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Effects of Derivatives of Sulfur Dioxide on Micronuclei Formation in Mouse Bone Marrow Cells *In Vivo*

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Sulfur dioxide (SO_2) is a common air pollutant. Inhaled SO_2 is hydrated to produce sulfurous acid in the respiratory tract, which subsequently dissociates to form its derivatives-bisulfite and sulfite (1:3 M/M, in neutral fluid). The derivatives can be absorbed in blood or other body fluids (Shapiro, 1977). Several studies have shown that the frequencies of chromosomal aberrations (CA), sister chromatid exchanges (SCE), and micronuclei (MN) in peripheral blood lymphocytes from workers chronically exposed to SO_2 in factories were higher than the controls (Schneider and Kalkins 1970; Beckman and Nordenson 1986; Meng and Zhang 1990a,b; Yadav and Kaushik 1996). Recent studies have confirmed that the SO_2 derivatives (bisulfite and sulfite) induce CA, SCE and MN in cultured human blood lymphocytes in vitro, and that these increases occur in a dose-dependent manner (Meng and Zhang 1992). The detection of MN frequency in polychromatic erythrocytes (PCE) of mouse bone marrow as well as in peripheral blood lymphocytes is a very sensitive index of damage produced by ionizing radiation (Jenssen and Ramel 1976; Chaubey *et al.* 1978; Morales-Ramirez *et al.* 1994; Abrasson-Zetterberg *et al.* 1995) and by chemical mutagens (Mavournin et al. 1990). In the present study, we evaluated SO_2 derivatives (sodium bisulfite and sodium sulfite) for their ability to induce MN in PCE cells of mouse bone marrow *in vivo*, and we also tested SO_2 derivatives for their potential to inhibit or enhance MN formation induced by mutagens cyclophosphamide (CP) or mitomycin C (MMC) in the mouse PCE cells *in vivo*.

MATERIALS AND METHODS

Approximately 6 week old male and female Kunming mice were supplied by the Division of Laboratory Animals, China Institute for Radiation Protection, Taiyuan. The mice weighed between 20 and 25 g on the day of the experiment. Animals were housed in the departmental animal facility for 4 days after receipt prior to each experiment. The animal room was maintained at 22±2℃ with 50% humidity and time-controlled lighting (12 h of light per day). The animals

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were fed standard rodent pellet diet (purchased from China Institute for Radiation Protection, Taiyuan) and water ad libitum. Animals were randomly numbered and then assigned to different treatment groups.

Sodium bisulfite, sodium sulfite, CP, MMC and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Four groups of 10 mice each received an intraperitoneal (i.p.) dose of the SO_2 derivatives (the mixture of sodium sulfite and sodium bisulfite, 3:1 M/M) (20, 100, 500, 750 mg/kg body weight) dissolved in saline; one negative control group of 10 mice each received i.p. 25 μl of saline; one positive control group of 10 mice each received i.p. dose of CP (50 mg/kg body weight). After 24 h, the treatment was repeated, but the positive control did not receive a second dose of CP; So, total doses of the derivatives were: 20 x 2, 100 x 2, 500 x 2, 750 x 2 mg/kg body weight. All first injections were on the left side of each animal, and second injections on the right. Based on our pilot study with the derivatives at the i.p. dose of 1000 x 2 mg/kg, which resulted in lethality to 50% mice (data not shown), the maximum dose of 750 x 2 mg/kg (0.75 LD_{50}) and the minimum dose of 20 x 2 mg/kg (0.02 LD_{50}) were selected. Saline was administered in a constant volume of approximately 1 ml/kg body weight. Animals were killed by cervical dislocation 24 h after the second injection. The bone marrow was removed by flushing each femur with 0.1 ml of fetal bovine serum (FBS). The cells from each femur were then pooled and centrifuged at 1500 rpm for 10 min. After most of the supernatant was discarded, the cell pellet was resuspended in the remaining FBS, and slide smears were prepared. To determine the relationship between MN formation and time after exposure to the SO_2 derivatives, 7 groups of 10 mice each received twice the i.p. dose of the derivatives (500 mg/kg), animals were killed 12, 24, 36, 48, 60,and 72 h after second injection. One negative control group of 6 mice each received i.p. 25 μl of saline, animals were killed 24 h after second injection, and then micronuclei slides were prepared.

Six groups of 10 mice each received the SO_2 derivatives on the left side and/or CP on the right side as follows: (1) negative saline control group, (2) CP group (50 mg/kg), (3) CP (50mg/kg) add SO_2 derivative (500 mg/kg) group, (4) CP group (100 mg/kg), (5) CP (100 mg/kg) add SO_2 derivative (500 mg/kg) group, (6) SO_2 derivative (500mg/kg) group. Second i.p. injections of SO_2 derivatives were carried out 24 h after first i.p. injections. The doses of CP alone were administered only once. Animals were killed 24 h after second injections, and then the micronuclei slides were prepared.

Seven groups of 10 mice each received the SO_2 derivatives on the left side and/or MMC on the right side as follows: (1) negative saline control group, (2)

DMSO control group (4 ml/kg), (3) MMC group (0.50 mg/kg), (4) MMC (0.50 mg/kg) add SO_2 derivative (500 mg/kg) group, (5) MMC group (1.00 mg/kg), (6) MMC (1.00 mg/kg) add SO_2 derivative (500 mg/kg) group, (7) SO_2 derivative (500mg/kg) group. Second i.p. injections were carried out 24 h after first i.p. injections. The doses of MMC were given only once. Animals were killed 24 h after second injections, and then the micronuclei slides were prepared.

Slides were prepared from the bone marrow according to the method outlined by Schmid (1976). One slide was prepared from each mouse. After smearing, all slides were air dried, fixed in methanol for 10 min. and stained with 0.25% May-Grunwald stain for 5 min and rinsed with deionized water. The slides were then stained with a 20% Giemsa stain for 30 min, rinsed with deionized water, and air dried. After 1 day, coverslips were placed onto each slide and each slide was coded. From each mouse, 1000 PCE were examined. Only PCE with a homogeneous blue/grey color and no sign of RNA-reticulum were scored. Micronuclei (MN) within PCE were recognized by their characteristic size, rounded shape, and dark-blue color. The number of PCE containing MN (MNPCE) was recorded as a percentage of the total PCE counted. For all slides, the percentage of PCE was recorded to monitor for erythropoietic toxicity. All slides were scored blindly by the same observer. The statistical significance of the differences between the treated and control groups was determined using the Student *t*-test.

RESULTS AND DISCUSSION

Dose-response relationship: In the current study, SO_2 derivatives were tested for potential clastogenicity in both male and female mice using the mouse bone marrow micronucleus test. The results on SO_2 derivatives-induced MN are summarized in Table 1. The saline control group resulted in a background level of $0.23 \pm 0.05\%$ and $0.22 \pm 0.05\%$ MNPCE in male and female mice, respectively. For the bone marrow cells of both male and female mice, the frequencies of MNPCE increased significantly with dosages of SO_2 derivatives. CP (50mg/kg) was used as the positive control and resulted in $5.02 \pm 0.28\%$ and $5.01 \pm 0.42\%$ MNPCE in male and female mice, respectively. It was also shown that male mice were very similar to female in the responses to SO_2 derivatives and CP.

MN analysis of mouse bone marrow cells is one of the most useful methods to assess genetic effects of chemicals. However, a study on SO_2 derivatives -induced MN and its dose-response relationship has not previously been carried out *in vivo*. Although our data are still insufficient, this is the first experimental evidence that SO_2 derivatives induces MN in mouse bone marrow cells in a

Table 1. Induction effect of SO_2 derivatives on MN in mouse bone marrow PCE cells

Chemical, mg/kg,b.w.	Male mice(n=10)		Female mice(n=10)	
	MN,% ($\bar{X}$±SD)	MNPCE,% ($\bar{X}$±SD)	MN,% ($\bar{X}$±SD)	MNPCE,% ($\bar{X}$±SD)
Saline	0.23±0.05	0.23±0.05	0.22±0.05	0.22±0.05
SO_2-d				
2 x 20	0.50±0.08**	0.49±0.07**	0.52±0.07**	0.52±0.07**
2 x 100	0.69±0.09**	0.69±0.09**	0.67±0.10**	0.67±0.10**
2 x 500	1.06±0.14**	1.00±0.13**	1.08±0.14**	1.06±0.13**
2 x 750	0.93±0.06**	0.93±0.06**	0.90±0.07**	0.90±0.07**
CP 50	5.38±0.32**	5.02±0.28**	5.58±0.39**	5.01±0.42**

CP: cyclophosphamide; SO_2-d: SO_2-derivatives, a mixture of sodium sulfite and bisulfite (3:1,M/M); MNPCE: micronucleated PCE cells; Results at each dose were compared to those of the control (saline group) using Student's *t*-test, **p<0.01.

dose-dependent manner *in vivo*. These results have confirmed our previous observations that SO_2 is a clastogenic and genotoxic agent (Meng and Zhang 1992). The genetic effects of bisulfite have been examined in bacterial and mammalian cells. At high concentrations and at pHs between 5 and 6, bisulfite modifies DNA *in vitro* and deaminates cytosine to uracil (Shapiro 1977). Recently, we have reported that bisulfite increases frequencies of mutants in CHO-AS52 cells and causes deletion mutation of xathine-guanine phosphoribosyl transferase locus (gpt) (Meng and Zhang 1999). Several possibilities still remain to explain the mechanism of SO_2 derivatives-induced MN formation. It is possible the bisulfite induces MN formation via DNA modification and deamination of cytosine to uracil, an enzyme-catalyzed process that is proposed for mammalian cells. A second possible mechanism for SO_2 derivatives-induced MN may involve formation of sulphur- and oxygen-centred free radicals, either via autoxidation of bisulfite or by enzyme-catalyzed oxidation (Meng and Zhang 1992). The one-electron oxidation of bisulfite produces the sulfur trioxide radical anion, which reacts rapidly with molecular oxygen to form a peroxyl radical. Free radicals generated by SO_2 derivatives can damage nucleic acids, proteins, lipids and directly or indirectly induce MN formation and chromosome aberrations(Meng and Zhang 2001).

Time-response relationship: Table 2 indicates that the frequencies of MNPCE induced by SO_2 derivatives changed with time after the treatments with the derivatives. A significant increase of the %MNPCE in male and female mice were caused 12 h after exposure to SO_2 derivatives, the increase was the highest

Table 2. Time-effect on MN formation in mouse bone marrow PCE cells after exposure to SO_2 derivatives

Chemical	Time after Exposure (h)	Male mice(n=10) MN,% ($\bar{X}$ ±SD)	Male mice(n=10) MNPCE,% ($\bar{X}$ ±SD)	Female mice(n=10) MN,% ($\bar{X}$ ±SD)	Female mice(n=10) MNPCE,% ($\bar{X}$ ±SD)
Saline	24	0.23±0.03	0.23±0.03	0.21±0.02	0.21±0.02
SO_2-d					
	12	0.63±0.05**	0.63±0.05**	0.64±0.06**	0.62±0.05**
	24	1.00±0.06**	1.00±0.06**	1.10±0.06**	1.00±0.05**
	36	0.55±0.05**	0.55±0.05**	0.53±0.05**	0.53±0.05**
	48	0.53±0.06**	0.52±0.05**	0.52±0.06**	0.52±0.06**
	60	0.57±0.07**	0.57±0.07**	0.52±0.05**	0.50±0.05**
	72	0.38±0.07	0.38±0.07	0.35±0.08	0.35±0.08

SO_2-d: SO_2-derivatives (2x500mg/kg), the mixture of sodium sulfite and bisulfite (3:1,M/M); MNPCE: micronucleated PCE cells; Results at each group were compared to those of the control (saline group) using Student's t-test, **p<0.01.

24 h after treatment with the derivatives, the %MNPCE at 36, 48, 60 h after the treatment were very similar to that at 12 h after the treatment. However, the %MNPCE at 72 h after the treatment was similar to the background level. It was shown that the frequencies of MNPCE changed in time-dependent manner. Male mice were very similar to female mice in time-response relationships between MN formation and exposure to SO_2–derivatives. It is known that the bone marrow cells are cycling about once every 11-13 h and so 24, 48, and 72 h after the derivatives exposure in this study, the cells would pass through 2, 4 and 6 cell cycles on average, respectively. Thus, it was suggested that MN induced by the chemicals might be lost at the cell division. On the other hand, the cells with damaged seriously genetic material could not enter the next cycle and even deceased. So, the longer the duration after SO_2 derivatives-exposure, the lower the MN frequency, as table 2.

Effects of SO_2 derivatives on CP-induced MN: Table 3 indicates that for male mice, at the i.p. doses of 50 mg and 100 mg CP/kg , the %MNPCE were 5.02 and 9.98, respectively. However, at the i.p. doses of 50 mg CP/kg add 2 x 500 mg SO_2 derivatives/kg or 100 mg CP/kg add 2 x 500 mg SO_2 derivatives/kg, the %MNPCE were 3.68 and 6.20, respectively. These differences between the CP group and the CP + SO_2 derivative group were statistically significant ($P < 0.01$). Table 3 also shows that the effects of SO_2 derivatives on MN formation induced by CP in female mice were very similar to those in male mice. It is shown that there is an inhibition effect of SO_2 derivatives on MN formation induced by CP in the mouse bone marrow cells. It is suggested that SO_2 could inhibit

mutagenesis of some mutagens such as CP.

Effects of SO_2 derivatives on MMC-induced MN: For male mice, table 4 shows that at the i.p. doses of 0.5, and 1.0 mg MMC/kg, the %MNPCE were 1.98 and

Table 3. Effect of SO_2 derivatives on cyclophosphamide(CP)-induced MN in mouse bone marrow PCE cells

	Male mice(n=10)		Female mice(n=10)	
Chemical	MN,% ($\bar{X}$±SD)	MNPCE,% ($\bar{X}$±SD)	MN,% ($\bar{X}$±SD)	MNPCE,% ($\bar{X}$±SD)
Saline	0.23±0.03	0.23±0.03	0.24±0.04	0.24±0.04
SO_2-d	1.05±0.10	1.08±0.11	1.03±0.09	1.03±0.09
CP(50mg/kg)	5.38±0.32	5.02±0.28	5.23±0.28	5.23±0.28
CP(50mg/kg) add SO_2-d	4.02±0.40**	3.68±0.31**	4.05±0.38**	4.05±0.38**
CP(100mg/kg)	10.57±0.71	9.98±0.21	11.76±0.61	10.56±0.53
CP(100mg/kg) add SO_2-d	6.60±0.43**	6.20±0.22**	6.80±0.44**	6.20±0.21**

SO_2-d: SO_2-derivatives, the mixture of sodium sulfite and sodium bisulfite (3:1,M/M), its dose is 2 x 500mg/kg for each mouse. MNPCE: micronucleated PCE cells; Results at CP group were compared to those of CP add SO_2-d group using Student's t-test, **p<0.01.

Table 4. Effect of SO_2 derivatives on mitomycin C(MMC)-induced MN in mouse bone marrow PCE cells

	Male mice(n=10)		Female mice(n=10)	
Chemical	MN,% ($\bar{X}$±SD)	MNPCE,% ($\bar{X}$±SD)	MN,% ($\bar{X}$±SD)	MNPCE,% ($\bar{X}$±SD)
Saline	0.22±0.06	0.22±0.06	0.24±0.05	0.24±0.05
SO_2-d	1.08±0.10	1.04±0.12	1.03±0.11	1.05±0.12
DMSO(4 mL/kg)	0.46±0.07	0.46±0.07	0.45±0.06	0.42±0.06
MMC(0.5mg/kg)	1.98±0.06	1.98±0.06	2.00±0.06	2.00±0.06
MMC(0.5mg/kg) add SO2-d	2.74±0.07**	3.60±0.06**	2.86±0.08**	2.86±0.08**
MMC(1.0mg/kg)	4.50±0.20	4.23±0.16	4.42±0.10	4.25±0.08
MMC(1.0mg/kg) add SO2-d	6.40±0.34**	6.40±0.30**	6.20±0.22**	6.22±0.22**

SO_2-d: SO_2-derivatives, the mixture of sodium sulfite and sodium bisulfite (3:1,M/M), its dose is 2 x 500mg/kg for each mouse. MNPCE: micronucleated PCE cells; Results at MMC group were compared to those of MMC add SO_2-d group using Student's t-test, **p<0.01.

4.23, respectively. At the i.p. doses of 0.5 mg MMC/kg add 2 x 500 mg SO_2 derivatives/kg or 1.0 mg MMC/kg add 2 x 500 mg SO_2 derivatives/kg, the

%MNPCE were 3.60 and 6.40, respectively. These differences between the MMC group and the MMC add SO_2 derivative group were statistically significant ($P < 0.01$). Table 4 also shows that the effects of SO_2 derivatives on MN formation induced by MMC in female mice were very similar to those in male mice. These results show that there is an enhanced effect of SO_2 derivatives on MN formation induced by MMC in mouse bone marrow cells. It implies that SO_2 could enhance mutagenesis of some mutagens such as MMC. In the current study, SO_2 derivatives enhanced mutagenesis of MMC, but inhibited mutagenesis of CP on the mouse bone marrow cells. It implies that effects of SO_2 on mutagens are very complicated. SO_2 could be a comutagen of some mutagens such as MMC, and a inhibitor of other mutagens such as CP. The mechanism of the roles of SO_2 derivatives on MN formation induced by MMC or CP remains unclear, more studies are needed. SO_2 derivatives, which itself can undergo autoxidation and enzyme-catalyzed oxidation, are reducing agents; SO_2 derivatives are also oxidants, because their one-electron oxidation can produce the sulfur trioxide radical anion, the latter reacts rapidly with molecular oxygen to form a peroxyl radical. We suggested that SO_2 and its derivatives are a comutagen or an inhibitor of mutagens depends on the chemical property and the in vivo metabolic pathways of the mutagens and their metabolic products.

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