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Evaluation of Genotoxicity on Plant-Derived Dietary Sulfur

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Abstract The potential genotoxicity of methylsulfonylmethane, a crystalline organic sulfur, derived from chemically modified lignin from plants was evaluated using *in vitro* and *in vivo* assays. In the bacterial reverse mutation test using *Salmonella typhimurium* TA98, TA100, TA1535, and TA1538, methylsulfonylmethane did not induce any significant increase of His' revertants. In the *in vitro* chromosome aberration test using Chinese Hamster Lung (CHL) cells, no aberration effects were seen. In the *in vivo* evaluation using a micronucleus test, negative results were obtained. Accordingly, the results indicated that methylsulfonylmethane is not genotoxic and its use is unlikely to present a potential hazard.

Key words: Genotoxicity, dietary sulfur, lignin, micronucleus

Methylsulfonylmethane is a sulfur compound that is plentiful in fresh fruits and vegetables, milk, fish, and grains, yet quickly destroyed when foods are processed. It appears in all living organisms and is biologically active, and there is a minimum concentration of methylsulfonylmethane that must be maintained in the body to preserve the normal function and structure [13, 14]. Methylsulfonylmethane is an important source of sulfur, and experiments using methylsulfonylmethane containing radiolabeled sulfur (^{35}S) have shown that methylsulfonylmethane releases its sulfur to form collagen, keratin, and the basic ingredients of hair and nails, as well as the essential amino acids, methionine and cysteine, and serum proteins [10, 25]. As such, the importance of methylsulfonylmethane as a source of sulfur has been clearly underestimated. Furthermore, it also has preventive and therapeutic properties [3, 6, 21, 24]; for example, the production of methylsulfonylmethane based on the oxidation of DMSO [5]. DMSO is a popular dietary supplement [7] used for various therapeutic purposes, including inflammation [20], allergies [4], arthritis [16, 17, 21, 27], interstitial cystitis [24], cancer prevention [8], and the maintenance of normal keratin levels in hair, skin, and nails [22]. Nonetheless, although methylsulfonylmethane is already used in many countries as a source of sulfur in functional food, the toxicity of methylsulfonylmethane has only been determined for acute and subchronic conditions [12]. Accordingly, the present study assessed the genotoxicity of methylsulfonylmethane using a bacterial reverse mutation test, *in vitro* chromosome aberration test, and *in vivo* micronucleus test.

Highly purified methylsulfonylmethane was used in these studies. For the *in vitro* assays, stock solutions were prepared in sterile distilled water just prior to use. For the *in vivo* assays, the methylsulfonylmethane was formulated in 0.5% w/v carboxymethylcellulose for oral administration. The stock was diluted to deliver the required dose. The sodium azide, mitomycin C, and methyl methane sulfonate were purchased from Sigma Chemicals (St. Louis, MI, U.S.A.), the 9-aminoacridine, 2-nitrofluorene, benzo[a]pyrene, and 2-aminoanthracene purchased from Aldrich Chemical Co. Ltd. (Milwaukee, WI, U.S.A.), and the *S. typhimurium* strains, TA98, TA100, TA1535, and TA1538 provided from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The mice and rats were purchased from Jung-Ang Lab. Animal, Inc. (Seoul, Korea). The S9 fraction was prepared essentially as described by Maron and Ames [19]. The rats were administered Aroclor 1254 (500 mg/kg) [18]. Five days later, the livers of the pretreated rats were removed and homogenized with three times the volume of 0.15 M KCl. The samples were centrifuged at 9,000 ×*g* for 10 min, and S9 derived from the supernatant. The composition of the S9 mix was as follows: 10% (v/v) S9, 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, and 0.1 M phosphate buffer (pH 7.4). In the chromosome aberration assay, 30% S9 was added to the S9 mix.

The reverse mutation tests were carried out using *S. typhimurium* strains TA98, TA100, TA1535, and TA1538 [1, 9, 11, 15]. A 0.1 ml volume of the methylsulfonylmethane

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Table 1. Reverse mutation test of methylsulfonylmethane in *S. typhimurium*.

Compound[a]	Dose (μg/plate)	S9 Mix	No. of His^{+} revertants/plate			
			TA1535	TA1538	TA98	TA100
Solvent control		-	22±3	30±4	35±3	179±9
Methylsulfonylmethane	10,000	-	18±3	25±3	34±7	184±15
	5,000	-	16±4	33±3	33±4	176±10
	2,500	-	14±3	24±3	30±5	170±11
SAZ	0.5	-	922±190	NT	NT	877±77
9-AA	50	-	NT	1,236±563	NT	NT
2-NF	1	-	NT	NT	579±261	NT
Solvent control		+	17±3	16±7	57±6	189±15
Methylsulfonylmethane	10,000	+	20±7	17±4	55±7	191±10
	5,000	+	21±5	14±5	53±7	199±9
	2,500	+	16±4	19±7	58±9	180±7
2-AA	0.5	+	NT	NT	119±4	NT
	1	+	171±65	162±41	NT	674±322

[a]SAZ: sodium azide; 9-AA: 9-aminoacridine hydrochloride; 2-NF: 2-nitrofluorene; 2-AA: 2-aminoanthracene.

solutions (100, 50, and 25 mg/ml) was mixed with 0.1 ml of the 10-h cultured bacteria, and then the bacterial culture was mixed with 0.5 ml of the S9 mix or a 0.1 M phosphate buffer (pH 7.4) and incubated for 20 min at 37°C. After incubation, a top agar (2 ml) was added and the mixture overlaid on a minimal glucose agar plate. After 48 h of incubation at 37°C, the revertant colonies were counted. The treatments were performed in the presence and absence of metabolic activation in two independent experiments. Three replicate plates per dose of methylsulfonylmethane were used in each part of the study, and three doses evaluated within each experiment. As shown in Table 1, in the reverse mutation assay (Ames test) using bacterial cells, there was no increase in the revertant number as a result of methylsulfonylmethane treatment for any of the *S. typhimurium* strains with or without the presence of the S9 mix. It is also noteworthy that uniformly negative results were obtained with the *S. typhimurium* strains. The many revertants in the positive controls suggest the sensitivity of this experimental system. For these reasons, it was concluded that methylsulfonylmethane was nonmutagenic in the bacterial test systems.

The *in vitro* chromosome aberration assay was carried out using CHL cells to evaluate the potential of methylsulfonylmethane damaging chromosomes [23]. Three independent experiments were carried out in the absence or presence of the rat liver S9 fraction. The cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum. The cells were incubated in a 95% air and 5% CO_2 atmosphere at 37°C. The cells were plated at a density of 2.5×10^5 cells on 6-cm plates and grown for 22 h, and then colcemid (0.25 g/ml) was added 2 h before harvest. When using the S9 mix, the medium was changed for a fresh one after 6 h of incubation. After 24 h of incubation, the cells were harvested by trypsinization (0.25%) and centrifugation (1,000 rpm, 5 min). The harvested cells were then resuspended in a 0.075 M KCl solution, incubated at 37°C for 15 min, and fixed with a cold solution (methanol:acetic acid=3:1) by repeating resuspension and centrifugation 3 times. Thereafter, the fixed cells were dried on a clean glass slide, stained with Giemsa (10% in PBS, pH 7.4) for 15 min, washed with water, and dried. One-hundred metaphase cells were observed at a 1,000× magnification. The chromosomal aberrations recorded were a gap (chromatid and chromosome gap), ctb (chromatid break), cte (chromatid exchange), csb (chromosome break), cse (chromosome exchange), and frg (fragmentation). Any increase in the aberration frequency was compared with the negative controls.

After treatment with methylsulfonylmethane, no significant increase in the number of aberrant cells was observed (Table 2). In the presence and absence of the S9 mix, the chromosome aberration produced by treatment with methylsulfonylmethane (1.25, 2.5, 5 mg/ml) was less than 2%. Conversely, in the presence of the positive controls, MMS and B(a)P, a significant increase in the number of aberrant cells was observed in the presence and absence of the S9 mix. Accordingly, these results indicated that methylsulfonylmethane did not increase the chromosome aberration when compared with the negative control.

The *in vivo* micronucleus assay was carried out in mouse bone marrow, where methylsulfonylmethane was administered to groups of mice using an oral gavage on a single occasion at doses of 1,250, 2,500, and 5,000 mg/kg. The vehicle control groups received carboxymethylcellulose at an equivalent oral volume of 0.5% w/v, whereas the positive control groups received an i.p. dose of mitomycin C at 4 mg/kg. The animal groups were all sacrificed 48 h after treatment and bone marrow smears prepared. After staining, the incidence of micronuclei in the polychromatic

Table 2. Chromosome aberration test of methylsulfonylmethane in CHL cells.

Compound[a]	Dose (mg/ml)	S9 mix	Time (h)[b]	No. of cells scored	No. of aberrations[c]						Aberrant cells (%)[d]		
					Gap	ctb	cte	csb	cse	frg	num	TA	TAG
Methylsulfonylmethane	0	-	24	100	3	0	0	0	0	0	1	1	4
	5	-	24	100	0	0	1	0	1	0	0	2	2
	2.5	-	24	100	0	0	0	0	0	0	0	0	0
	1.25	-	24	100	0	0	0	0	0	0	0	0	0
MMS	0.02	-	24	100	4	8	29	0	1	0	0	30	34
Methylsulfonylmethane	0	+	6-18	100	0	0	0	0	0	0	0	0	0
	5	+	6-18	100	2	0	0	0	0	0	0	0	2
	2.5	+	6-18	100	2	0	0	0	0	0	0	0	2
	1.25	+	6-18	100	0	0	0	0	0	0	0	0	0
B[a]P	0.02	+	6-18	100	8	8	14	0	1	0	1	21	27

[a]MMS: methylmethane sulfonate; B[a]P: benzo[a]pyrene.
[b]Treatment time.
[c]gap: chromatid gap+chromosome gap; ctb: chromatid break; cte: chromatid exchange; csb: chromosome break; cse: chromosome exchange; frg: fragmentation; num: numerical aberration.
[d]TA: total structural aberration; TAG: total structural aberration, including gap.

erythrocytes was scored (2,000/mouse). After the oral administration of methylsulfonylmethane, the frequency of micronucleated polychromatic erythrocytes (MNPCE) in the methylsulfonylmethane-treated and vehicle control groups was compared using a mouse bone marrow micronucleus assay. No significant difference in body weight was noted between the methylsulfonylmethane-treated mice and the solvent control (data not shown). Table 3 shows the number of micronucleated polychromatic erythrocytes, where the frequency in the solvent control was 0.4±0.5, the frequency in the positive control was 12.2±1.2, which was significantly higher, and the frequency after methylsulfonylmethane treatment with 1,250, 2,500, and 5,000 mg/kg was 0.1±0.5, 0.5±0.5, and 0.4±0.5, respectively. Therefore, the methylsulfonylmethane treatment did not cause any change in the frequency of micronucleated polychromatic erythrocytes when compared with the solvent control. Consequently, the micronucleus assay results confirmed that methylsulfonylmethane at doses up to 5,000 mg/kg did not exhibit any genotoxicity in mouse bone marrow.

Humans naturally obtain methylsulfonylmethane through vegetables, fruits, and animal products, such as eggs and milk. Yet, since methylsulfonylmethane is volatile, it is invariably lost during cooking or food processing [2, 8, 10], meaning that current eating trends provide less organic sulfur, which may cause health problems. Organic sulfurs, such as DMSO and DMS, are produced from the continuous oxidation of lignin, which is usually obtained from the waste liquid of paper manufacturing, and because of the therapeutic properties of DMSO, it is used for medical purposes. Although the toxicity of DMSO has already been investigated from various aspects [26], confirming a low toxicity and high efficacy, DMSO has an unpleasant smell. Thus, scientists have attempted to find a derivative of DMSO, eventually producing methylsulfonylmethane based on the oxidation of DMSO [5]. Methylsulfonylmethane has almost identical medical properties with DMSO [3, 4, 6, 17, 20, 21, 24]. Therefore, several countries are already using methylsulfonylmethane for various medical purposes, including food supplements and lotions, *etc*. Nonetheless, despite its popular and frequent utilization by the general public, little genotoxicity data have been published on methylsulfonylmethane.

Accordingly, the present study investigated the genotoxicity of methylsulfonylmethane using a bacterial reverse mutation

Table 3. Results of a micronucleus test in mice treated with methylsulfonylmethane.

Test compound[a]	Dose (mg/kg)	No. of mice tested	Exposure (h)	MNPCEs(/1,000 PCEs)
Control	0	6	48	0.4±0.5
Methylsulfonylmethane	1,250 mg/kg	6	48	0.1±0.5
	2,500 mg/kg	6	48	0.5±0.5
	5,000 mg/kg	6	48	0.4±0.5
Mitomycin C	2 mg/kg	6	48	12.2±1.2*

MNPCs: Micronucleated polychromatic erythrocytes; PCEs: Polychromatic erythrocytes.
*: Significantly different from mice treated with vehicle ($p<0.05$ by chi-square test).

test, *in vitro* chromosome aberration test, and *in vivo* micronucleus test. Methylsulfonylmethane was not found to cause any genotoxicity under the test conditions. Yet, further toxicity tests under different conditions are still required to confirm the safety of using methylsulfonylmethane.

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