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Sister Chromatid Exchange in Cultured Human Lymphocytes Treated With Carbosulfan, Ethly Carbamate and Ethly Methanesulfonat Separately and in Mixtures

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Abstract: The aim of this study was to investigate the ability of Carbosulfan (the effective ingredient in Marshal, a carbamate insecticide/nematocide), Ethyl Carbamate (EC=carcinogen and Ethyl Methanesulfonat (EMS=mutagen) separately and in mixtures to induce sister chromatid exchange (SCE) in human lymphocytes and to nivestigate whether the test substances, as a mixture had any synergistic or antogonistic effect. Carbosulfan separately weakly induced SCE during the 48 hour treatment. Although EC separately could not induce SCE, EMS separately induced dose-dependently SCE at 24 and 48 hour treatments. EC increased the RI, while Carbosulfan and EMS decreased it during the 48 hour treatment. The mixtures of Carbosulfan+EC and Carbosulfan-EMS induced the SCE at all concentrations and treatment times and decreased the RI compared with normal and solvent controls. In addition, there was synergism between the mixtures of Carbosulfan and EC and also between the mixtures of Carbosulfan and EMS on the induction of SCE and also on the decrease in the RI.

Key Words: Carbosulfan, Ethyl Carbamate, Ethyl Methanesulfonat, Synergistic Effect, SCE.

Kültürü Yapılmış İnsan Periferal Lenfositlerinde Carbosulfan, Ethyl Carbamate ve Ethyl Methanesulfonat'ın Tek Başlarına veya Birlikte Kullanıldıkları Zaman Kardeş Kromatid Değişimi Üzerindeki Etkileri

Özet: Bu çalışmanın amacı, Marshal insektisidi/nematosidinin etkin maddesi olan Carbosulfan, kanserojen bir madde olan Ethyl Carbamate (EC) ve mutajen bir madde olan Ethyl Methanesulfonat'in (EMS) tek tek veya karışım halinde insan periferal lenfositlerinde kardeş kromatid değişimi (KKD) ve replikasyon indeksi (RI) üzerindeki etkilerini araştırmak, test maddelerinin karışım halinde herhangi bir sinerjitik veya antagonist etkilere sahip olup olmadıklarını saptamaktır. Tek başına Carbosulfan KKD'i sadece 48 saatlik muamele süresinde zayıf bir şekilde artırmıştır. Tek başına EC, KKD'i artırmazken, EMS ise KKD'i her iki muamele süresinde ve doza bağlı olarak artırmıştır. Carbosulfan RI'ni sadece 48 saatlik muamele süresinde düşürmüş, EC RI'ni yine 48 saatlik muamele süresinde hafif bir şekilde artırmış, EMS ise yine 48 saatlik muamele süresinde ve sadece en yüksek konsantrasyonda RI'ni

düşürmüştür. Carbosulfan+EC ve Carbosulfan+EMS karışım halinde KKD sayısını kontrollara nazaran artırmışlar ve RI'ni düşürmüşlerdir. Ayrıca test maddeleri karışım halinde KKD'ni sinerjitik bir şekilde artırdıkları, genel olarak RI'ni de sinerjitik olarak düşürdükleri saptanmıştır.

Anahtar Sözcükler: Carbosulfan, Ethyl Carbamate, Ethyl Methanesulfonat, Sinerjik Etki, SCE

Introduction

We are living in hazardous conditions due to combinations of pollutants namely pesticides, nuclear chemicals, drugs and airborn pollutants. Humans are exposed to these pollutants as a mixture because of their living conditions. Most of these pollutants are mutagenic or carcinogenic and have synergism on the induction of chromosomal abnormalities and sister chromatid exchanges. A lot of mutgenic and carcinogenic chemicals have induced the formation of chromosomal aberrations and SCE in Chinese hamster ovary (CHO) cells (1). The carbamate pesticides namely aldicarb, nitroso-aldicarb, benomyl, propoxur and nitroso propoxur induced SCE and decreased the RI in human lymphocytes (2-5). On the other hand, Marshal, Carbosulfan and MBC (methyl-2-benzimidazole carbamate) did not induce SCE but, Marshal and Carbosulfan decreased the RI in human lymphocytes (6, 7).

EC (Ethyl Carbamate=Urethan) induced SCE dose-dependently in mice following in vitro exposures and in the bone marrow cells, alveolar macrophages and regenerating liver cells of mice (8) and also EC induced SCE in Chinese hamster, Golden hamster, rats and mice bone marrow cells (9). EC weakly induced SCE in C. hamster V79-4 cells (10) and in human lymphocytes without metabolic activation (11).

EMS (Ethyl Methanesulfonat) induced SCE dose-dependently in CHO cells (1, 12, 13) and in marine fish following in vivo exposures (14). EMS also induced SCE and slightly decreased the RI in human lymphocytes (15, 16). Stetka and Wolff (17) the reported that EMS induced SCE in rabbit peripheral lymphocytes following intraperitoneal administration.

On the other hand, human beigns are living in hazardous conditions due to mixtures of the chemicals mentioned above. A lot of these chemicals induced SCE as a mixture. Dolara et al. (18) reported the mixture of 4 pesticides namely dimethoate, omethoate, deltamethryn and benomyl, induced SCE while these pesticides separately did not induce SCE. SCE was induced in lymphocytes of pesticide sprayer workers who were smokers (19). In addition, SCE was not induced in lymphocytes of pesticide packing workers (20) and in rural populations occupationally exposed to pesticides (21). A significant increase in frequency of SCE was found in traffic policemen (20), in workers occuptionally exposed to a chemical mixture in the tyre industry (22), in men environmentally and occupationally exposed to airborn pollutants (23) and also in workers exposed to occupational clastogens (24). Sasiadek (22) reported the RI was lower in the lymphocytes of workers occupationally exposed to a chemical mixture in the tyre industry.

As shown in these results, the chemicals exhibited synergism on induction of SCE when the populations were exposed to a mixture of these chemicals. To investigate the effects of the mixtures of pesticides with carcinogens and the mixtures of pesticides with mutagens on the induc-

tion of SCE, Carbosulfan was used as the pesticide, EC was used as the carcinogen and EMS was used as the mutagen, because Carbosulfan is a carbamate pesticide. Carbamate pesticides have been used extensively in the Çukurova region which is one of the most important agricultural regions in Turkey. An estimated 275.000 kg of Carbamate was used in this region in 1995. In the same year 155.500 kg of Carbosulfan (trade name Marshal) was used in the province of Adana of Çukurova. The aim of this study was to investigate the effects of Carbosulfan, EC and EMS separately and in mixtures on SCE in human lymphocytes and also to investigate whether the chemicals have a synergistic or antagonist effect when used as a mixture.

Material and Method

Human peripheral blood was used as the test system and Carbosulfan (extensively used in the Çukurova region as an insecticide/nematocide), EC carcinogen, and EMS (mutagen) were used as the test substance. The chemical structure and formula of the test substances are shown in Figure 1.

1- Addition of the blood and the test substances to the medium

Whole blood (0.2 ml) from two healthy donors (one male, one female, non smokers age 30) was added to 2.5 ml Chromosome Medium B (Biochrom cat. no: F5023) supplemented with 10 µg/ml Bromodeoxyuridine (BrdUrd). The cultures were incubated at 37°C for 72 hours. The cells were treated with $2.5x10^{-5}$, $5x10^{-5}$ and 10^{-4} M concentrations of Carbosulfan; $2x10^{-3}$, $4x10^{-3}$ and $8x10^{-3}$ M concentrations of EC and $5x10^{-4}$, 10^{-3} and $2x10^{-3}$ M concentrations of EMS for 24 hours (the test substances were added 48 hours after initiating the culture) and 48 hours (the test substances were added 24 hours after initiating the culture). A normal control and solvent control (for carbosulfan 80% ethanol, for EC distilled water and for EMS 50% ethanol) were present at each treatment time. Then the cells were treated with mixtures of the test substances as shown below:

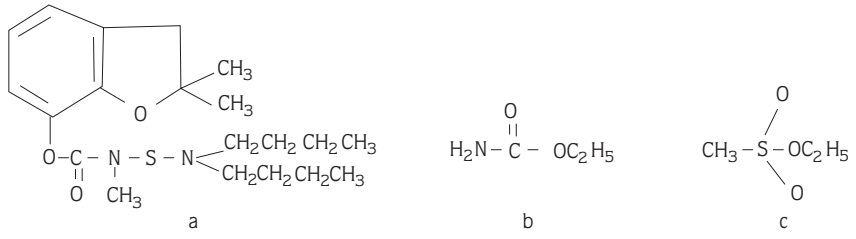

Figure 1. a) Carbosulfan (2, 3-dihydro-2, 2-dimethyl-7-benzofuranyl ((dibutyl amino) thio) methylcarbamate) (CAS registry number: 55285-14-8)
b) Ethyl Carbamate (Urethan) (CAS registry number: 51-79-6)
c) Ethyl Methanesulfonat (Methanesulfonic acid ethylester) (CAS registry number: 62-50-0)

Carbosulfan + $2x10^{-3}$ M EC

1. tube: control, untreated
2. tube: 80% ethanol 10 µl+d. water 10 µl
3. tube: $2.5x10^{-5}$ M C + $2x\ 10^{-3}$ M EC
4. tube: $5x10^{-5}$ M C + $2x10^{-3}$ M EC
5. tube:10^{-4} M C + $2x10^{-3}$ M EC

Carbosulfan + $5x10^{-4}$ M EMS

1. tube: control, untreated
2. tube: 80% ethanol 10 µl + 50% ethanol 10 µl
3. tube: $2.5x10^{-5}$ M C + 10^{-4} M EMS
4. tube: $5x10^{-5}$ M C + $5x10^{-4}$ M EMS
5. tube: 10^{-4} M C + $5x10^{-4}$ M EMS

Carbosulfan + $4x10^{-3}$ M EC

1. tube: control, untreated
2. tube: 80% ethanol 10 µl+d. water 10 µl
3. tube: $2.5x10^{-5}$ M C + $4x10^{-3}$ M EC
4. tube: $5x10^{-5}$ M C + $4x10^{-3}$ M EC
5. tube: 10^{-4} M C + $4x10^{-3}$ M EC

Carbosulfan + 10^{-3} M EMS

1. tube: control, untreated
2. tube: 80% ethanol 10 µl+50% ethanol 10 µl
3. tube: $2.5x10^{-5}$ M C +10^{-3} M EMS
4. tube: $5x10^{-5}$ M C + 10^{-3} M EMS
5. tube: 10^{-4} M C + 10^{-3} M EMS

Carbosulfan + $8x10^{-3}$ M EC

1. tube: control, untreated
2. tube: 80% ethanol 10 µl+d. water 10 µl
3. tube: $2.5x10^{-5}$ M C + $8x10^{-3}$ M EC
4. tube: $5x10^{-5}$ M C + $8x10^{-3}$ M EC
5. tube: 10^{-4} M C + $8x10^{-3}$ M EC

Carbosulfan + $2x10^{-3}$ M EMS

1. tube: control, untreated
2. tube: 80% ethanol 10 µl+50% ethanol 10 µl
3. tube: $2.5x10^{-5}$ M C + $2x10^{-3}$ M EMS
4. tube: $5x10^{-5}$ M C + $2x10^{-3}$ M EMS
5. tube: 10^{-4} M C + $2x10^{-3}$ M EMS

2. Harvesting the cells and preparing permanent slides

Thu human lymphocytes were treated with mixtures of the test substances for 24 and 48 hours. Colchicine (0.06 µg/ml) was present for the last 2 hours of the culture. To collect the cells, the cultures were centrifuged (1200 rpm, 15 min), treated with hypotonic solution (0.4 % KCl) for 30 min at 37°C, and then fixed in fixative (mehanol: glacial acetic acid 3:1) for 20 min at room temperature. The treatment with fixative was repeated 3 times. The cells were spread on glass slides and air dried. The slides were processed by modifed fluorescence plus Giemsa technique according the method of Speit (25), Speit and Haupter (26).

3. Slide investigations

For the occurence of the number of sister chromatid exchange, a total of 50 cells (25 cells from the male and 25 cells from the female donor) under second metaphases were examined. The second metaphases were examined at 1000x magnification. This data was used to determine the mean number of the SCE (SCE/cell). In addition, for the occurence of the replication

index (RI), a total of 200 cells (100 cells from each donor) were scored. The metaphases was classified as being in the first (M_1), second (M_2) and third (M_3) mitosis. The replication index was calculated according to Schneider et al. (1981) from Lin et al. (27).

4. Statistical significance

The significance between the mean SCE, RI and their controls was determined using the t-test. Therefore, the results obtained from mixtures of the test substances were compared with the results obtained from the test substances separately for determined synergism or antagonism between mixtures of Carbosulfan and EC and also between Carbosulfan and EMS. For example, the result obtained from $2.5x10^{-5}$ M Carbosulfan + $2x10^{-3}$ M EC was compared with the results obtained from both $2.5x10^{-5}$ M Carbosulfan and $2x10^{-3}$ M EC separately.

Dose-response relationships were determined from the correlation and regression coefficients and the corresponding regression lines for the mean SCE.

Results

1. The effects of the test substances separately and as a mixture on SCE and the RI in human lymphocytes

Carbosulfan induced SCE only during the 48 hour treatment compared with the controls but was not dose-dependent (Table 1). Carbosulfan also decreased the RI only at the two highest concentrations for 48 hours compared with the controls (Table 1). EC could not effect the frequency of the SCE compared with the controls (Table 1). However, it slightly increased the RI only during the 48 hour (Table 1). EMS induced the SCE at all concentrations and treatment times. This increase was dose-dependent (Table 1, Figure 2). A cell with a 91 SCE is shown in Figure 3. However, EMS decreased the RI only at the highest concentration during the 48 hour treatment (Table 1).

The mixture of Carbosulfan and EC induced SCE at all concentrations and treatment times compared with the controls (Table 2). The increase in the frequency of SCE was dose-dependent in cultures treated with mixtures of Carbosulfan and $4x10^{-3}$ M EC for 24 and 48 hours and also in cultures treated with mixtures of Carbosulfan and $8x10^{-3}$ M EC for 48 hours (Figure 4). When compared with the controls, the mixtures of Carbosulfan and $2x10^{-3}$ M EC decreased the RI only at two the highest concentrations during the 24 and 48 hour treatments (Table 2). However, the mixtures of Carbosulfan and $4x10^{-3}$ M EC decreased the RI at all concentrations and treatment times (Table 2). Therefore, the mixtures of Carbosulfan and $8x10^{-3}$ M EC decreased the RI only at the two highest concentrations during the 48 hour teratment (Table 2).

The mixtures of Carbosulfan and EMS induced the SCE at all concentrations and treatment times compared to the controls (Table 3). This increase was dose-dependent in the cultures treated with mixtures of Carbosulfan and $5x10^{-4}$ M EMS (Figure 5) and also in the cultures treated with mixtures of Carbosulfan and $2x10^{-3}$ M EMS (Figure 6) during the 24 and 48 hour treatments. The mixtures of Carbosulfan and $5x10^{-4}$ M EMS decreased the RI only at two the

Table 1. The frequency of the SCE and the RI in cultured human lymphocytes treated with Carbosulfan, EC and EMS separately for 24 and 48 hours*

Test Substances	Treatment Times (Hour)	Concentrations (M)	Min-Max. SCE	SCE/Cell±SE	M_1	M_2	M_3	RI±SE
Carbosulfan	24	Control	2-14	7.70±0.501	40	76	84	2.22±0.185
		80% Ethanol 10 µl	2-14	7.82±0.470	41	79	80	2.19±0.170
		$2.5x10^{-5}$	2-14	7.52±0.429	45	68	87	2.21±0.196
		$5x10^{-5}$	3-22	9.02±0.515	49	75	76	2.13±0.222
		10^{-4}	3-16	8.68±0.427	73	72	55	1.91±0.202
	48	Control	2-16	7.80±0.477	40	73	87	2.23±0.084
		80% Ethanol 10 µl	3-16	8.30±0.464	33	83	84	2.25±0.003
		$2.5x10^{-5}$	4-17	10.44±0.480a4b4	45	87	68	2.11±0.130
		$5x10^{-5}$	2-18	9.98±0.510a4b3	85	103	12	1.63±0.072a3b3
		10^{-4}	5-25	11.00±0.500a4b4	181	18	1	1.10±0.003a4b4
EC	24	Control	2-14	7.14±0.440	50	83	67	2.08±0.107
		D. Water 10 µl	2-15	7.64±0.490	49	89	62	2.06±0.095
		$2x10^{-3}$	2-15	8.00±0.450	66	92	42	1.88±0.092
		$4x10^{-3}$	2-17	7.92±0.460	63	97	40	1.89±0.124
		$8x10^{-3}$	3-19	8.46±0.530	72	96	32	1.80±0.150
	48	Control	3-16	7.90±0.460	40	73	87	2.23±0.084
		D. Water 10 µl	3-15	8.48±0.430	43	82	75	2.16±0.058
		$2x10^{-3}$	5-19	8.96±0.440	30	62	108	2.39±0.035a1b2
		$4x10^{-3}$	4-16	8.66±0.420	31	82	87	2.28±0.003a4b4
		$8x10^{-3}$	4-17	9.02±0.450	30	66	104	2.37±0.058b1
EMS	24	Control	2-16	6.78±0.380	33	75	92	2.29±0.153
		50% Ethanol 10 µl	2-16	6.98±0.460	36	67	97	2.30±0.030
		$5x10^{-4}$	3-19	10.34±0.510a4b4	49	75	76	2.14±0.118
		10^{-3}	6-18	11.94±0.470a4b4	48	67	85	2.18±0.118
		$2x10^{-3}$	8-28	15.60±0.660a4b4	49	70	81	2.16±0.098
	48	Control	3-17	7.74±0.450	35	60	105	2.35±0.017
		50% Ethanol 10 µl	3-17	8.60±0.430	47	80	73	2.13±0.040
		$5x10^{-4}$	12-33	21.66±0.720a4b4	34	75	91	2.28±0.136
		10^{-3}	24-49	34.64±0.970a4b4	37	105	58	2.10±0.061a1
		$2x10^{-3}$	49-91	63.92±1.390a4b4	77	110	13	1.68±0.115a2b1

* A total of 50 cells were scored for SCE and 200 cells were scored for the RI

a: Significant from control; b: Significant from solvent control;

a1b1: P≤0.05, a2b2: P≤0.01, a3b3: P≤0.005, a4b4: P≤0.001.

highest concentrations during the 24 and 48 hour treatments however the mixtures of Carbosulfan and 10^{-3} M EMS decreased the RI at all concentrations and treatment times (Table 3). The mixtures of Carbosulfan and $2x10^{-3}$ M EMS decreased the RI only at the highest concentrations during the 24 hour treatment, while they decreased the RI at all concentrations during the 48 hour treatment (Table 3).

No statistical differences were found between the frequency of SCE obtained from the male and female donors. However, the SCE frequency of the female donor was generally higher than that of the male donor.

Table 2. The frequency of the SCE and the RI in cultured human lymphocytes treated with mixtures of Carbosulfan, and EC for 24 and 48 hours*

Test Substances	Treatment Times (Hour)	Concentrations (M)	Min-Max. SCE	SCE/Cell±SE	M_1	M_2	M_3	RI±SE
Carbosulfan+	24	Control	2-17	7.22±0.480	44	104	52	2.04±0.005
$2x10^{-3}$ M EC		Solvent Control**	4-13	7.64±0.340	48	94	58	2.05±0.017
		$2.5x10^{-3}$ EC	4-18	9.44±0.460a4b4	49	96	55	2.03±0.069
		$5x10^{-5}$ C+$2x10^{-3}$ EC	4-21	10.76±0.530a4b4	57	108	35	1.89±0.023a2b2
		10^{-4} C+$2x10^{-3}$ EC	5-24	10.58±0.550a4b4	95	89	16	1.60±0.077a1b1
Carbosulfan+	48	Control	3-15	7.74±0.470	33	52	115	2.41±0.051
$2x10^{-3}$ M EC		Solvent Control**	3-14	8.48±0.450	32	73	95	2.31±0.031
		$2.5x10^{-5}$ C+10^{-3} EC	5-19	11.06±0.460a4b4	38	104	58	2.10±0.109
		$5x10^{-5}$ C+$2x10^{-3}$ EC	6-20	10.90±0.430a4b4	58	109	33	1.87±0.072a2b2
		10^{-4} C+$2x10^{-3}$ EC	6-24	11.12±0.580a4b4	164	34	2	1.19±0.011a4b4
Carbosulfan+	24	Control	3-15	8.42±0.360	44	108	48	2.02±0.002
$4x10^{-3}$ M EC		Solvent Control**	2-16	7.64±0.420	41	102	57	2.08±0.040
		$2.5x10^{-5}$ C+$4x10^{-3}$ EC	5-18	9.76±0.450a2b4	56	113	31	1.87±0.002a4b4
		$5x10^{-5}$ C+$4x10^{-3}$ EC	5-17	10.06±0.390a4b4	71	112	17	1.73±0.005a4b4
		10^{-4} C+$4x10^{-3}$ EC	5-19	10.96±0.380a4b4	121	73	6	1.42±0.129a1b1
Carbosulfan+	48	Control	3-14	8.42±0.440	32	60	108	2.38±0.069
$4x10^{-3}$ M EC		Solvent Control**	3-14	7.92±0.410	31	59	110	2.39±0.014
		$2.5x10^{-5}$ C+$4x10^{-3}$ EC	7-17	10.52±0.390a4b4	37	100	63	2.13±0.086a1b1
		$5x10^{-5}$ C+$4x10^{-3}$ EC	5-21	10.58±0.470a4b4	47	106	47	2.00±0.080a1b1
		10^{-4} C+$4x10^{-3}$ EC	2-18	10.72±0.460a4b4	166	30	4	1.19±0.023a4b4
Carbosulfan+	24	Control	2-11	7.52±0.310	37	91	72	2.17±0.069
$8x10^{-3}$ M EC		Solvent Control**	3-16	7.58±0.400	35	91	74	2.19±0.106
		$2.5x10^{-5}$ C+$8x10^{-3}$ EC	4-19	8.86±0.450a4b2	44	88	68	2.12±0.098
		$5x10^{-5}$ C+$8x10^{-3}$ EC	5-17	10.48±0.440a4b4	76	76	48	1.86±0.184
		10^{-4} C+$8x10^{-3}$ EC	5-15	10.00±0.390a4b4	87	74	39	1.76±0.225
Carbosulfan+	48	Control	4-15	8.84±0.430	35	82	83	2.24±0.011
$8x10^{-3}$ M EC		Solvent Control**	3-14	8.32±0.380	38	62	100	2.31±0.063
		$2.5x10^{-5}$ C+$8x10^{-3}$ EC	5-19	10.62±0.500a4b4	28	107	65	2.18±0.043
		$5x10^{-5}$ C+$8x10^{-3}$ EC	5-24	11.18±0.500a4b4	52	92	56	2.02±0.052a1b1
		10^{-4} C+$8x10^{-3}$ EC	6-21	11.96±0.500a4b4	156	43	1	1.22±0.037a4b4

* A total of 50 cells were scored for SCE and 200 cells were scored for the RI

** 80% Ethanol 10 µl + sterile distilled water 10 µl

a: Significant from control; b: Significant from solvent control.

a1b1: P≤0.05, a2b2: P≤0.01, a3b3: P≤0.005, a4b4: P<0.001

Table 3. The frequency of the SCE and the RI in cultured human lymphocytes treated with mixtures of Carbosulfan, and EMS for 24 and 48 hours*

Test Substances	Treatment Times (Hour)	Concentrations (M)	Min-Max. SCE	SCE/Cell±SE	M_1	M_2	M_3	RI±SE
Carbosulfan+ $5x10^{-4}$ M EMS	24	Control	3-17	7.54±0.410	40	94	66	2.13±0.034
		Solvent Control**	1-17	6.94±0.430	41	89	70	2.14±0.026
		$2.5x10^{-5}$ C+$5x10^{-4}$ EMS	4.21	10.28±0.610a4b4	44	97	59	2.07±0.060
		$5x10^{-5}$ C+$5x10^{-4}$ EMS	4-19	10.80±0.540a4b4	65	109	26	1.80±0.089a1b1
		10^{-4} C+$5x10^{-4}$ EMS	4-20	11.28±0.490a4b4	77	97	26	1.74±0.077a1b1
Carbosulfan+ $5x10^{-4}$ M EMS	48	Control	2-16	8.46±0.470	38	83	79	2.20±0.008
		Solvent Control**	3-14	8.56±0.680	46	74	80	2.17±0.017
		$2.5x10^{-5}$ C+$5x10^{-4}$ EMS	14-36	23.86±0.680a4b4	52	112	36	1.92±0.103
		$5x10^{-5}$ C+$5x10^{-4}$ EMS	16-37	24.84±0.710a4b4	73	108	19	1.73±0.011a4b4
		10^{-4} C+$5x10^{-4}$ EMS	18-42	27.58±0.840a4b4	173	26	1	1.14±0.040a4b4
Carbosulfan+ 10^{-3} M EMS	24	Control	3-17	7.38±0.450	35	69	96	2.30±0.066
		Solvent Control**	2-17	7.02±0.420	32	68	100	2.34±0.086
		$2.5x10^{-5}$ C+10^{-3} EMS	5-21	10.88±0.490a4b4	55	79	66	2.05±0.049a1b2
		$5x10^{-5}$ C+10^{-3} EMS	6-29	12.36±0.600a4b4	76	66	58	1.91±0.075a1b2
		10^{-4} C+10^{-3} EMS	6-26	12.02±0.510a4b4	68	100	32	1.77±0.002a4b4
Carbosulfan+ 10^{-3} M EMS	48	Control	2-14	7.78±0.420	39	74	87	2.24±0.028
		Solvent Control**	3-16	8.34±0.430	38	85	77	2.19±0.031
		$2.5x10^{-5}$ C+10^{-3} EMS	26-57	38.48±1.030a4b4	60	125	15	1.77±0.020a4b4
		$5x10^{-5}$ C+10^{-3} EMS	28-58	42.52±1.060a4b4	123	66	11	1.43±0.129a2b2
		10^{-4} C+10^{-3} EMS	32-56	42.12±0.950a4b4	186	10	4	1.09±0.005a4b4
Carbosulfan+ $2x10^{-3}$ M EMS	24	Control	3-17	7.50±0.430	35	66	99	2.32±0.075
		Solvent Control**	2-17	7.52±0.450	31	66	103	2.36±0.066
		$2.5x10^{-5}$ C+$2x10^{-3}$ EMS	7-24	14.46±0.550a4b4	55	76	69	2.07±0.161
		$5x10^{-5}$ C+$2x10^{-3}$ EMS	9-34	16.40±0.730a4b4	57	84	59	2.01±0.109
		10^{-4} C+$2x10^{-3}$ EMS	13-30	19.10±0.480a4b4	77	95	28	1.75±0.072a3b3
Carbosulfan+ $2x10^{-3}$ M EMS	48	Control	3-16	7.70±0.400	38	76	86	2.24±0.024
		Solvent Control**	3-16	7.92±0.460	42	87	71	2.14±0.060
		$2.5x10^{-5}$ C+$2x10^{-3}$ EMS	51-93	72.38±0.460a4b4	111	88	1	1.45±0.052a4b4
		$5x10^{-5}$ C+$2x10^{-3}$ EMS	60-110	81.10±0.760a4b4	160	39	1	1.20±0.020a4b4
		10^{-4} C+$2x10^{-3}$ EMS***	-	-	182	9	9	1.13±0.031a4b4

* A total of 50 cells were scored for SCE and 200 cells were scored for the RI

** 80% Ethanol 10 µl + 50% Ethanol 10 µl

*** Not scorable due to excessive toxicity

a: Significant from control; b: Significant from solvent control,

a1b1: P≤0.05, a2b2: P≤0.01, a3b3: P≤0.005, a4b4: P<0.001

Table 4. The synergistic effects of Carbosulfan and EC on SCE and the RI in human lymphocytes during the 24 hour treatment

Test Substances	Treatment Times (Hour)	Concentrations (M)	SCE/Cell±SE	RI±SE
Carbosulfan+ $2x10^{-3}$ M EC	24	$2.5x10^{-5}$ M C+$2x10^{-3}$ M EC	9.44±0.44a4b3	2.03±0.069
		$5x10^{-5}$ M C+$2x10^{-3}$ M EC	10.76±0.53a4b4	1.89±0.023a3
		10-4 M C+$2x10^{-3}$ M EC	10.58±0.55a4b4	1.60±0.078a1b1
		$2.5x10^{-5}$ M C	7.52±0.24	2.21±0.196
		$5x10^{-5}$ M C	9.02±0.51	2.13±0.222
		10^{-4} M C	8.68±0.42	1.91±0.202
		$2x10^{-3}$ M EC	8.00±0.45	1.88±0.092
Carbosulfan+ $4x10^{-3}$ M EC	24	$2.5x10^{-5}$ M C+$4x10^{-3}$ M EC	9.76±0.46a4b4	1.87±0.003a4b2
		$5x10^{-5}$ M C+$4x10^{-3}$ M EC	10.06±0.39a2b4	1.73±0.006a4b4
		$10x^{-4}$ M C+$4x10^{-3}$ M EC	10.96±0.38a4b4	1.42±0.130a1b1
		$2.5x10^{-5}$ M C	7.52±0.24	2.21±0.196
		$5x10^{-5}$ M C	9.02±0.51	2.13±0.222
		10^{-4} M C	8.68±0.42	1.91±0.202
		$4x10^{-3}$ M EC	7.92±0.46	1.89±0.124
Carbosulfan+ $8x10^{-3}$ M EC	24	$2.5x10^{-5}$ M C+$8x10^{-3}$ M EC	8.86±0.45a3	2.12±0.098b1
		$5x10^{-5}$ M C+$8x10^{-3}$ M EC	10.48±0.44a3b4	1.86±0.185
		10^{-4} M C+$8x10^{-3}$ M EC	10.39±0.38a3b4	1.76±0.225
		$2.5x10^{-5}$ M C	7.52±0.24	2.21±0.196
		$5x10^{-5}$ M C	9.02±0.51	2.13±0.222
		10^{-4} M C	8.68±0.42	1.91±0.202
		$8x10^{-3}$ M EC	8.46±0.53	1.80±0.150

a: Significant from Carbosulfan separately, b: Significant from EC separately
a1b1: P≤0.05, a2b2: P≤0.01, a3b3: P≤0.005, a4b4: P≤0.001.

2. The synergistic effects of the test substances on SCE and the RI in human lymphocytes

There was a synergism between Carbosulfan and EC on induction of SCE when compared with Carbosulfan and EC during the 24 hour treatment (Table 4). The mixtures of 10^{-4} M Carbosulfan+$2x10^{-3}$ M EC, $5x10^{-5}$ Carbosulfan+$4x10^{-3}$ M EC and 10^{-4} M Carbosulfan+$4x10^{-3}$ M EC synergistically decreased the RI during the 24 hour treatment. There was no synergism or antagonism between Carbosulfan and $8x10^{-3}$ M EC during the 24 hour treatment (Table 4).

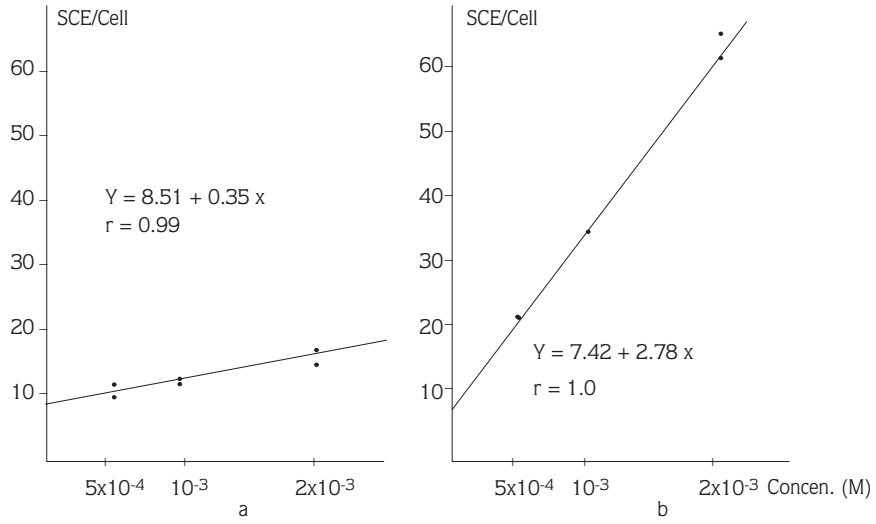

Figure 2. Correlation coefficients and regression lines of SCE/cell in human lymphocytes treated with EMS for 24 (a) and 48 (b) hours

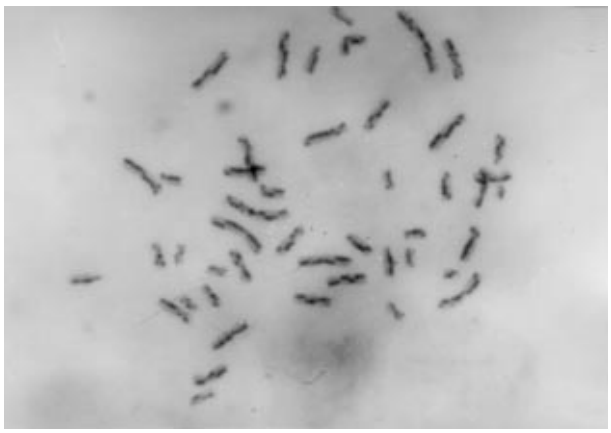

Figure 3. A cell had a 91 SCE ($2x10^{-3}$ M EMS, 48 h).x1000

During the 48 hour treatment, $5x10^{-5}$ M Carbosulfan+$2x10^{-3}$ M EC, $5x10^{-5}$ M Carbosulfan+$8x10^{-3}$ M EC and 10^{-4} M Carbosulfan+$8x10^{-3}$ M EC synergistically induced SCE (Table 5). However, there was no synergism or antagonism between Carbosulfan and EC on the RI during the 48 hour treatment (Table 5).

There was not any synergism or antagonism between Carbosulfan and EMS on the frequency of SCE during the 24 hour treatment, except the mixtures of 10^{-4} M Carbosulfan+$5x10^{-4}$ M

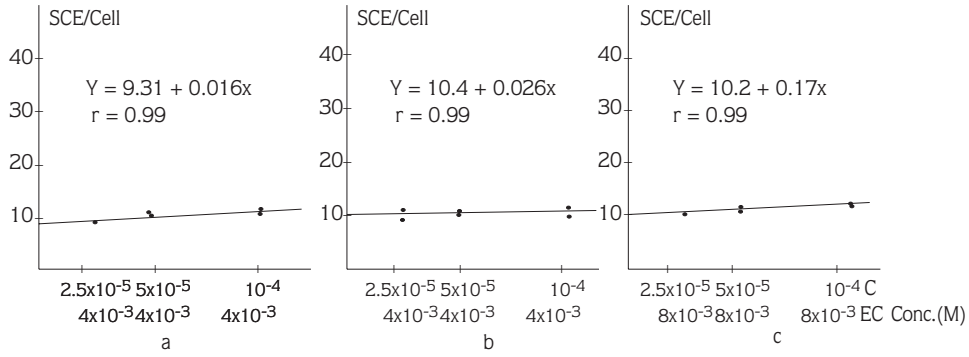

Figure 4. Correlation coefficients and regression lines of SCE/cell in human lymphocytes treated with a mixture of Carbosulfan+4x10^{-3} M EC for 24 (a) and 48 (b) hours and with a mixture of Carbosulfan 8x10^{-3} M EC for 48 hours (c).

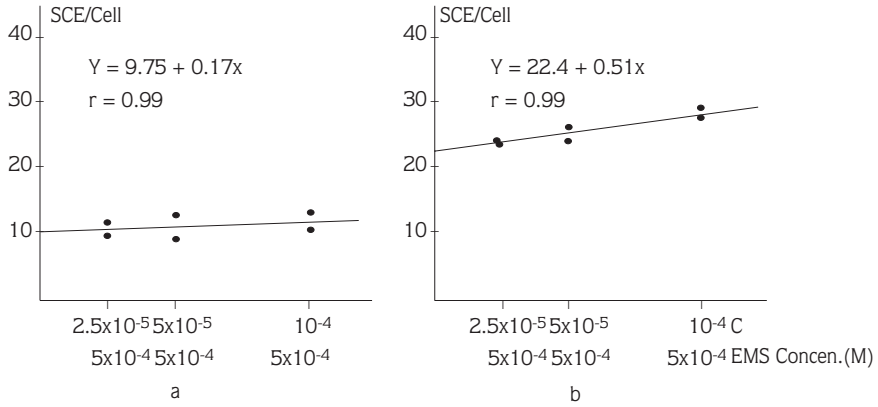

Figure 5. Correlation coefficients and regression lines of SCE/cell in human lymphocytes treated with a mixture of Carbosulfan and 5x10^{-4} M EMS for 24 (a) and 48 (b) hours

EMS and 10^{-4} M Carbosulfan+2x10^{-3} M EMS which synergistically induced the SCE during this treatment time (Table 6). The mixtures of Carbosulfan and 10^{-3} M EMS synergistically decreased the RI. In addition the mixture of 5x10^{-5} M Carbosulfan+5x10^{-4} M EMS and 10^{-4} M Carbosulfan+2x10^{-3} M EMS also synergistically decreased the RI during the 24 hour treatment (Table 6).

Table 5. The synergistic effect of Carbosulfan and EC on SCE and the RI in human lympochytes during the 48 hour treatment

Test Substances	Treatment Times (Hour)	Concentrations (M)	SCE/Cell±SE	RI±SE
Carbosulfan+ $2x10^{-3}$ M EC	48	$2.5x10^{-5}$ M C+$2x10^{-3}$ M EC	11.06±0.46b4	2.10±0.110
		$5x10^{-5}$ M C+$2x10^{-3}$ M EC	10.90±0.43a1b4	1.87±0.072a1b1
		10^{-4} M C+$2x10^{-3}$ M EC	11.12±0.58b4	1.19±0.012a2b4
		$2.5x10^{-5}$ M C	10.44±0.48	2.11±0.130
		$5x10^{-5}$ M C	9.98±0.51	1.63±0.072
		10^{-4} M C	11.00±0.50	1.10±0.003
		$2x10^{-3}$ M EC	8.96±0.44	2.39±0.035
Carbosulfan+ $4x10^{-3}$ M EC	48	$2.5x10^{-5}$ M C+$4x10^{-3}$ M EC	10.52±0.39b4	2.13±0.087
		$5x10^{-5}$ M C+$4x10^{-3}$ M EC	10.58±0.47b4	2.00±0.081a1b1
		$10x^{-4}$ M C+$4x10^{-3}$ M EC	10.72±0.46b4	1.19±0.023a1b4
		$2.5x10^{-5}$ M C	10.44±0.48	2.11±0.130
		$5x10^{-5}$ M C	9.98±0.51	1.63±0.072
		10^{-4} M C	11.00±0.50	1.10±0.003
		$4x10^{-3}$ M EC	8.66±0.42	2.28±0.003
Carbosulfan+ $8x10^{-3}$ M EC	48	$2.5x10^{-5}$ M C+$8x10^{-3}$ M EC	10.62±0.50b3	2.18±0.043b1
		$5x10^{-5}$ M C+$8x10^{-3}$ M EC	11.18±0.57a1b4	2.02±0.052a2b2
		10^{-4} M C+$8x10^{-3}$ M EC	11.96±0.50a1b4	1.22±0.038b4
		$2.5x10^{-5}$ M C	10.44±0.48	2.11±0.130
		$5x10^{-5}$ M C	9.98±0.51	1.63±0.072
		10^{-4} M C	11.00±0.50	1.10±0.003
		$8x10^{-3}$ M EC	9.02±0.45	2.37±0.058

a: Significant from Carbosulfan separately, b: Significant from EC separately
a1b1: P≤0.05, a2b2: P≤0.01, a3b3: P≤0.005, a4b4: P≤0.001.

The mixtures Carbosulfan and EMS synergistically induced the SCE at all concentrations during the 48 hour treatment (Table 7). There was synergism on the decrease of the RI in cultures treated for 48 hours with mixtures of Carbosulfan+10^{-3} M EMS and Carbosulfan+$2x10^{-3}$ M EMS (Table 7).

Table 6. The synergistic effect of Carbosulfan and EMS on SCE and the RI in human lympochytes during the 24 hour treatment

Test Substances	Treatment Times (Hour)	Concentrations (M)	SCE/Cell±SE	RI±SE
Carbosulfan+ $5x10^{-4}$ M EMS	24	$2.5x10^{-5}$ M C+$5x10^{-4}$ M EMS	10.28±0.61a4	2.07±0.061
		$5x10^{-5}$ M C+$5x10^{-4}$ M EMS	10.80±0.54a3	1.80±0.089a1b1
		10^{-4} M C+$5x10^{-4}$ M EMS	11.28±0.49a4b1	1.74±0.078b1
		$2.5x10^{-5}$ M C	7.52±0.24	2.21±0.196
		$5x10^{-5}$ M C	9.02±0.51	2.13±0.222
		10^{-4} M C	8.68±0.42	1.91±0.202
		$5x10^{-4}$ M EMS	10.34±0.51	2.14±0.118
Carbosulfan+ 10^{-3} M EMS	24	$2.5x10^{-5}$ M C+10^{-3} M EMS	10.88±0.49a4b1	2.05±0.049a1b1
		$5x10^{-5}$ M C+10^{-3} M EMS	12.36±0.60a4	1.91±0.075a1b1
		$10x^{-4}$ M C+10^{-3} M EMS	12.02±0.51a4	1.77±0.003a4b4
		$2.5x10^{-5}$ M C	7.52±0.24	2.21±0.196
		$5x10^{-5}$ M C	9.02±0.51	2.13±0.222
		10^{-4} M C	8.68±0.42	1.91±0.202
		10^{-3} M EMS	11.94±0.47	2.18±0.118
Carbosulfan+ $2x10^{-3}$ M EMS	24	$2.5x10^{-5}$ M C+$2x10^{-3}$ M EMS	14.46±0.55a4b1	2.07±0.165
		$5x10^{-5}$ M C+$2x10^{-3}$ M EMS	16.40±0.73a4	2.01±0.110
		10^{-4} M C+$2x10^{-3}$ M EMS	19.10±0.48a4b4	1.75±0.72b1
		$2.5x10^{-5}$ M C	7.52±0.24	2.21±0.196
		$5x10^{-5}$ M C	9.02±0.51	2.13±0.222
		10^{-4} M C	8.68±0.42	1.91±0.202
		$2x10^{-3}$ M EC	15.60±0.66	2.16±0.098

a: Significant from Carbosulfan separately, b: Significant from EMS separately

a1b1: P≤0.05, a2b2: P≤0.01, a3b3: P≤0.005, a4b4: P≤0.001.

Table 7. The synergistic effect of Carbosulfan and EMS on SCE and the RI in human lympochytes during the 48 hour treatment

Test Substances	Treatment Times (Hour)	SCE/Cell±SE Concentrations (M)	RI±SE	
Carbosulfan+ $5x10^{-4}$ M EMS	48	$2.5x10^{-5}$ M C+$5x10^{-4}$ M EMS	23.86±0.68a4b3	1.92±0.104b1
		$5x10^{-5}$ M C+$5x10^{-4}$ M EMS	24.84±0.70a4b4	1.73±0.012a3b4
		10^{-4} M C+$5x10^{-4}$ M EMS	27.68±0.84a4b4	1.14±0.040b4
		$2.5x10^{-5}$ M C	10.44±0.48	2.11±0.130
		$5x10^{-5}$ M C	9.98±0.51	1.63±0.072
		10^{-4} M C	11.00±0.50	1.10±0.003
		$5x10^{-4}$ M EMS	21.66±0.72	2.28±0.136
Carbosulfan+ 10^{-3} M EMS	48	$2.5x10^{-5}$ M C+10^{-3} M EMS	38.48±1.03a4b4	1.77±0.020a4b4
		$5x10^{-5}$ M C+10^{-3} M EMS	42.42±1.06a4b4	1.43±0.130a1b1
		$10x^{-4}$ M C+10^{-3} M EMS	42.12±0.95a4b4	1.09±0.006b4
		$2.5x10^{-5}$ M C	10.44±0.48	2.11±0.130
		$5x10^{-5}$ M C	9.98±0.51	1.63±0.072
		10^{-4} M C	11.00±0.50	1.10±0.003
		10^{-3} M EMS	34.64±0.97	2.10±0.061
Carbosulfan+ $2x10^{-3}$ M EMS	48	$2.5x10^{-5}$ M C+$2x10^{-3}$ M EMS	72.38±1.46a4b4	1.45±0.052a4b1
		$5x10^{-5}$ M C+$2x10^{-3}$ M EMS	81.10±1.76a4b4	1.20±0.020a4b4
		10^{-4} M C+$2x10^{-3}$ M EMS	-- -- --	1.13±0.032b4
		$2.5x10^{-5}$ M C	10.44±0.48	2.11±0.130
		$5x10^{-5}$ M C	9.98±0.51	1.63±0.072
		10^{-4} M C	11.00±0.50	1.10±0.003
		$2x10^{-3}$ M EMS	63.92±1.39	1.68±0.115

a: Significant from Carbosulfan separately, b: Significant from EMS separately

a1b1: P≤0.05, a2b2: P≤0.01, a3b3: P≤0.005, a4b4: P≤0.001.

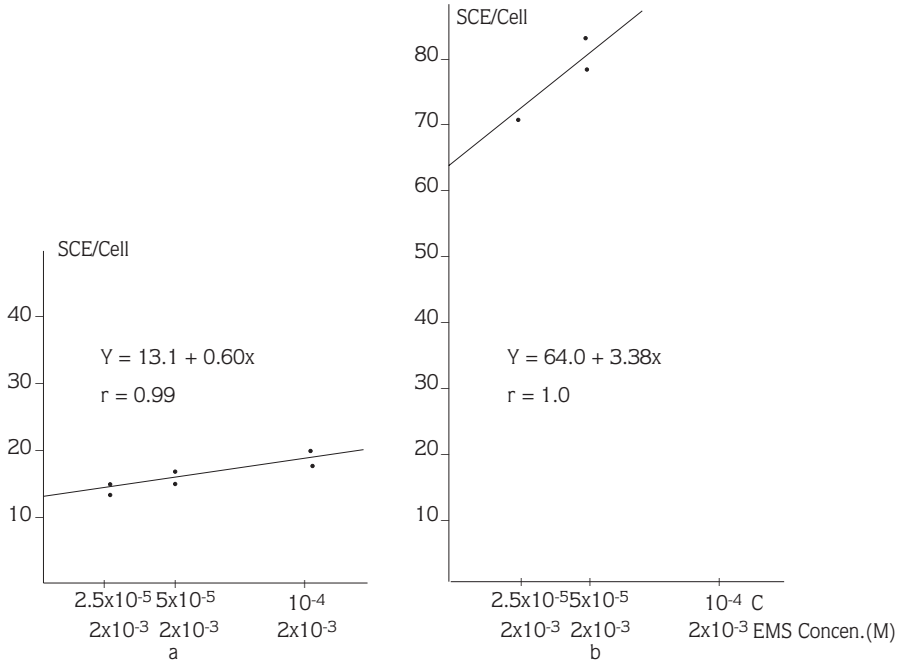

Figure 6. Correlation coefficients and regression lines of SCE/cell in human lymphocytes treated with a mixture of Carbosulfan and $2x10^{-3}$ M EMS for 24 (a) and 48 (b) hours

Discussion

In this study, Carbosulfan separately did not affect the frequency of SCE and the RI during the 24 hour treatment. However, Carbosulfan slightly induced SCE and decreased the RI during the 48 hour treatment. Carbamate pesticides namely aldicarb, benomyl and propoxur and their nitroso derivatives induced SCE. However, MBC did not induce it in human lymphocytes (2-5, 7). In our previous study we reported that Marshal and its effective ingredient Carbosulfan did not induce SCE in human lymphocytes when a male donor was used (6). However, in this study we used male and female donors. The SCE frequency of the female donor was generally higher than that of the male donor. There was no statistical cignificance. It can be concluded that the differences between the results of this study and the previous study arose from the number of donors and especially from the SCE frequency of the female donor. It was clearly shown that a lot of donors have to be used for in vitro studies. As shown, most of the carbamate pesticides induced SCE. For occurence of SCE, the double-strands of DNA must break and it is necessary to exchange the identic molecules of the DNA. The carbamate pesticides namely Marshal (Carbosulfan), aldicarb, benomyl and nitroso propoxur also decreased the RI in human lymphocytes (2, 4-6). As shown, the carbamate pesticides are able to decrease the RI by preventing the replication of DNA at the S phase of the interphase nucleus.

In this study, EC separately did not induce the frequency of SCE at all concentrations and treatment times but, EC slightly increased the RI only during the 48 hour treatment. Popescu et al. (10) have reported that EC slightly induced the SCE in C. hamster V79-4 cells. However, EC induced SCE dose-dependently in $BD2F_1$ mice and there was a correlation between the carcinogenic effect of EC and the induction of SCE (8). EC increased the frequency of SCE in C. hamster, G. hamster, rats and mice. This increase was related to the species (9). Csukas et al. (11) have reported that urethan (EC) and hidroxy urethan induced SCE in cultured human lymphochytes. As shown, EC is able to induce SCE following in vivo exposures higher than the in vitro exposures, because of urethan and hidroxy urethan metabolized to S-ethoxy carbonyl groups under in vivo conditions. According to Nerry, 1969 hidroxy urethan and its esters act directly on cytosine under physiological conditions, carboxyethylating it, i.e., they could act directly on DNA (according to Nerry, 1969 from Bateman, 1976) (28). Boyland and Williams (29) have reported that the labelled urethan appeared to be ethly ester of cytosine-5-carboxylic acid formed by the reaction of urethan appeared to be ethyl ester of cytosine-5-carboxylic acid plays a part in carcinogenesis is unknown, but Berenblum et al. (1959) aftre testing urethane derivatives for skin initiating action and lung carcinogenesis have suggested that the carcinogenic activity was dependent on the intact ethoxy carbonyl group of urethan (according to Berenblum et al., 1959 from Boyland and Williams, 1969) (29). As a result, the carcinogenic activity of EC resulted from the derivatives of EC which occurred following in vivo exposures. Because of that, EC is able to induce SCE following in vivo exposures higher than in vitro exposures. During the 48 hour treatment, the increases in the RI have been the result of the synergistic effects between EC and phytohemagglutinin (PHA), a mitogen, in culture conditions. Especially after 48 hours, EC and PHA displayed synergism on the DNA replication. To discover whether EC has a mitogenic effect or not, it must be tested in cultures wtihout PHA.

In this study, EMS separately induced SCE at all concentrations and treatment times. This result was supported by the other researchers' results (1, 12-17). EMS is known as an alkylating agent which causes alkylation of cellular substances. Kojima et al. (30) have reported that EMS may react by different kinds of SN_1-type (substitution, nucleophilic unimolecular) and SN_2-type (substitution, nucleophilic bimolecular) mechanism. According to this result, EMS is able to alter the coupling of the nucleotids by alkylating and therefore EMS is able to cause CA and SCE. Most of the alkylating agents like EMS, cause mutations at concentrations which are not cytotoxic. Therefore EMS could not decrease the RI except at the highest concentration. Maddock et al. (14) have reported that EMS did not decrease the RI in marine fish following in vivo exposures, Topaktaş and Speit (16) have also reported that EMS slightly decreased the RI in human lymphocytes, but was not dose-dependent.

In this study, the mixtures of Carbosulfan-EC and Carbosulfan-EMS synergistically induced SCE in human lymphocytes. The increases in SCE in the cultures treated with mixtures of Carbosulfan and EMS was higher than the cultures treated with mixtures of Carbosulfan and EC. Dolara et al. (18) have reported that 4 pecticides separately did not induce SCE, but the mixtures of these pesticides induced SCE in human lymphocytes. SCE was also induced in lymphocytes of smoking pesticide sprayers (19). However, it was not induced in the lymphocytes of

workers packing pesticides or in the lymphocytes of rural populations exposed to pesticides (20, 21). These results showed that the exposure route and time exposed to pesticides are important and also showed that the mixture of pesticides are able to induce SCE synergistically. On the other hand, the frequency of SCE was hig- her in the lymphocytes of populations exposed to occupational and environmental pollutants (20, 22, 23, 24, 31). As shown, the frequency of SCE was synergistically increased in populations exposed to mixtures of pesticides, and it was higher in populations exposed to mixtures of pesticides with mutagens. In addition, SCE was higher in populations exposed to mixtures of occupational and environmental pollutants. In this study, althought Carbosulfan separately slightly induced the SCE, but EC did not induce it. Generally, the mixtures of Carbosulfan and EC synergistically induced SCE. The in vivo effects of Carbosulfan and EC were higher than the in vitro effects. Due to this result, it can be concluded that most probably the chemicals will exhibit synergism when the human population is exposed to mixtures of pesticides and mutagens and also to pesticides and carcinogens. In this study, generally the mixtures of Carbosulfan and EC synergistically decreased the RI, except the mixture of Carbosulfan and $8x10^{-3}$ M EC. However Carbosulfan separately decreased the RI. It was understood that $8x10^{-3}$ M EC blocked the effect of Carbosulfan on the RI. For this reason, the mixture of Carbosulfan and $8x10^{-3}$ M EC did not decrease the RI. The mixtures of Carbosulfan and EMS synergistically decreased the RI. This result also showed that Carbosulfan and EMS as a mixture, could prevent DNA replication. It was reported that mixtures of pesticides and the mixtures of other chemicals decreased the RI (19, 22, 23).

As a result, it can be concluded that the combined treatment of Carbosulfan with a carcinogen (EC) or with a mutagen (EMS) might cause the highest genotoxic risk for humans because of nitrosated derivates and metabolities of Carbosulfan and EC following in vivo exposures and also because of the alkylating action of EMS. According to the results obtained from this study, it was celarly shown that mutagens and carcinogens caused the greatest genetic damage in the regions in which pesticides were extensively used. For this reason, it is necessary to inform the populations exposed to environmental pollutants and rural populations exposed to pesticides. And it is also necessary to be careflul when exposed to these pollutants for the health of future generations.

Acknowledgements

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