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Induction of sister chromatid exchanges by cypermethrin and carbosulfan in bone marrow cells of mice *in vivo*

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The public health effects of pesticides cannot be denied. However, the undesired effects of chemical pesticides have been recognized as a serious public health concern during the past decades. The present study describes the genotoxic effects of two pesticides, namely cypermethrin and carbosulfan, in a murine test system *in vivo*. The test parameter used was analysis of sister chromatid exchanges (SCE) in bone marrow cells. Both cypermethrin (5, 10 and 20 mg/kg) and carbosulfan (1.25, 2.5 and 5 mg/kg) induced significant increases in the frequency of SCEs ($P < 0.001$). However, no significant dose–response correlation could be found for either of the pesticides. Carbosulfan induced a cell cycle delay, as evidenced by an increase in average generation time accompanied by accumulation of cells in the first division cycle, but cypermethrin did not induce any such response. The present study indicates that carbosulfan has a higher potential to cause genetic alterations than cypermethrin in mice and may also pose a mutagenic risk to human beings.

Introduction

Pesticides have made valuable contributions to human health by increasing food and fiber production and by reducing the occurrence of vector-borne diseases (Blindauer *et al.*, 1999). Unfortunately, while the acute toxicity of most pesticides is well documented (Ecobichon *et al.*, 1990), information on chronic human illness resulting from pesticide exposure is not as sound (Wilkinson, 1990). It has been estimated that occupational exposure accounts for ~4% of all human cancers (Doll and Peto, 1981).

Cypermethrin is a very active synthetic pyrethroid insecticide and is used to control pests of a variety of crops. Although considered to be safe for mammals (Perry *et al.*, 1998), literature on *in vivo* genotoxicity of cypermethrin is limited. Giray *et al.* (2001) reported that cypermethrin exposure of rats resulted in free radical-mediated tissue damage and reduced the total glutathione (GSH) level by 20%. Hemming *et al.* (1993), in a study using partially hepatectomized male Sprague–Dawley rats, reported that cypermethrin as a single agent enhanced the development of *N*-nitrosodiethylamine (NDEA)-initiated GGT-positive foci in the liver at non-hepatotoxic doses. They suggested that cypermethrin could act as a tumor promoter.

Cypermethrin has been reported to induce gene mutations in male germ cells of *Drosophila* (Batiste-Alentorn *et al.*, 1986) and genotoxicity and sperm abnormality in mice (Bhunya and Pati, 1988). Surrallés *et al.* (1995b), studying whole blood and isolated human lymphocyte cultures, reported that cypermethrin has a weak genotoxic activity *in vitro*. In contrast, the same research group (Surrallés *et al.*, 1995a) reported that cypermethrin did not increase the ratio of excision repairable DNA lesions converted to micronuclei. Puig *et al.* (1989) reported that although cypermethrin affected the cell cycle causing a decrease in the proliferative rate index (PRI) at concentrations >10 μg/ml, it did not induce chromosome aberration or sister chromatid exchanges (SCE) in cultured human peripheral blood lymphocytes.

Carbosulfan belongs to the benzofuranyl methylcarbamate group of insecticides/acaricides. It is proposed as an effective agent in the control of pyrethroid-resistant mosquitoes (Guillet *et al*., 2001). Carbosulfan, as with other carbamates, is extremely toxic to mammals and its toxicity is mediated through inhibition of acetylcholine esterase, a respiratory enzyme (Renzi and Krieger, 1986). Very few studies have been carried out on the potential cytogenetic effects of carbosulfan. Studying the genotoxicity of Marshal and its effective ingredient carosulfan, Topaktas and Rencüzogullari (1993) reported that both test agents significantly induced the formation of chromosome aberrations in human peripheral lymphocytes *in vitro*. In another study from the same laboratory (Topaktas *et al.*, 1996) an increase in chromosome aberrations and decrease in mitotic index in rat bone marrow cells following carbosulfan treatment has also been reported. Stehrer-Schmid and Wolf (1995a) reported that carbosulfan induced a positive micronucleus response in polychromatic erythrocytes in bone marrow cells of mice at different expression times. However, in the same study carbosulfan was reported to have no influence on the frequency of gene conversion and reverse mutation in the yeast *Saccharomyces cerevisiae* strains D7 and D61.M. A dose-dependent reduction in porcine brain tubulin polymerization following carbosulfan treatment has also been reported by the same research group (Stehrer-Schmid and Wolf, 1995b). In a recent report, Rencüzogullari and Topaktas (2000) reported that a mixture of carbosulfan with ethyl methanosulfate (EMS) or ethylcarbamate (EC) shows a synergistic effect in inducing chromosome aberrations in human peripheral lymphocytes.

Carbofuran, a structural analog of carbosulfan, has been reported to be teratogenic and embryotoxic (Gupta, 1994). It has also been shown to be mutagenic in *Salmonella typhimurium* following metabolic activation with S9 (Moriya *et al.*, 1983). Increased frequencies of micronuclei, chromosome aberrations and sperm abnormalities, a decreased mitotic index in mice and a weak mutagenic response to carbofuran in Chinese hamster cells have also been reported (Georges-Gridelet *et al.*, 1982; Wojcliechowski *et al.*, 1982; Chauhan *et al.*, 2000).

The frequency of SCEs in eukaryotic cells is increased by exposure to genotoxic agents that induce DNA damage (e.g.

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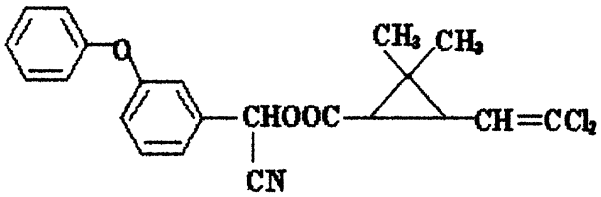

Fig. 1. Chemical structure of cypermethrin.

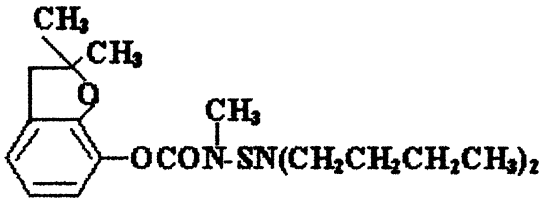

Fig. 2. Chemical structure of carbosulfan.

alkylated bases and crosslinks) capable of interfering with DNA replication (Tucker *et al.*, 1993). The currently used method for the detection of SCEs requires DNA replication in the presence of bromodeoxyuridine (BrdU) for two consecutive cell cycles, with scoring conducted in second division metaphase cells. The SCE assay yields quantifiable data from every cell scored, which increases the efficiency of data collection. The readily quantifiable nature of SCEs with a high sensitivity for revealing toxicant–DNA interactions and the demonstrated ability of genotoxic chemicals to induce significant increases in SCEs has resulted in this end-point being used as an indicator of DNA damage following exposure to genotoxic agents (Dolara *et al.*, 1992; Yager *et al.*, 1993; Giri and Chatterjee, 1998; Shaham *et al.*, 2001; Giri *et al.*, 2002a). Further, due to incorporation of BrdU in the present test procedure for SCEs, valuable information on cell cycle kinetics can be obtained by identifying cells in the M1, M2 and M3 division cycles of mitosis. The present studies were undertaken to evaluate the potential of cypermethrin and carbosulfan to induce SCEs in bone marrow cells of mice *in vivo*.

Materials and methods

Animals

Swiss albino mice, 10–12 weeks old and weighing 20–25 g, obtained from the Pasteure Institute (Shillong, India), were used as test animals. The animals were maintained in a closely inbred colony under conventional laboratory conditions at a room temperature of 25.0 ± 5.0°C on a 12 h dark/12 h light cycle. Food (standard food pellets) and water were provided *ad libitum.* Healthy and sexually mature animals, where bone marrow is expected to be actively dividing, were utilized for the experiments. Animals comprising both sexes were randomly divided into treatment groups without any sex bias and three animals were used per dose.

Laboratory reagents

Mitomycin C (MMC) was obtained from Kyowa Hakko Co. (Tokyo, Japan) and used as the positive control. 5-BrdU tablets (50 mg) were obtained from Boehringer-Mannheim (Mannheim, Germany). Hoechst 33258 was purchased from Sigma Chemical Co. (St Louis, MO). Giemsa stain was procured from Glaxo India Ltd. (Mumbai, India). All other chemicals used were of analytical grade. The buffer for the reagent solutions and stains were always prepared in glass-distilled water.

Test chemicals and treatments

Cypermethrin [cyano(3-phenoxyphenyl)methyl-3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylate; CAS no. 52315-07-8; chemical formula $C_{22}H_{19}Cl_2NO_3$; purity 99%] (Figure 1) was obtained from United Phosphorous Ltd. (Mumbai, India). Carbosulfan [2,3-dihydro-2,2-dimethyl-7-benzofuranyl [(dibutylamino)thio]methylcarbamate; CAS no. 55285-14-8; chemical formula $C_{20}H_{32}N_2O_3S$; purity 97% minimum] (Figure 2) was obtained from Rallis India Ltd. (Mumbai, India). The highest sub-lethal acute dose was selected by trial as suggested by Krishna and Hayashi (2000) so that the animals receiving the highest dose showed minimal toxicity.

Cypermethrin was used in three sub-lethal acute doses (5, 10 and 20 mg/kg) via the i.p. route. Carbosulfan was used in three sub-lethal acute doses (1.25, 2.5 and 5 mg/kg) via the i.p. route. The control animals received equal volumes of normal saline.

SCE assay

The SCE assay was done as described by Goto *et al.* (1975). Ten minutes prior to the various treatments, mice weighing between 20 and 22 g were s.c. implanted with BrdU tablets (50 mg) under light ether anesthesia. Prior to killing, the animals were treated i.p. with colchicine (4 mg/kg) for 90 min. The animals were killed by cervical dislocation after 24 h treatment and both the femora were dissected out and cleaned of any adhering muscles. The bone marrow was collected in 0.56% KCl at 37°C. A single cell suspension was made by gentle agitation and incubated at 37°C for 18 min in a water bath. After the incubation period, the material was centrifuged at 1000 r.p.m. for 5 min, the supernatant was decanted and the cell pellet was thoroughly mixed with the residual supernatant by gentle agitation. The cells were fixed in cold acetic acid/methanol (1:3 v/v). Centrifugation and fixation were repeated twice with an interval of 30 min. Finally, immediately before preparation of slides, the fixed material was again centrifuged and resuspended in a small volume of fixative by gentle flushing until a cloudy suspension resulted. A few drops of the cell suspension were dropped onto a clean grease-free slide previously chilled in 50% ethanol, burnt in a smokeless flame (spirit lamp) for a while and stored for 1–2 days in dust-free conditions before staining.

For staining, the slides were treated for 10 min with Hoechst 33258 (50 μg/ml) in the dark at room temperature, rinsed in distilled water, mounted in 2× SSC (NaCl, sodium citrate, pH 6.8) and kept in direct sunlight under moist conditions for 30–40 min, depending upon the intensity of the sunlight. Then the slides were rinsed and stained in 3% buffered Giemsa (pH 7.0) for 6 min, air dried and mounted in DPX. At least 50 well-spread second division metaphases identifiable by their uniform differential staining pattern and containing the normal chromosome complement were analyzed for the presence of SCEs. Every switch of staining between the sister chromatids was scored as one SCE.

In addition to the analysis of SCEs, cells were also analyzed for the relative frequency of first division metaphases (identifiable by uniform staining of both the sister chromatids), second division metaphases and third and subsequent division metaphases (identifiable by a non-uniform pattern of staining). This information was used to evaluate cell proliferation kinetics as described by Tice *et al.* (1976, 1979). Replication index (RI) or proliferation index is the average number of replications completed by metaphase cells and is calculated as follows:

RI = [(% first division metaphases) + 2×(% second division metaphases) + 3×(% subsequent division metaphases)]/100.

The average generation time (AGT) was determined as the ratio of BrdU duration (h) to RI.

Statistical analysis

Student's *t*-test was used to compare the level of significance of the results for the pesticide-treated groups and the untreated control as well as among the various treated groups. Regression analysis was carried out to determine the dose–response correlations.

Results

Cypermethrin

Cypermethrin induced a significant increase in the frequency of SCEs ($P < 0.001$) in the bone marrow cells as compared with the untreated control at all the three doses (5, 10 and 20 mg/kg) tested (Table I). However, no significant difference could be found in the frequency of SCEs among the treated groups. An increasing trend in the frequency of M1 cells was observed as compared with the control value. However, this did not attain statistical significance. Similarly, although a slightly increasing trend in the AGT of cells was noted in the treated series as compared with the control, this was not statistically significant (Table I). The linear regression analysis (Figure 3) revealed a lack of correlation between the dose of cypermethrin and the frequency of SCEs ($r = 0.6254$, $P > 0.05$).

Table I. Effect of cypermethrin on cell cycle kinetics and the frequency of SCEs in the bone marrow cells of mice

Dose (mg/kg)	TM	M1	M2	M3	M1 (%)		RI	AGT (h)		Cells scored[a]	SCE/M	
						Mean ± SD			Mean ± SD			Mean ± SD
Control	181	18	154	9	09.94		1.950	12.30		50	2.80	
	156	14	135	7	08.97		1.955	12.27		50	3.08	
	185	24	152	9	12.97	10.46 ± 2.18	1.918	12.51	12.36 ± 0.12	50	3.32	3.06 ± 0.26
MMC, 2.0	240	91	146	3	37.91		1.633	14.69		50	9.16	
	187	86	095	6	45.98		1.572	15.26		50	9.34	
	210	89	117	4	42.38	42.09 ± 4.04	1.595	15.04	14.99 ± 0.28	50	9.42	9.30 ± 0.13
Cypermethrin, 5.0	155	21	131	3	13.54		1.883	12.76		50	4.68	
	143	17	121	5	11.88		1.916	12.52		50	4.58	
	182	25	144	8	13.73	13.05 ± 1.01	1.906	12.59	12.62 ± 0.12	50	4.94	4.73 ± 0.18[b]
10.0	162	19	137	6	11.72		1.919	12.50		50	4.90	
	155	22	128	5	14.19		1.890	12.69		50	5.04	
	176	21	146	9	11.93	12.61 ± 1.36	1.931	12.42	12.53 ± 0.13	50	4.64	4.86 ± 0.20[b]
20.0	178	22	147	9	12.35		1.926	12.46		50	5.16	
	166	22	138	6	13.25		1.903	12.61		50	4.90	
	185	29	145	11	12.67	12.75 ± 0.45	1.902	12.61	12.56 ± 0.08	50	5.28	5.11 ± 0.19[b]

Control, only normal saline was given. TM, total metaphases; AGT, average generation time; M, metaphase; RI, replication index; SCE, sister chromatid exchange. Statistical analysis Student's *t*-test ($n = 3$).
[a]Total cells scored for SCE.
[b]Significantly different from control ($P < 0.001$).

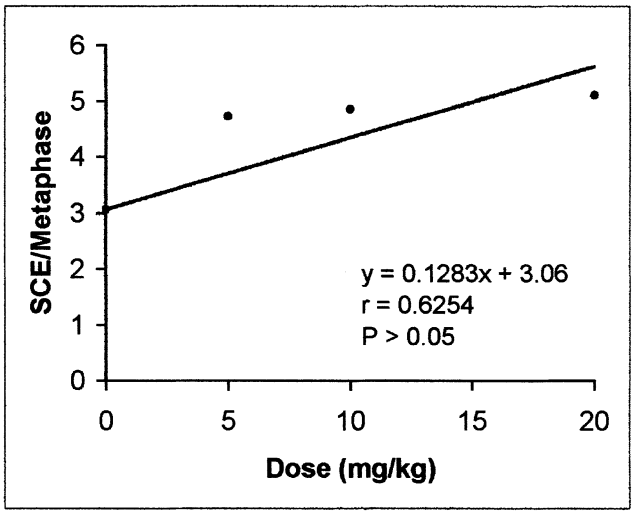

Fig. 3. Graph showing regression line and correlation coefficient (*r*) of SCEs in bone marrow cells of mice following i.p. treatment with cypermethrin for 24 h.

Carbosulfan

All the acute doses (1.25, 2.5 and 5 mg/kg) of carbosulfan induced significantly higher frequencies of SCEs ($P < 0.001$) in bone marrow cells (Table II). Among the treated series, a significant difference in the frequency of SCEs was noted between 1.25 versus 2.5 mg/kg ($P < 0.01$), 1.25 versus 5 mg/kg ($P < 0.001$) and 2.5 versus 5 mg/kg ($P < 0.001$) in the carbosulfan-treated groups (Table II). The dose–response analysis (Figure 4) revealed that no significant association existed between the dose of carbosulfan and the frequency of SCEs ($r = 0.9281$, $P > 0.05$).

Following 2.5 and 5 mg/kg carbosulfan treatment, significant increases ($P < 0.05$ and $P < 0.001$, respectively) in the AGT of bone marrow cells were found. Concomitant with AGT, a significant increase in the percentage of M1 cells following 2.5 and 5 mg/kg carbosulfan ($P < 0.05$ and $P < 0.01$, respectively) was also noted (Table II).

Discussion

Introduced commercially about 30 years ago, synthetic pyrethroids account for more than 30% of insecticides used worldwide in agricultural, domestic and veterinary applications and have a high potential for human exposure (Eisler, 1992; Perry *et al.*, 1998). Cypermethrin is reported to cause free radical-mediated tissue damage and reduce total GSH in rats (Giray *et al.*, 2001). Cypermethrin at non-hepatotoxic doses has also been reported to enhance the development of NDEA-initiated GGT-positive foci in rat liver (Hemming *et al.*, 1993). However, literature on the genotoxicity of cypermethrin is limited.

In the present study, we found that all the three doses of cypermethrin (5, 10 and 20 mg/kg) induced significant increases in the frequency of SCEs (Table I). However, the increase was not dose dependent (Figure 3). Puig *et al.* (1989) reported that although cypermethrin affected the cell cycle causing a decrease in the PRI, it did not induce SCE in cultured human peripheral blood lymphocytes. This difference could be due to the different test systems used and/or possible production of genotoxic metabolites during *in vivo* metabolism. It has been reported that *in vivo* genotoxicity tests are very helpful in the consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA repair processes (Krishna and Hayashi, 2000). Further, in contrast to earlier reports (Puig *et al.*, 1989), we did not find any significant difference in either the percentage of cells in the first division cycle (M1) or in AGT as compared with the untreated control (Table I). Therefore, it is evident from the present findings that the lack of a dose–response correlation for the induction of SCEs as observed presently does not arise due to the cytotoxic action of cypermethrin or a delay in the cell cycle, which accumulates more cells in M1. Bhunya and Pati (1988), using similar concentrations of cypermethrin through the i.p. as well as s.c. routes, reported a dose-dependent increase in the frequency of chromosome aberrations following cypermethrin treatment in mice. The lack of a dose–response correlation for the frequency of SCEs indicates that different mechanisms may be involved

Table II. Effect of carbosulfan on cell cycle kinetics and the frequency of SCEs in the bone marrow cells of mic

Dose (mg/kg)	TM	M1	M2	M3	M1 (%)		RI	AGT (h)		Cells scored	SCE/M	
						Mean ± SD			Mean ± SD			Mean ± SD
Control	181	18	154	9	9.94		1.950	12.30		50	2.80	
	156	14	135	7	8.97		1.955	12.27		50	3.08	
	185	24	152	9	12.97	10.46 ± 2.18	1.918	12.51	12.36 ± 0.12	50	3.32	3.06 ± 0.26
MMC, 2.0												
	240	91	146	3	37.91		1.633	14.69		50	9.16	
	187	86	095	6	45.98		1.572	15.26		50	9.34	
	210	89	117	4	42.38	42.09 ± 4.04	1.595	15.04	14.99 ± 0.28	50	9.42	9.30 ± 0.13
Carbosulfan, 1.25	167	18	141	8	10.77		1.940	12.37		50	6.20	
	168	21	142	5	12.50		1.904	12.60		50	5.88	
	176	24	145	8	13.63	12.30 ± 1.44	1.920	12.50	12.49 ± 0.11	50	6.10	6.09 ± 0.21[a,b,c]
2.5	135	19	111	5	14.07		1.896	12.65		50	7.38	
	152	23	121	8	15.13		1.901	12.62		50	7.10	
	180	31	143	6	17.22	15.47 ± 1.60[d]	1.861	12.89	12.72 ± 0.17[d]	50	7.26	7.24 ± 0.14[a,b]
5.0	206	35	167	4	16.99		1.849	12.97		50	9.20	
	177	32	143	2	18.07		1.830	13.11		50	8.91	
	190	36	149	5	18.94	18.00 ± 0.97[e]	1.836	13.07	13.07 ± 0.07[a]	50	9.60	9.23 ± 0.35[a,c]

Control, only normal saline was given. TM, total metaphases; AGT, average generation time; M, metaphase; RI, replication index; SCE, sister chromatid exchange. Statistical analysis Student's *t*-test ($n = 3$).
[a]Significantly different from control, $P < 0.001$.
[b]Values with similar superscripts are significantly different from each other, $P < 0.01$.
[c]Values with similar superscripts are significantly different from each other, $P < 0.001$.
[d]Significantly different from control, $P < 0.05$.
[e]Significantly different from control, $P < 0.01$.

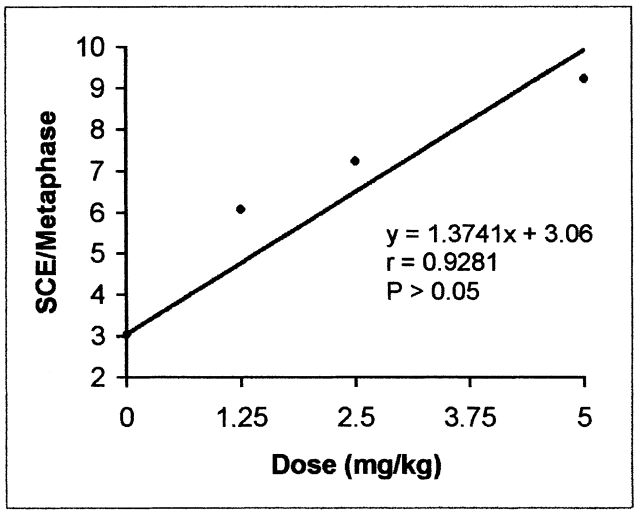

Fig. 4. Graph showing regression line and correlation coefficient (*r*) of SCEs in bone marrow cells of mice following i.p. treatment with carbosulfan for 24 h.

in the formation of SCEs and chromosome aberrations. The possible involvement of separate mechanisms for induction of SCEs and structural chromosome aberrations has been reported in other studies (Giri and Chatterjee, 1998; Giri *et al.*, 2002a).

SCEs arise from reciprocal exchange of DNA at apparently identical loci of the sister chromatids of a duplicated chromosome in response to a damaged DNA template (Tice and Hollaender, 1984a,b; Tucker *et al.*, 1993). The frequency of SCEs in eukaryotic cells is increased by exposure to genotoxic agents that induce DNA damage capable of interfering with DNA replication (Tucker *et al.*, 1993) and has been used to identify genotoxic agents (Dolara *et al.*, 1992; Yager *et al.*, 1993; Giri and Chatterjee, 1998; Shaham *et al.*, 2001; Giri *et al.*, 2002a). The significant increase in the frequency of SCEs induced by cypermethrin may further indicate its potential to interact with cellular DNA.

A structure–activity relationship for synthetic pyrethroids has been shown for their electrophysiological and behavioral effects on rats (Verschoyle and Aldridge, 1980). It is now well established that the sodium channel is the primary target site of pyrethroids, which brings about depolarization of the nerve membrane by prolonging the sodium current during excitation (Perry *et al.*, 1998). Metabolism of cypermethrin is considered to be both oxidative and hydrolytic, and both pathways involve esterase cleavage (Chang and Jordan, 1982; Perry *et al.*, 1998). However, the mechanism of action of synthetic pyrethroids on nucleic acids is yet to be elucidated. Therefore, at present it is difficult to comment on the structure–genotoxicity relationship for cypermethrin. Independent of the nature of molecular interactions that may occur, the present data clearly indicate that cypermethrin possesses the potential, at least to a limited extent, to cause alterations in the cellular DNA in mammalian cells *in vivo*.

Carbosulfan, a benzofuranyl methylcarbamate pesticide, has been widely used in agriculture and is also reported to be very effective against pyrethroid-resistant mosquitoes (Guillet *et al.*, 2001). Very few published reports could be found on the potential cytogenetic effects of carbosulfan (Topaktas and Rencüzogullari, 1993; Stehrer-Schmid and Wolf, 1995a,b; Topaktas *et al.*, 1996; Rencüzogullari and Topaktas, 2000). Carbamate pesticides such as aldicarb, benomyl and propoxur and their nitroso derivatives have been reported to induce SCEs (Rencüzogullari and Topaktas, 1998). On the other hand, methyl-2-benzimidazole carbamate, a carbamate fungicide, did not induce SCE in human lymphocytes *in vitro* (Cid and Matos, 1984; Banduhn and Obe, 1985; Cid *et al.*, 1990; Georgieva *et al.*, 1990). In the present study, all three doses (1.25, 2.5 and 5 mg/kg) of carbosulfan induced significant increases in the frequency of SCEs (Table II) following 24 h treatment, but no dose–response correlation could be found

(Figure 4). The lack of a dose–response correlation could be due to possible cytotoxicity at higher doses (resulting in death of highly damaged cells) and/or a cell cycle delay (an increase in AGT; Table II), retaining cells for longer in the M1 phase of the cell cycle, during which DNA repair takes place. It has been reported that adducts formed by mutagenic agents interfere with DNA replication and induce cell cycle delay (Sognier and Hittelman, 1986). Carbosulfan has been reported to induce a cytotoxic response when given at 5 mg/kg through the i.p. route (Giri *et al.*, 2002b).

In higher eukaryotes, carbosulfan has been reported to decrease the RI and mitotic index in a dose-dependent manner (Rencüzogullari and Topaktas, 1996). Rencüzogullari and Topaktas (1998) reported that carbamate pesticides decrease RI by preventing the replication of DNA in S phase. In the present study, we also found that 2.5 and 5 mg/kg carbosulfan treatment resulted in a significant increase in AGT (decrease in RI) with a concurrent accumulation of cells at the M1 phase of the cycle at 24 h treatment (Table II).

Carbosulfan is reported to be non-mutagenic in *S.typhimurium* strains TA97, TA98, TA100 and TA102, but it induced mitotic aneuploidy in *S.cerevisiae* strain D61.M (Wiedenmann *et al.*, 1990). Carbosulfan has been reported to increase chromosomal aberrations in human peripheral blood lymphocytes and bone marrow cells of rats (Topaktas and Rencüzogullari, 1993; Topaktas *et al.*, 1996). The present findings provide further evidence in support of a mutagenic potential of carbosulfan.

In conclusion, the results of the present study indicate that cypermethrin and carbosulfan have definite interactions with DNA metabolism in mice, resulting in SCEs, indicating potential mutagenic effects. Human exposure to these agents should be restricted.

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