

# Genotoxic Effects of $\alpha$ -Endosulfan and $\beta$ -Endosulfan on Human HepG2 Cells

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$\alpha$ -Endosulfan and  $\beta$ -endosulfan are isomers of endosulfan, a pesticide used worldwide. In this study, we examined the genotoxicity of  $\alpha$ - and  $\beta$ -endosulfan *in vitro* with a HepG2 cell line. We used sister chromatid exchanges (SCE), micronuclei (MN), and DNA strand breaks as detected by single-cell gel electrophoresis (SCG) assays as biomarkers to judge the genotoxicity of  $\alpha$ - and  $\beta$ -endosulfan at concentrations from  $1 \times 10^{-12}$  M to  $1 \times 10^{-3}$  M. After treating HepG2 cells for 48 hr with  $\beta$ -endosulfan, SCE showed a significant increase at concentrations from  $1 \times 10^{-7}$  M to  $1 \times 10^{-5}$  M, and MN showed a significant increase at concentrations from  $5 \times 10^{-5}$  M to  $1 \times 10^{-3}$  M.  $\alpha$ -Endosulfan failed to show significant effects in both the SCE and MN assays. After treating HepG2 cells with  $\alpha$ - or  $\beta$ -endosulfan for 1 hr, DNA strand breaks were significantly induced by  $\alpha$ -endosulfan at concentrations from  $2 \times 10^{-4}$  M to  $1 \times 10^{-3}$  M, and by  $\beta$ -endosulfan at  $1 \times 10^{-3}$  M. The results of this study suggest that both  $\alpha$ - and  $\beta$ -endosulfan are genotoxic to HepG2 cells and that the genotoxicity of  $\beta$ -endosulfan seems stronger than that of  $\alpha$ -endosulfan. **Key words:**  $\alpha$ -endosulfan,  $\beta$ -endosulfan, genotoxicity, HepG2, micronuclei, MN, single-cell gel electrophoresis, SCG, sister chromatid exchanges, SCE. *Environ Health Perspect* 108:559–561 (2000). [Online 2 May 2000]

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Endosulfan is a synthetic chlorinated cyclodiene that is an environmental endocrine disruptor (1). It was introduced into the earth's environment in 1956 as a general use insecticide, and it was primarily used to protect food crops such as tea, fruits, vegetables, and grains as well as wood from a wide variety of insects and mites through contact poison. Commercially used endosulfan is generally composed of its two isomers— $\alpha$ -endosulfan and  $\beta$ -endosulfan.

Endosulfan is toxic not only to insects, but also to fish, animals, and humans (2,3). Autopsy examinations have revealed its damage to liver, lung, and brain (3). However, the data regarding its genotoxicity (4), especially that of its two isomers, are limited. Perhaps because its carcinogenicity and genotoxicity have not been confirmed, endosulfan is still widely used and continues to pollute the human environment not only in developing countries but in developed countries as well (5).

In this study, we observed the influence of  $\alpha$ - and  $\beta$ -endosulfan on the frequency of sister chromatid exchanges (SCE), micronuclei (MN), and the DNA damage assessed by single-cell gel electrophoresis (SCG) in HepG2, a target cell line that expresses estrogen receptors (6) and is able to metabolize xenobiotics like S9 *in vitro* or *in vivo* (7).

## Materials and Methods

**Materials.** All reagents used in this experiment were of analytical grade.

We obtained  $\alpha$ -endosulfan [ $\alpha$ -1,4,5,6,7,7-hexachlorbicyclo-(2.2.1)-5-hepten-2,3-bis(methylen)sulfite;  $C_9H_6Cl_6O_3S$ ] from Wako Pure Chemical Industries, Osaka,

Japan. We obtained  $\beta$ -endosulfan [ $\beta$ -1,4,5,6,7,7-hexachlorbicyclo-(2.2.1)-5-hepten-2,3-bis(methylen)sulfite;  $C_9H_6Cl_6O_3S$ ] from Riedel-deHaen, Seelze, Germany. We dissolved the endosulfans in DMSO (E. Merck, Darmstadt, Germany) to prepare a 0.5-M stock solution.

**Cell culture.** We obtained HepG2 cells from the cell bank of the Institute of Physical and Chemical Research of Japan Wako-Shi, Saitoma, Japan; cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (Biosciences PTY Ltd., Australia) and 1% penicillin-streptomycin (Life Technologies, Grand Island, NY). Before use, HepG2 cells were taken from fluid nitrogen and washed 2 times with fresh culture medium. After culturing for two passages in 37°C and 5% CO<sub>2</sub>, we prepared the HepG2 cells for use in the experiments.

**SCE and MN.** HepG2 cells ( $2 \times 10^5$ ) were divided into 5 mL culture medium and cultured for 48 hr. After discarding 2.5 mL supernatant, we added 2.5 mL new culture medium containing different concentrations of either  $\alpha$ - or  $\beta$ -endosulfan for both the SCE and MN assays. We added 5-bromo-2'-deoxyuridine (Sigma) in the SCE assay (final concentration 40  $\mu$ M). The cells were cultured for another 48 hr. Six hours before collection, we added colcemid (Sigma) to the SCE assay (final concentration  $2 \times 10^{-7}$  M). We used 0.2% DMSO as the vehicle control and mitomycin C (Sigma) as the positive control.

After 48-hr treatment in both SCE and MN assays, we collected the HepG2 cells with 0.25% trypsin-EDTA (Life Technologies).

The cells were then treated with 0.075 M KCl for 10 min and fixed with Carnoy's solution (methanol:acetic acid 3:1, v/v) for 30 min. The cells were washed twice with Carnoy's solution and stored in methanol containing 1% (v/v) acetic acid.

For SCE measurement, we stained the slides using the fluorescence-plus-Giemsa differential staining method. We blindly evaluated SCE in 30 cells per concentration for each experiment. The final result was the combination of two independent experiments ( $n = 60$  metaphases). For MN measurement, we stained the slides with acridine orange (40  $\mu$ g/mL; Sigma) solution, and we used the frequency of the micronucleated cells per 1,000 HepG2 cells as the end point.

We judged the MN according to the following criteria: size (diameter one-tenth to one-half of the main nucleus size), number ( $\leq 3$  MN/cell), number of nuclei (only one main nucleus in the cells counted), attributes (the micronuclei must be round and have a clear boundary), color (the micronuclei must have the same color and staining degree as the main nucleus), separation (the micronuclei must be clearly separated from the main nucleus), and location (the micronuclei must be in the same cell plasma with the main nucleus).

To assess the effects of  $\alpha$ - and  $\beta$ -endosulfan on the cell-cycle kinetics of the cells, we scored the proliferation index (PI) on the same slides used for counting SCE. We evaluated cell-cycle kinetics by the proportion of the first ( $X_1$ ), second ( $X_2$ ), and third ( $X_3$ ) division cells in 100 consecutive metaphases for each independent experiment. We calculated the PI according to the following formula:

$$PI = (1 \times X_1) + (2 \times X_2) + (3 \times X_3).$$

**SCG.** We divided  $2 \times 10^5$  HepG2 cells into 5 mL culture medium and cultured them for 48 hr. After 2.5 mL supernatant was discarded, we added 2.5 mL of a new culture

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medium containing different concentrations of  $\alpha$ - or  $\beta$ -endosulfan. We used 2% DMSO as the vehicle control. After 1 hr of treatment, the cells were collected with 0.25% trypsin-EDTA and adjusted to  $1 \times 10^6$  cells/mL cell suspension after thorough mixing. We mixed 25  $\mu$ L cell suspension with 75  $\mu$ L 0.75% low-melting agarose (Nusieve GTG, FMC BioProducts, Rockland, NY) and then placed it on pre-cleaned frosted micro slides (Matsunami Glass Ind., LTD, Kishiwata, Japan) that were first covered with 80  $\mu$ L 0.5% normal-melting agarose (Sigma). To make this layer adhere to the slide more effectively, we used 20  $\mu$ L 0.5% normal melting agarose to cover the slide and dried it beforehand. We immediately covered the mixed-cell suspension with a coverglass, and then kept the slides at 4°C for 10 min to allow solidification of the agarose. After gently removing the coverglass, we covered the slides with a third layer of low-melting agarose using a coverglass, and then kept it at 4°C for another 10 min to allow solidification of the agarose. After gently removing the coverglass, we immersed the slides in a lysing solution for 1 hr and then moved them to the electrophoretic buffer to allow 20 min for the unwinding of DNA strands, as described by Singh et al. (8). The electrophoresis time was 20 min under 25 V and 300 mA using an electrophoresis compact power supply (ATTO Corporation, Tokyo, Japan). After staining with 20  $\mu$ g/mL ethidium bromide (Sigma), we measured the DNA strand breaks under a fluorescent microscope using a DNA SCG test system (Keio Electronic Ind. Co., Ltd, Ibaraki, Japan). We examined all of the slides 5 hr after staining, and only the cells in the central part of the slides were detected. In the present study, we used the cell tail length to represent the degree of DNA damage to the HepG2 cells.

**Statistics.** We used Dunnett's test in SPSS statistical software (SPSS, Inc., Chicago, IL) for the SCE and SCG assays. We used the chi-square test in SPSS statistical software for the MN assay. For all three assays, the results of two independent experiments were combined for the analysis.

**Table 1.** The effects of  $\alpha$ - and  $\beta$ -endosulfan on the frequency of SCEs in HepG2 cells.

Concentration (M)	No.	$\alpha$ -Endosulfan		$\beta$ -Endosulfan	
		SCE <sup>a</sup>	PI	SCE <sup>a</sup>	PI
Control	60	0.206 ± 0.062	1.99	0.191 ± 0.085	2.02
1 × 10 <sup>-12</sup>	60	0.203 ± 0.078	2.00	0.200 ± 0.047	2.08
1 × 10 <sup>-11</sup>	60	0.216 ± 0.068	2.00	0.204 ± 0.058	2.01
1 × 10 <sup>-10</sup>	60	0.204 ± 0.071	2.04	0.199 ± 0.113	1.93
1 × 10 <sup>-9</sup>	60	0.204 ± 0.076	2.00	0.196 ± 0.075	1.99
1 × 10 <sup>-8</sup>	60	0.206 ± 0.060	2.02	0.220 ± 0.070	2.01
1 × 10 <sup>-7</sup>	60	0.197 ± 0.065	2.02	0.248 ± 0.080*	2.02
1 × 10 <sup>-6</sup>	60	0.205 ± 0.053	1.91	0.252 ± 0.072**	1.97
1 × 10 <sup>-5</sup>	60	0.217 ± 0.059	2.00	0.272 ± 0.065**	1.93

<sup>a</sup>Data are presented as mean ± SD. \*Statistically significant as compared to control (Dunnett's test,  $p < 0.05$ ). \*\*Statistically significant difference as compared to control (Dunnett's test,  $p < 0.01$ ).

## Results

**Influence of  $\alpha$ - and  $\beta$ -endosulfan on the frequency of SCE in HepG2 cells.** We treated HepG2 cells with concentrations of  $\alpha$ - and  $\beta$ -endosulfan ranging from  $1 \times 10^{-12}$  M to  $1 \times 10^{-5}$  M for 48 hr. Because HepG2 is an aneuploid cell line (modal number = 55 chromosomes; range = 50–60 chromosomes), we calculated SCE per chromosome. As shown in Table 1,  $\beta$ -endosulfan caused a significant increase in SCE at concentrations from  $1 \times 10^{-7}$  M to  $1 \times 10^{-5}$  M. In contrast,  $\alpha$ -endosulfan failed to show any significant effect. SCE frequency was approximately 0.19/chromosome in 0.2% DMSO (vehicle control) versus 0.27/chromosome in the highest concentration ( $1 \times 10^{-5}$  M) of  $\beta$ -endosulfan. Positive controls treated with  $1 \times 10^{-7}$  M mitomycin C resulted in 0.484/chromosome ( $p < 0.01$ ).

In these experiments, the addition of  $\alpha$ - and  $\beta$ -endosulfan did not cause any significant change in the proliferation index as compared to the cultures with the 0.2% DMSO controls.

**Influence of  $\alpha$ - and  $\beta$ -endosulfan on the frequency of MN in HepG2 cells.** We used the frequency of micronucleated HepG2 cells to represent the effects of  $\alpha$ - and  $\beta$ -endosulfan on MN induction in the HepG2 cells. As shown in Table 2, after treating HepG2 cells for 48 hr with  $\beta$ -endosulfan at concentrations from  $5 \times 10^{-5}$  M to  $1 \times 10^{-3}$  M, the frequency of micronucleated cells was significantly increased; the frequency at  $1 \times 10^{-3}$  M was approximately 6 times that of the control. Although we failed to find any significant increase of MN in HepG2 cells treated with  $\alpha$ -endosulfan, we found slight increases in MN cells at higher concentrations ( $5 \times 10^{-5}$  M to approximately  $1 \times 10^{-3}$  M). In the present study, mitomycin C ( $1 \times 10^{-6}$  M) showed a much stronger potency to induce MN (116 micronucleated cells in 2,000 cells) than did  $\beta$ -endosulfan ( $p < 0.01$ ).

**Influence of  $\alpha$ - and  $\beta$ -endosulfan on induction of DNA strand breaks as evaluated by SCG assay in HepG2 cells.** We measured the tail length of HepG2 cells treated with

different concentrations of  $\alpha$ - and  $\beta$ -endosulfan. As shown in Table 3, after 1 hr treatment,  $\alpha$ -endosulfan induced significant increases in DNA strand breaks at concentrations from  $2 \times 10^{-4}$  M to  $1 \times 10^{-3}$  M, as did  $\beta$ -endosulfan at  $1 \times 10^{-3}$  M.

## Discussion

Endosulfan is an insecticide with estrogenic activity that is toxic to many fish and mammals. Some reports suggested that it could accumulate in aquatic animals (9) and cause human fatalities (3). The genotoxicity of its two isomers, however, has not been confirmed.

To study the genotoxicity of endosulfan, we used HepG2 cells *a*) because endosulfan is hepatotoxic (3) and *b*) because the metabolic property of human HepG2 cells will offer a chance to examine the effects of its metabolites on those cells. The result will thus be more comparable to findings in an *in vivo* study. Finally, because the HepG2 cell line is well reported in the genotoxic studies using SCE, MN, and SCG assays (10,11), we believe it is reasonable to use such cells to examine the genotoxicity of endosulfan.

Our repeated *in vitro* experiments showed that both  $\alpha$ - and  $\beta$ -endosulfan induced DNA strand breaks as detected by SCG assay. Nevertheless, HepG2 cells seem more sensitive to  $\alpha$ - than to  $\beta$ -endosulfan, as shown by the cell tail length. Because we used a dry-layer gel technique on the slides, the three layers of gel were easily prepared. Using trypsin-EDTA to collect the HepG2 cells kept the individual cells well separated and evenly distributed in the second layer of gel.

For the SCE and MN assays, only  $\beta$ -endosulfan showed significant effects on the cell line, and our present results correspond to the increased frequency of SCE in blood lymphocytes from workers using pesticides including endosulfan (12) and to the increased SCE induced by endosulfan ( $1 \times 10^{-6}$  M, mixture of  $\alpha$ -endosulfan and  $\beta$ -endosulfan) in human lymphoid cells (13). The result of MN induced by  $\beta$ -endosulfan

**Table 2.** Effects of  $\alpha$ - and  $\beta$ -endosulfan on the induction of MN in HepG2 cells.<sup>a</sup>

Concentration (M)	Micronuclei <sup>b</sup>	
	$\alpha$ -Endosulfan	$\beta$ -Endosulfan
Control	20	13
1 × 10 <sup>-7</sup>	18	14
1 × 10 <sup>-6</sup>	17	13
5 × 10 <sup>-6</sup>	14	17
1 × 10 <sup>-5</sup>	16	22
5 × 10 <sup>-5</sup>	26	39*
1 × 10 <sup>-4</sup>	26	51*
5 × 10 <sup>-4</sup>	26	74*
1 × 10 <sup>-3</sup>	30	82*

<sup>a</sup> $n = 2,000$ . <sup>b</sup>Data represent the number of HepG2 cells with at least one micronucleus. \*Statistically significant as compared to control (chi-square test,  $p < 0.01$ ).

**Table 3.** The effect of  $\alpha$ - and  $\beta$ -endosulfan on DNA strand breaks as detected by the SCG assay in HepG2 cells.

Concentration (M)	$\alpha$ -Endosulfan		$\beta$ -Endosulfan	
	No.	Tail length ( $\mu\text{m}$ ) <sup>a</sup>	No.	Tail length ( $\mu\text{m}$ ) <sup>a</sup>
Control	116	49.28 $\pm$ 16.57	123	40.26 $\pm$ 8.26
2 $\times$ 10 <sup>-5</sup>	119	54.73 $\pm$ 18.08	99	42.36 $\pm$ 7.31
1 $\times$ 10 <sup>-4</sup>	110	53.37 $\pm$ 16.73	99	39.64 $\pm$ 7.34
2 $\times$ 10 <sup>-4</sup>	128	58.32 $\pm$ 16.08*	108	43.68 $\pm$ 9.58
5 $\times$ 10 <sup>-4</sup>	118	60.13 $\pm$ 15.86*	90	44.33 $\pm$ 11.33
1 $\times$ 10 <sup>-3</sup>	108	63.56 $\pm$ 32.73*	126	50.27 $\pm$ 13.38*

<sup>a</sup>Data are presented as mean  $\pm$  SD. \*Statistically significant as compared to control (Dunnett's test,  $p < 0.01$ ).

is also in agreement with an *in vivo* study of endosulfan (14).

In our present study, all three end points were from two independent experiments, and the results were sufficiently repeated. Although both  $\alpha$ - and  $\beta$ -endosulfan showed genotoxicity to HepG2 cells at different concentrations by different end points, neither of them showed any apparent effects on cell cycle kinetics in the cell line.

Of the three biomarkers used in this study, SCE and MN were more sensitive in detecting the genotoxicity of  $\beta$ -endosulfan, but SCG was less sensitive. For  $\alpha$ -endosulfan, only SCG showed genotoxicities. The concentration of  $\alpha$ -endosulfan needed to induce DNA strand breaks was apparently lower than that of  $\beta$ -endosulfan. Thus, our results suggest that  $\beta$ -endosulfan more readily induced SCE and MN, whereas  $\alpha$ -endosulfan more easily induced DNA strand breaks, as detected by SCG in the HepG2 cells.

We have limited data regarding the mechanisms of the genotoxicity of  $\alpha$ - and  $\beta$ -endosulfan. Clastogenic activity of the two compounds and/or their metabolites may exist, although only sparse data are available so far (15,16). In addition, because spindle poisoning is reported to relate to MN formations such as bisphenol and diethylstilbestrol (17), whether  $\beta$ -endosulfan actually induced MN through spindle poisoning should be examined in future studies.

Another possible target of research might be the estrogenic effects of  $\alpha$ - and  $\beta$ -endosulfan (18), given the existence of estrogen receptors in HepG2 cells (19). Many environmental estrogenic disruptors, such as diethylstilbestrol, reportedly induce SCE either *in vitro* or *in vivo*, and it seems that their effects tend to be restricted to cells with abundant estrogen receptors (20,21). Endosulfan (a

mixture of  $\alpha$ - and  $\beta$ -endosulfan) is able to combine with estrogen receptors and exert biologic effects (22), and although we have no direct evidence, it is difficult to exclude the possibility that  $\alpha$ - and  $\beta$ -endosulfan bind with the estrogen receptors in HepG2 cells (19,23) to induce genotoxicity in these cells.

Because  $\alpha$ - and  $\beta$ -endosulfan can be metabolized by HepG2 cells, the genotoxicity we found in the cell line may include their metabolites. Further research is needed to determine whether  $\alpha$ - and  $\beta$ -endosulfan or their metabolites are responsible for the observed genotoxicity.

Our findings are based on a study using a human hepatoma HepG2 cell line so as to extrapolate the results to humans. However, further studies with normal human cells and human subjects exposed to the same agents are needed.

In conclusion, the present study has shown for the first time that both  $\alpha$ - and  $\beta$ -endosulfan are genotoxic to HepG2 cells and that the genotoxicity of  $\beta$ -endosulfan is more potent than that of  $\alpha$ -endosulfan. Although the underlying mechanism is still beyond our knowledge, the clastogenicity and estrogenicity of the two isomers suggest the need for further studies.

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