

# Protective Effects of Thiol Compounds on Chromate-induced Toxicity *In Vitro* and *In Vivo*

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The effects of thiol compounds (L-cysteine ethyl ester, 2,3-dimercaptosuccinic acid, or 2,3-dimercapto-1-propanesulfonic acid) on the toxicity induced by chromate (potassium dichromate) were investigated in HeLa cells and mice. Chromate-induced cytotoxicity evaluated by inhibition of cell growth and chromium content of the cells was diminished by all of the thiol compounds tested when the cells were incubated in the medium with both chromate and one of the thiol compounds. In mice injected ip with a thiol compound immediately after injection of chromate, mortality, ornithine carbonyl transferase activity in the serum, and chromium content in the liver were diminished remarkably compared with mice injected with chromate alone. These thiol compounds also caused an increase of urinary chromium excretion. These results suggest that the thiol compounds tested are useful for treating chromate-induced toxicity when they are given immediately after intake of the metal. — Environ Health Perspect 102(Suppl 3):247–250 (1994).

Key words: chromate, L-cysteine ethyl ester, 2,3-dimercaptosuccinic acid, 2,3-dimercapto-1-propanesulfonic acid, HeLa cells, mice, cytotoxicity, thiol compounds

## Introduction

A number of thiol compounds are available for the treatment of heavy-metal intoxication. For example, cysteine, penicillamine, 2,3-dimercaptosuccinic acid, 2,3-dimercapto-1-propanesulfonic acid, and dithiothreitol are effective in treating poisoning by compounds of cadmium (1,2), mercury (3,4), and other heavy metals. Susa (5) reported that DL-penicillamine diminished chromate-induced cytotoxicity, which was closely related to the reduction of chromium uptake by the cultured HeLa cells. Furthermore, Susa et al. (6) reported that combined administration of chromate and DL-penicillamine caused not only diminished chromium accumulation within the tissues, but also increased urinary excretion of chromium, and thus, DL-penicillamine prevented the lethal effects of chromium in mice.

Ascorbic acid and thiol-containing molecules such as cysteine, cysteamine, glutathione, unithiol, penicillamine, dithiothreitol, mercaptoethanol, lipoic acid, 2,3-dimercaptosuccinic acid, and thiolactic acid effectively reduce chromate under physiologic conditions (7). This finding suggests the possibility that thiol-containing compounds may be useful for the prevention and treatment of chromium poisoning.

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The purpose of this study is to investigate the effectiveness of L-cysteine ethyl ester, 2,3-dimercaptosuccinic acid, and 2,3-dimercapto-1-propanesulfonic acid on the toxicity induced by chromate *in vitro* and *in vivo*.

## Materials and Methods

### Cells and Cell Culture

HeLa cells (Flow Laboratories, Rockville, MD) were grown in a monolayer at 37°C with Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 2 mM glutamine, kanamycin (60 µg/ml) and 10% calf serum (Irvine Scientific, Santa Ana, CA), in an atmosphere of 5% CO<sub>2</sub> to 95% air.

### Evaluation of Cytotoxicity

The cells were seeded at  $5 \times 10^5$  cells per 60 mm glass petri dish with 5 ml of medium. One day after incubation, the medium was exchanged for a fresh medium containing either chromate alone, of both chromate and one of the thiol compounds, in which the cells were incubated for 3 days. For the control experiment, the cells were incubated in the medium with neither chromate nor thiol compounds, in the same manner as described above. The medium was not changed during exposure to the chemicals. After 3 days of additional incubation, the viable cells were counted by the trypan-blue exclusion test and the growth-inhibitory ratio, Y, for each dose of test chemical was calculated using the equation

$$Y(\%) = (C-T)/(C-CO) \times 100$$

where T is the cell count for each dose after 3 days incubation, C is the cell count for the control after 3 days, CO is the cell count at the start of chemical treatment.

### Evaluation of Chromate Reduction

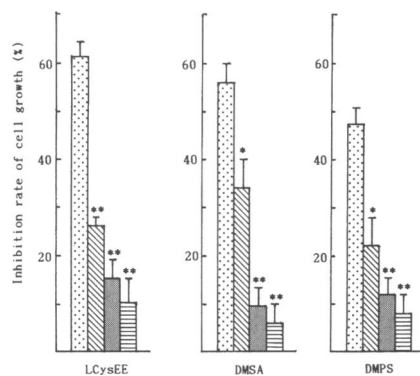
A solution of chromate dissolved in distilled water was mixed with one of the solutions of thiol compounds, also dissolved in distilled water. After the mixture was incubated at 37°C for 5 min, the amount of chromate in the mixture was determined by the diphenylcarbazid method.

### Chromium Uptake by the Cells

The cells were seeded at  $1 \times 10^6$  cells per 100-mm plastic petri dish with 10 ml of the medium. Three days after incubation, the medium was exchanged for serum-free medium containing chromate alone or both chromate and one of the thiol compounds, in which the cells were then incubated for 6 hr. The medium was not changed during exposure to the chemicals. After 6 additional hours of incubation, the medium was discarded and the cell layer was rinsed twice with phosphate-buffered saline (PBS). The cells were then scraped from the dishes with a rubber policeman and suspended in an aliquot of PBS to analyze the cellular chromium.

### Animals and Treatment

Male ddY mice weighing 25 to 30 g were used for the study. Mice were injected ip with one of the thiol compounds immediately after the ip injection of chromate.



**Figure 1.** Effects of several thiol compounds on the growth-inhibitory effects of chromate in HeLa cells. After 24 hr of incubation, the medium was exchanged for a medium containing 5.0  $\mu\text{M}$  chromate alone (dotted) or chromate and thiol compound (25  $\mu\text{M}$ ; diagonal slash, 50  $\mu\text{M}$ ; shaded, 100  $\mu\text{M}$ ; horizontal slash) in which the cells were then incubated for 3 days. The viable cells were counted using trypan blue exclusion test after 3 days exposure to the compounds. Control cells were incubated in medium with neither chromate nor thiol compounds. Results from the cells incubated in medium with a thiol compound alone (25 to 100  $\mu\text{M}$ ) were not significantly different from the control result. Each value represents the mean  $\pm$  SE of four replicate cultures for each exposure concentration. Significantly different from chromate alone; \* $p < 0.05$ ; \*\* $p < 0.01$ .

The mice were then placed in individual metabolism cages so that we could collect urine and feces separately.

At the times required after injection of chromate, the mice were killed by decapitation, and the liver and kidney were excised for determination of chromium content.

### Evaluation of Hepatotoxicity

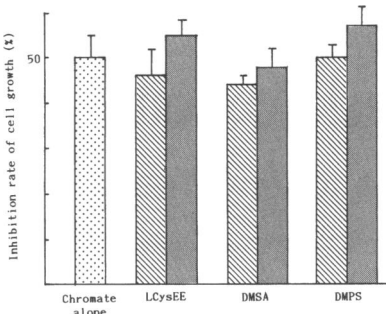
The activity of ornithine carbamyl transferase (OCT) in serum, an indicator of liver injury, was measured using a test-kit from Wako Pure Chemical Ind.

### Determination of Chromium

The amount of chromium was estimated in the cells, tissues, urine, and feces following digestion with  $\text{HNO}_3$  using an atomic absorption spectrophotometer.

### Chemicals

The chemicals used were potassium dichromate as chromate (Kanto Chemical Co., Inc., Tokyo, Japan), L-cysteine ethyl ester (LCysEE, Nakarai Chemical Ltd., Kyoto, Japan), 2,3-dimercaptosuccinic acid (DMSA, Nakarai Chemical Ltd.), and 2,3-dimercapto-1-propanesulfonic acid sodium salt (DMPS, Sigma Chemical Company, St. Louis, MO). All chemicals employed were of commercial reagent-grade quality. Each chemical was dissolved in distilled water just prior to use at 100 times the



**Figure 2.** Effects of pre- or posttreatment of several thiol compounds on the growth-inhibitory effect of chromate in HeLa cells. After 24 hr of incubation, the medium was exchanged for a fresh medium and a thiol compound (100  $\mu\text{M}$ ) was added to the medium 1 hr before (diagonal slash) or after (shaded) addition of 5  $\mu\text{M}$  chromate (dotted; chromate alone), in which the cells were then incubated for 3 days. The viable cells were then counted using the trypan blue exclusion test after 3 days exposure to the compounds. Control cells were incubated in medium with neither chromate nor thiol compounds. Each value represents the mean  $\pm$  SE of four replicate cultures for each exposure.

final concentration and then sterilized by Millipore filtration (0.45  $\mu\text{m}$ ). These solutions were further diluted to final concentrations with the culture medium.

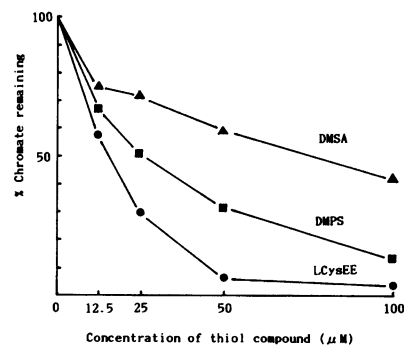
The difference between the mean values for the data were evaluated by the Student's *t*-test for equal variance or Welch's *t*-test for unequal variance. A *p*-value less than 0.05 was considered to be statistically significant.

## Results

### Effects of Thiol Compounds on Chromate-induced Cytotoxicity

When the cells were incubated in the medium with 5.0  $\mu\text{M}$  chromate alone, or with both chromate and one of the thiol compounds (25–100  $\mu\text{M}$ ) for 3 days, the cell-growth inhibition induced by chromate was diminished with increased concentration of the thiol compounds (Figure 1).

In the second experiment, the medium was exchanged for fresh medium one day



**Figure 3.** Reduction of chromate by several thiol compounds. A solution of chromate (10  $\mu\text{M}$ ) in distilled water was mixed with one of the solutions of a thiol compound (12.5–100  $\mu\text{M}$ ) in distilled water. After the reaction mixture was incubated at 37°C for 5 min, the chromate levels were determined by diphenylcarbazid method. The amount of chromate remaining was expressed as the percentage of the control containing chromate alone.

after incubation and the thiol compounds (100  $\mu\text{M}$ ) were added to the medium 1 hr before or after addition of chromate (5  $\mu\text{M}$ ), in which the cells were then incubated for 3 days. The growth-inhibitory ratios (%) of the cells obtained at 3 additional days of incubation are shown in Figure 2. A significant difference in the growth-inhibitory ratio induced by chromate was not observed between the cultures with both chromate and thiol compound.

### Reduction of Chromate by Thiol Compounds

All of the thiol compounds tested produced a concentration-related reduction of chromate. With a solution containing both 10  $\mu\text{M}$  chromate and 100  $\mu\text{M}$  thiol compounds, chromate concentration in the solution decreased to 3% (LCysEE), 43% (DMSA), and 13% (DMPS) of that of a solution containing chromate alone (Figure 3).

### Effects of Thiol Compounds on Chromate Uptake by Cells

The chromium content of the cells decreased with all of the thiol compounds; significant differences were observed for more than 25

**Table 1.** Effects of combined intraperitoneal administration of thiol compounds and chromate on chromate-induced lethality in mice.<sup>a</sup>

Dose, mg/kg		Cumulative no. of dead mice		
		Time after injection, hr		
Chromate	Thiol compound	24	48	72
None	None (control)	0/10	0/10	0/10
40	None	7/10	10/10	—
40	LCysEE 500	0/10	0/10	2/10
40	DMSA 500	3/10	6/10	7/10
40	DMPS 500	5/10	6/10	6/10

<sup>a</sup>Chromate was injected intraperitoneally into the mice with or without LCysEE, DMSA, or DMPS. Control group of animals were injected with saline only.

$\mu\text{M}$  of LCysEE and DMPS, and for more than  $50 \mu\text{M}$  of DMSA (Figure 4).

In the other experiment, thiol compounds were added to the medium 1 hr before or after chromate ( $5 \mu\text{M}$ ), and the chromium content of the cells was measured at 6 hr after addition of the chromate. As shown in Figure 5, the chromium content of the cells decreased slightly with DMPS before and after chromate treatment. However, no significant changes were induced by LCysEE or DMSA.

**Effects of Thiol Compounds on Chromate-induced Toxicity in Mice**

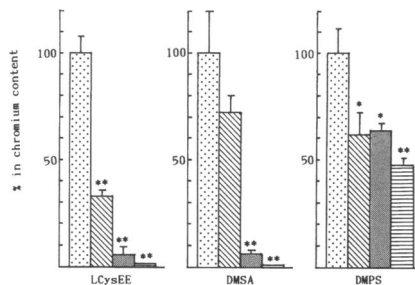
In the mice that received  $40 \text{ mg Cr/kg}$  ip, 100% mortality was observed after 48 hr. When the mice received LCysEE, DMSA, or DMPS at a dose of  $500 \text{ mg/kg}$  immediately after the injection of chromate ( $40 \text{ mg/kg}$ ), mortality diminished to 20%, 70%, and 60% respectively at 72 hr after administration (Table 1).

In the mice that received  $20 \text{ mg/kg}$  ip chromate with one of the thiol compounds at a dose of  $300 \text{ mg/kg}$ , the chromium content in liver and kidney diminished remarkably compared with that of the mice administered chromate alone. These thiol compounds caused increased urinary chromium excretion, and LCysEE caused diminished fecal chromium excretion. All of the thiol compounds suppressed the increase of serum OCT activity induced by chromate (Figure 6).

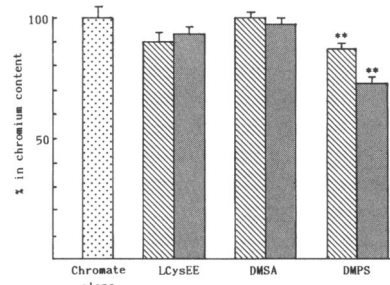
**Discussion**

It is known that chelating agents containing a sulfhydryl group, such as penicillamine or cysteine, form a stable trivalent chromium complex by reactive chelation with sodium chromate (8).

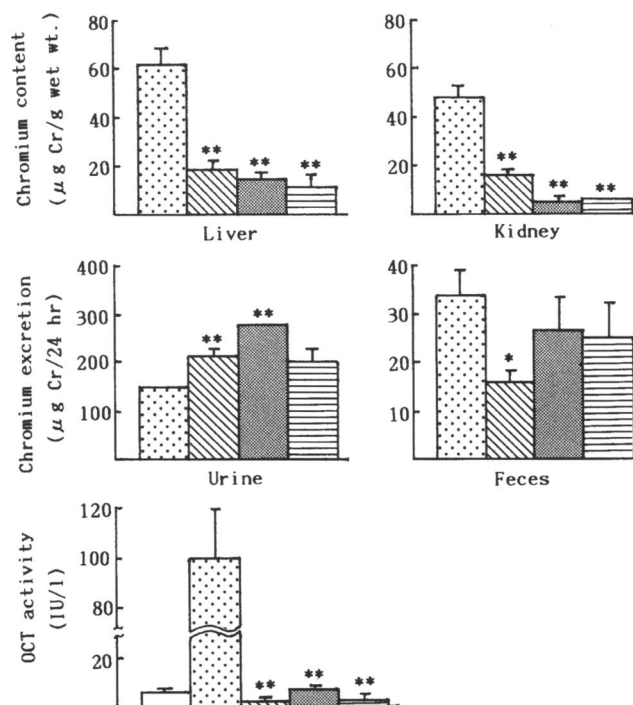
In this experiment, LCysEE, DMSA, and DMPS were effective agents for diminishing chromate-induced cytotoxicity and decreasing the chromium content of the cells. They also exhibited a chromate-reducing ability. These results suggest that chromate-induced cytotoxicity is diminished as a result of the reduction of chromium uptake by the cells accompanying the reduction of chromate because of the cell membrane impermeability to the trivalent chromium (9). However, addition of these thiol compounds to the medium before or after treatment with chromate did not restore chromate-induced cytotoxicity or remarkably diminished the cellular chromium content. Toohey (10) reported that added sulfhydryl compounds, such as cysteine, thioethanolamine, and dithiothreitol, oxidize rapidly in the medium tissue



**Figure 4.** Effects of several thiol compounds on chromium uptake by HeLa cells from chromate-containing medium during a 6-hr incubation period. After 3 days incubation, the medium was exchanged for a medium containing  $10 \mu\text{M}$  chromate alone (dotted) or chromate and a thiol compound ( $25 \mu\text{M}$ ; diagonal slash,  $50 \mu\text{M}$ ; shaded,  $100 \mu\text{M}$ ; horizontal slash), in which the cells were then incubated for 6 hr. The chromium content of the cells was estimated by atomic absorption spectroscopy. The chromium content was expressed as percentage of each control incubated with chromate alone. Each value represents the mean  $\pm$  SE of four replicate cultures for each exposure concentration. The chromium content of control in medium with  $10 \mu\text{M}$  chromate alone was approximately  $0.36 \mu\text{g Cr/mg}$  cell protein at 6 hr of incubation. Significant difference from chromate alone: \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Figure 5.** Effects of pre- or posttreatment of several thiol compounds on chromium uptake by HeLa cells from chromate-containing medium during a 6-hr incubation period. After 3 days incubation, the medium was exchanged for a fresh medium and  $100 \mu\text{M}$  thiol compound was added to the medium 1 hr before (diagonal slash) or 1 hr after (shaded) addition of  $5 \mu\text{M}$  chromate (dotted; chromate alone), in which the cells were then incubated for 6 hr. The content was expressed as a percentage of the control incubated with chromate alone. Each value represents the mean  $\pm$  SE of four replicate cultures for each exposure. Significantly different from chromate alone; \*\* $p < 0.01$ .



**Figure 6.** Effects of combined intraperitoneal administration of thiol compounds and chromate on tissue chromium content, chromium excretion, and serum ornithine carbamyl transferase (OCT) activity, as an indicator of liver cell damage, in the mice. Mice were injected ip with  $300 \text{ mg/kg}$  LCysEE (diagonal slash), DMSA (shaded) or DMPS (horizontal slash) immediately after ip injection of  $20 \text{ mg/kg}$  chromate (dotted; chromate alone). Control groups ( $\square$ ) of animals were injected only saline instead of the chemicals tested. Each value represents the mean  $\pm$  SE obtained from five mice 24 hr after administration. Significantly different from mice injected chromate alone; \* $p < 0.05$ ; \*\* $p < 0.01$ .

culture system containing serum and cells. From this point of view, it might be suspected that these thiol compounds oxidize rapidly in the medium, or that chromate is taken up rapidly by cell, and thus, these compounds added before or after treatment with chromate could not protect chromate-induced cytotoxicity.

In the mice that received chromate ip with LCysEE, DMSA, or DMPS, it becomes clear that chromium-induced lethality and chromium content in liver

and kidney remarkably diminished. Furthermore, these thiol compounds were able to increase the urinary chromium excretion and to diminish the increase of serum-OCT activity induced by chromate injection. These results suggest the possibility that combined administration of chromate and thiol compounds may cause not only diminished chromium accumulation within the tissues, but also increased urinary chromium excretion; thus, these compounds may prevent chromium-induced

toxicity in mice. This mechanism of protection supports experiments *in vitro* on the interaction of chromate and thiol compounds at the cellular level.

In conclusion, the thiol compounds tested in this experiment are useful for treating chromate-induced toxicity when given immediately after exposure, and a portion of this effect may be due to the reduction of chromate uptake by the cells or tissues that accompanies chromate reduction.

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