case is that of parthenin which presented a strong capacity to induce micronuclei *in vivo*.¹²⁾ These divergent results suggest the relevance of the chemical structure in the biological effect of sesquiterpenes, and indicate as well the importance of using various test models to reach a valid conclusion. Concerning this last point, Morales *et al.* compared the effect of 11 sesquiterpenoids in the Ames and the V79/HGPRT tests, and they found congruence in only four of them.¹³⁾

During its metabolism, BC can originate hydroperoxides as primary oxidation products; these, however, are rapidly converted to caryophyllene oxide, which is a low reactive and more stable product.⁴⁾ This process, together with efficient DNA repair and detoxification by mouse, may be involved in the absence of a significant number of micronuclei induced by the chemical. In summary, the results obtained in the present experiment suggest using BC safely for industrial and

therapeutic purposes, although this should be confirmed in other test models.

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