Environmental and Molecular Mutagenesis 32:251–259 (1998)

Studies on the Genotoxicity of Molybdenum Salts in Human Cells In Vitro and in Mice In Vivo

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Molybdenum is an essential element in plants and animals as a cofactor for enzymes. Molybdenum trioxide is used in metallurgical processes, in cosmetics as a pigment, and in contact lens solution, yet limited information is available on molybdenum genotoxicity. In the present study the micronucleus (MN) assay in human lymphocytes and mouse bone marrow and the dominant lethal assay in mice were used to assess the genotoxic effects of molybdenum salts in vitro and in vivo. Two salts of molybdenum were tested in whole blood cultures. Ammonium molybdate was more potent than sodium molybdate in causing a dose-dependent decrease in viability and replicative index and an increase in MN formation in binucleated lymphocytes ($P < 0.001$). A dose–response in both kinetochore-positive MN (caused by chromosome lagging) and kinetochore-negative MN (associated with chromosome breakage) was observed. Based on the results of a toxicity study of sodium molybdate, two doses, 200 and 400 mg/kg, were assessed in the bone marrow MN assay in mice (two i.p. injections 24 and 48 hr prior to euthanasia). A modest but statistically significant increase in MN frequency in polychromatic erythrocytes was observed ($P < 0.05$). The same treatment protocol was used to analyze dominant lethality. A dose-dependent increase in postimplantation loss represented mostly by early resorptions was observed the first week after treatment ($P = 0.003$). These preliminary data suggest that sodium molybdate induces dominant lethality at the postmeiotic stage of spermatogenesis. Overall, molybdenum salts produced moderately positive results both in vitro in human cells and in vivo in mice. Environ. Mol. Mutagen. 32:251–259, 1998

Key words: molybdenum; human lymphocytes; micronuclei; mouse bone marrow; postimplantation loss; dominant lethality

INTRODUCTION

Molybdenum is an essential microelement which serves as a cofactor in enzymes in humans and other species [US EPA, 1975]. Molybdenum salts and other compounds are used in metallurgical processes as additives to steel and corrosion-resistant alloys and occupational exposure occurs at roasting plants [Walravens et al., 1979]. Molybdenum is also an environmental pollutant produced from uranium processing and combustion processes, and was recently introduced as a component of contact lens solution and a color additive in cosmetics [ACGIH, 1995]. Human molybdenum intake in the United States has been estimated to be 120–350 mg/day [Tsongas et al., 1980; Goyer, 1996], with a NOEL at 500 mg/day [Chappell, 1974].

The lethal dose for different species has been estimated to be between 100 and 800 mg/day, depending on the route of exposure and chemical structure of the molybdenum compound [Maresh et al., 1940; Miller and Engel, 1960; Mills and Davis, 1987]. Pneumoconiosis developed in 3 out of 19 people who had been chronically exposed to metallic molybdenum and molybdenum trioxide [Mogilevskaja, 1963]. A gout-like disease has been reported in people living in areas with high levels of molybdenum in the soil [Kovalsky and Vorotnitskaya, 1970; Pitt, 1976]. Teart disease, a form of wasting and copper deficiency, has been observed in cattle grazing on pastures high in molybdenum [Mills and Davis, 1987]. A recent National Toxicology Program study [NTP, 1997] has shown that exposure of mice and rats to molybdenum trioxide by inhalation caused alveolar inflammation and hyaline degeneration of the respiratory epithelia. Equivocal evidence of carcinogenic activity of molybdenum trioxide in rats was obtained in the same 2-year inhalation

Contract grant sponsor: National Institute of Environmental Health Sciences Center at Berkeley; Contract grant number: NIH P30 ESO1896; Contract grant sponsor: NIH; Contract grant number: P42 ES04705 from the National Institute of Environmental Health Sciences, using funds provided by US EPA.

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Received 29 September 1997; Revised and accepted 19 July 1998

study with exposure to 30 or 100 mg/m^3 6 days a week [NTP, 1997]. In a three-generation reproductive study, mice were chronically exposed to 10 ppm molybdenum in drinking water, which resulted in early death of mice in F1, F2, and F3, litters with no live pups, and increased maternal death [Schroeder and Mitchener, 1971]. In rats which received 80 or 140 ppm sodium molybdate in feed for several weeks, fewer litters and impaired growth of pups were observed [Jeter and Davis, 1954].

Only limited and conflicting information is available on the genotoxicity of molybdenum compounds. Ammonium molybdate (10^{-5} M, 24 hr) induced chromosome aberrations and sister-chromatid exchanges (SCEs) in human lymphocytes in vitro [Bobyleva et al., 1991]. These indices of cytogenetic damage were also increased in lymphocytes of workers with more than 10 years of occupational exposure to molybdenum [Babaian et al., 1980; Bobyleva et al., 1993]. Molybdenum trioxide (8 and 15 mg/kg), but not ammonium molybdate (2.5 and 7.5 mg/kg), induced chromosome damage in mouse bone marrow [Chopikashvili et al., 1991]. The same authors reported dominant lethal mutations were induced and survival in Drosophila decreased when 2–4 g ammonium molybdate were added per kg of feed [Chopikashvili et al., 1991]. On the other hand, compounds of molybdenum were not mutagenic in *Escherichia coli* [Venitt and Levy, 1974], *Bacillus subtilis* [Kada et al., 1980], and *Salmonella typhimurium,* with or without metabolic activation [Zeiger et al., 1992; NTP, 1997]. Negative results were also obtained with 0.5–10 mkg/ml molybdenum trioxide in cytogenetic tests (SCEs and chromosome aberrations) in cultured Chinese hamster ovary cells [NTP, 1997].

Since the information available on the genotoxicity of molybdenum is conflicting, and there were no data on reproductive genotoxicity in mammals, the goal of the present study was to explore further the possible genotoxicity of molybdenum salts in human cells in vitro and laboratory mice in vivo. An additional reason for the study was that applications of molybdenum were recently expanded from metallurgy to include contact lens solutions, cosmetics, etc., which could immediately affect many people. Three well-established genotoxicity assays were employed. The micronucleus (MN) assay was performed in human lymphocytes in vitro and mouse bone marrow in vivo. Additionally, the dominant lethal assay in mice was applied to assess the reproductive genotoxicity of molybdenum in a mammalian system. Ammonium molybdate and sodium molybdate were chosen since they are formed immediately upon addition of molybdenum trioxide to an aqueous buffered solution. They also represent the likely products of molybdenum trioxide upon interaction with body fluids. Finally, ammonium molybdate and sodium molybdate are readily soluble and less toxic than other molybdenum compounds.

MATERIALS AND METHODS

Micronucleus Assay in Human Lymphocytes

Blood was collected from two healthy volunteers (donor #1 was a 27-year-old female, and donor #2 a 24-year-old male) in heparin-vacutainers and cultured for 72 hr in a humidified incubator with 5% CO_2 at 37°C. Culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco, Grand Island, NY), and 1% phytohemagglutinin (PHA, HA15, Burroughs-Wellcome, Greenville, NC).

Two salts, ammonium molybdate $(NH_4)_6\,Mo_7O_{24}\cdot 4H_2O$, and sodium molybdate ($Na_2MoO_4\cdot H_2O$) (Sigma, St. Louis, MO), were used to assess the genotoxicity of molybdenum. Both salts were diluted in PBS (Ca^{2+} and Mg^{2+} free) immediately before addition to the cultures (24 hr after they were initiated) and were present until the end of cultivation at 72 hr. Four doses of each salt were used to treat duplicate lymphocyte cultures in three experiments. Colchicine (0.05 µM), a known aneuploidogen, was used as a positive control (incubation for 24 hr). Cytochalasin B (Sigma) (6 µg/ml) was added to the cultures at 44 hr postinitiation as described by Fenech and Morley [1985]. Cytochalasin B prevents the cells from completing cytokinesis, resulting in the formation of multinucleated cells.

Prior to harvesting, cell viability was determined by trypan blue (0.16%) exclusion. Easy-Lyse Whole Blood Erythrocyte Lysing Kit (Leinco Technologies, Ballwin, MO) was employed to gently lyse erythrocytes while maintaining the viability of unfixed cells. Briefly, 0.2 ml lysing solution was added to 0.2 ml whole blood culture medium. After incubation at room temperature for 10 min, mixed solution was centrifuged and cells washed in 1 ml of buffer. After the supernatant was decanted, cells were ready for viability check.

Lymphocytes were isolated using Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient at 2,000 rpm for 35 min at room temperature. The lymphocyte layer was removed and washed in PBS at 1,200 rpm for 10 min, then spun directly (600 rpm, 10 min) onto glass slides using a cytocentrifuge (Shandon, Sewickley, PA). After air drying and before methanol fixation at room temperature for 15 min, slides were stored at −20°C in a sealed box and desiccated in an N_2 atmosphere.

Detailed procedures for performing the MN assay with antikinetochore antibody staining have been described elsewhere [Eastmond and Tucker, 1989; Yager et al., 1990]. Briefly, methanol-fixed slides were incubated for 5 min in PBS containing 0.1% Tween 20. Excess fluid was drained from the slide and 40–50 µl of the antikinetochore antibody (Chemicon, Temecula, CA) diluted 1:1 with PBS containing 0.2% Tween 20 was applied. The slide was then coverslipped and placed in a humidified chamber at 37°C for 1 hr. Following two washes in PBS containing 0.1% Tween 20 for 5 min each, excess fluid was again drained and the slides were covered with a 1:50 dilution of fluorescent goat anti-human IgG (Chemicon) and incubated again for 1 hr. Because the fluorescent-labeled antibodies fade upon exposure to light, this and all subsequent steps were conducted in yellow light. The slides were rinsed twice in buffer plus 0.1% Tween 20 and counterstained with DNA-dye 4′6-diamidino-2-phenylindole (DAPI) (2 µg/ml) in an antifade solution [Johnson and de Nogueira Araujo, 1981]. Slides were stored refrigerated for up to a week prior to analysis by microscopy. Replicative index (RI), a measure of cell division kinetics, was calculated by scoring at least 200 cells per dose or sample and counting the percent of cells containing 1, 2, 3, or more nuclei per individual. RI was calculated as follows: RI = [(1×% mononuclear cells) + (2 ×% binuclear cells) + (3 ×% tri) + (4 ×% tetra)]/100. The MN frequency was estimated in 1,000 binucleated cells of sodium and ammonium molybdate/dose/per experiment per donor, except cultures treated with colchicine or high levels of ammonium molybdate, which were cytotoxic and thus yielded smaller numbers of binucleated cells.

Micronuclei in Mouse Bone Marrow

Male C57BL/6J and C3H/J female mice (7–12 weeks of age) were purchased from BAK Universal (Fremont, CA). Males were housed singly in plastic cages. The animal room was maintained on a 12-hr light/dark cycle, 20–24°C, with 60–70% relative humidity. Mice were fed standard breeding granulated diet and water was supplied ad libitum. Animal husbandry was in accordance with the Guide for Care and Use of Laboratory Animals (NIH Publication no. 78-023, revised, 1985). Fifteen healthy C57BL/6J male mice (10–12 weeks) were randomly assigned to control and treatment groups. Treated males received intraperitoneal injections (≤0.7 ml) of sodium molybdate (Sigma) in sterile PBS for two consecutive days, while mice in the control group received concurrent injections of PBS. Dose levels (200 and 400 mg/kg) were chosen on the basis of range-finding data which determined the 50% of maximum tolerated dose [MacGregor et al., 1987].

Colchicine was used as a positive control (single i.p. injection of 0.8 mg/kg). Animals were sacrificed by CO_2 anesthesia followed by cervical dislocation 48 hr after final injection. The femoral bone marrow cells were flushed out with fetal bovine serum (~1 ml) and the cellular suspension was spun at 1,000 rpm for 5 min. Smears were prepared from the resulting pellet, following the procedure of Schmid [1976], and stained by May-Grunwald-Giemsa. The incidence of MN was assessed in an average of 2,000 polychromatic erythrocytes (PCE) per dose. The proportion between normochromatic erythrocytes and PCE was estimated in 400 cells from each slide. Slides from each dosepoint were randomized and scored at 630× under oil immersion (Nikon microscope) by two observers who were unaware of the identity of the slides. No significant difference between scorers was observed. All questionable MN were additionally assessed by a third scorer.

Dominant Lethal Assay in Mice

The dominant lethal assay was performed according to the standard protocol [Green et al., 1985; Shelby et al., 1986] with some modifications. Since there was no data on the reproductive genotoxicity of sodium molybdate in mice, we decided to prescreen the effects on the main periods of spermatogenesis in a pilot experiment using a minimal number of females. Our goal was to determine whether any reproductive genotoxicity might be observed in either postmeiotic, mitotic, or premeiotic germ cells before committing a significantly larger number of animals with daily sampling for 7–8 weeks, as would be required by standard dominant lethal protocol. C57BL/6J male mice were randomly selected from the same group of animals used for the MN assay and assigned to control (10 males) and treated groups (10 males for 200 mg/kg and 12 males for 400 mg/kg). A total of 166 untreated C3H/J female mice (10–12 weeks old) were housed 4–5 per cage and were mated with treated C57BL/6J mice after the end of treatment to sample various postmeiotic cells: spermatozoa (week 1), spermatids (week 3), spermatocytes (week 5), and spermatogonia (week 7) [Bateman and Epstein, 1971]. The mating activity of molybdenum-treated C57BL/6J males was estimated as a percent of females found with vaginal plugs after overnight placement of one male with two females. Females were sacrificed 15–16 days after vaginal plugs were found. The corpus lutea (CL) on both ovaries were counted under a dissecting Nikon stereomicroscope to assess the number of ovulations or potential fertility in each female. The uterine contents were inspected for the number of total implants, early resorptions (moles), and dead embryos. Early postimplantation loss was estimated by the number of moles per female, and late postimplantation loss by the number of dead embryos per female. Total postimplantation loss was calculated as a percent of dead implants, divided by the total number of implants, including moles, live and dead embryos.

Statistical Analysis

Dose–response curves for each combination of chemical and MN endpoint were fitted by various polynomial equations using the generalized linear model routine of **S** language [Chambers and Hastie, 1992]. Best-fit curves were selected by minimizing the *Akiki* information measure. The *P*-value for each dose–response was generated according to trend of the best-fit curve. Effects of potential confounding factors on the dose–response were also assessed by fitting more complex logistic regression models which considered donor and experiment in addition to dose. However, there was negligible experiment variability, while donors differed significantly ($P < 0.001$). Thus, the *P*-values reported for Figures 1 and 2 were calculated after adjustment for effects of experiment and donor variability. Pairwise comparisons were performed by Fisher's exact test for the frequency of bone marrow MN in treated and control animals. When we analyzed the effects of dose and time on postimplantation loss, we pooled the results from all animals of the same dose/week group and used the generalized linear model approach [Chambers and Hastie, 1992]. A value of $P < 0.05$ was used as the criterion for statistical significance.

RESULTS

Micronuclei Induced by Sodium and Ammonium Molybdate in Human Lymphocytes

Micronucleus (MN) analysis was utilized as a measure of cytogenetic damage in human lymphocytes using anti-kinetochore-antibody staining to characterize the mechanism of MN formation (Table I). Two salts of molybdenum were used for in vitro treatment of human lymphocytes for 48 hr in three independent experiments for two donors (one female and one male). The range of concentrations chosen for sodium molybdate was 0.1–5 mM, while the more cytotoxic ammonium molybdate was studied at 0.1–2 mM. Viability of treated cells varied between 74 and 63% for sodium molybdate and 68 and 61% for ammonium molybdate. A significant inhibition of proliferation, assessed by RI, was observed only at the highest concentration of 5 mM sodium molybdate (1.5 vs. 2.0 in control, $P < 0.01$). Ammonium molybdate, on the other hand, caused a significant dose-dependent decrease of RI at three out of four concentrations. The extent of ammonium molybdate-induced inhibition of proliferation was comparable with the positive control colchicine (1.1 vs. 2.0 in control cultures). This inhibition resulted in a limited number of binucleated cells available for scoring, thus a total of only 452 cells were analyzed for MN frequency at the highest concentration of ammonium molybdate for both donors. The statistical generalized linear model was used, which allowed comparison between donors, evaluation of the variability between experiments, as well as interaction between these factors and the dose. In all cases of total MN frequency, as well as kinetochore-positive and kinetochore-negative MN, there was no significant difference between experiments for either donor. However, the female donor had a consistently higher MN frequency than the male donor in all experiments, both in controls (14.7 MN cells per 1,000 vs. 8 MN cells,

TABLE I. Micronuclei Induced by Molybdenum Salts in Human Lymphocytes

Chemical treatment	(mM)	Donor	Scored BN cells	MN cells per 1,000	MN			Replicative index*	Viability (%)
					Total	MN+	MN−		
Control	0	F	3,000	14.7 ± 2.9	51	17	34	2.0 ± 0.0	87.3 ± 2.3
		M	3,000	8.0 ± 1.7	24	9	15	2.0 ± 0.1	79.6 ± 5.0
Colchicine	5×10^{-5}	F	1,562	92.5 ± 14.8	167	131	36	1.1 ± 0.0	78.0 ± 5.8
		M	1,303	88.0 ± 19.8	132	106	6	1.1 ± 0.1	71.3 ± 2.1
Sodium molybdate	0.1	F	3,000	19.3 ± 1.5	69	41	28	1.8 ± 0.1	74.0 ± 7.0
		M	3,000	17.3 ± 4.5	54	30	24	1.9 ± 0.2	69.0 ± 6.2
	0.5	F	3,000	22.6 ± 6.5	75	46	29	1.9 ± 0.1	71.6 ± 7.5
		M	3,000	19.6 ± 5.7	63	40	23	1.8 ± 0.0	67.3 ± 3.5
	1	F	3,000	31.6 ± 4.5	100	59	41	1.7 ± 0.1	69.7 ± 1.1
		M	3,000	20.6 ± 5.1	69	40	29	1.8 ± 0.1	66.0 ± 4.6
	5	F	3,000	32.0 ± 3.0	112	68	44	1.5 ± 0.0	67.6 ± 5.7
		M	3,000	17.0 ± 1.7	57	33	24	1.6 ± 0.1	62.6 ± 5.5
Ammonium molybdate	0.1	F	3,000	19.3 ± 2.5	63	42	21	1.8 ± 0.1	68.3 ± 4.0
		M	3,000	13.3 ± 3.1	43	29	14	1.8 ± 0.1	62.7 ± 2.5
	0.5	F	2,577	30.3 ± 12.9	91	50	41	1.7 ± 0.1	67.0 ± 6.6
		M	3,000	20.6 ± 3.8	64	37	27	1.6 ± 0.0	64.0 ± 5.3
	1	F	2,281	40.3 ± 2.1	101	61	40	1.3 ± 0.0	64.3 ± 6.8
		M	1,624	19.3 ± 10.0	39	18	21	1.2 ± 0.0	67.6 ± 4.6
	2	F	323	55.5 ± 9.2	21	14	7	1.1 ± 0.0	61.0 ± 4.2
		M	129	31	4	2	2	1.0 ± 0.0	62.5 ± 2.1

Abbreviations: MN, micronuclei; BN, binucleated cells; MN cells, micronucleated cells; +, kinetochore-positive; −, kinetochore negative.
*Replicative index = [(1 × % mononucleated cells) + (2 × % binucleated cells) + (3 × % tri- or >trinucleated cells)]/100.

respectively) and after molybdenum exposure ($P < 0.001$).

Both molybdenum salts induced a dose-dependent increase in the frequency of total MN in both donors ($P < 0.001$) (Fig. 1). The increase in MN per 1,000 cells was modest for sodium molybdate (a little more than twofold over the control level), and more noticeable for ammonium molybdate (an almost fourfold increase) compared to a nearly tenfold increase caused by colchicine.

The level of kinetochore-positive MN+ in control cultures was 4.3/1,000 cells, and of kinetochore-negative MN− 8.2/1,000 cells. Similar trends for induction of both types of MN were observed after sodium molybdate treatment (Fig. 2b), and by ammonium molybdate in the male donor (Fig. 2a). However, in the female donor (#1) noticeably more kinetochore-positive than kinetochore-negative MN were induced by ammonium molybdate, with a fivefold increase over control level at the highest dose. A statistical model based on all datapoints estimated that dose-effect for both MN+ and MN− was significant after treatment with either sodium molybdate or ammonium molybdate ($P < 0.001$). These data suggest that a plausible mechanism of molybdenum genotoxicity includes chromosome lagging, resulting in aneuploidy, and chromosome breakage.

Molybdenum Toxicity in Mice

A dose range-finding experiment was performed with sodium molybdate in mice (Table II). This experiment showed that mortality was not affected up to a dose of 200 mg/kg sodium molybdate. No statistically significant increase was observed at 400 mg/kg (85.2% survival), while at 500 mg/kg more than 50% of treated animals died within 24 hr after the second injection ($P < 0.01$). Based on this mortality data, all experiments with dominant lethality and bone marrow MN were performed with two doses of 200 and 400 mg/kg of sodium molybdate.

Bone Marrow Micronuclei Induced by Sodium Molybdate in Mice

The background ratio of poly- and normochromatic erythrocytes was 0.97, and was not significantly affected by sodium molybdate (1.06 and 0.83 at 200 and 400 mg/kg, respectively) (Table III). Colchicine treatment (0.8 mg/kg), on the other hand, was highly cytotoxic and caused a significant decrease in the PCE/NCE ratio (0.13, $P < 0.001$). The results of the bone marrow MN assay are shown in Table III. The control level was 2.8 MN cells/1,000 with no multiple MN detected. Sodium molybdate induced a statistically significant increase of MN in PCEs. Micronucleated cells with multiple MN were observed mostly in PCEs at 400 mg/kg. As a result, a regression of the MN frequency per 1,000 cells was statistically significant ($P < 0.05$), though the dose response for micronucleated cells did not reach statistical significance. The level of molybdenum-induced MN was smaller (twofold increase) than with colchicine treatment used as a positive control (3.5-fold increase, $P < 0.01$).

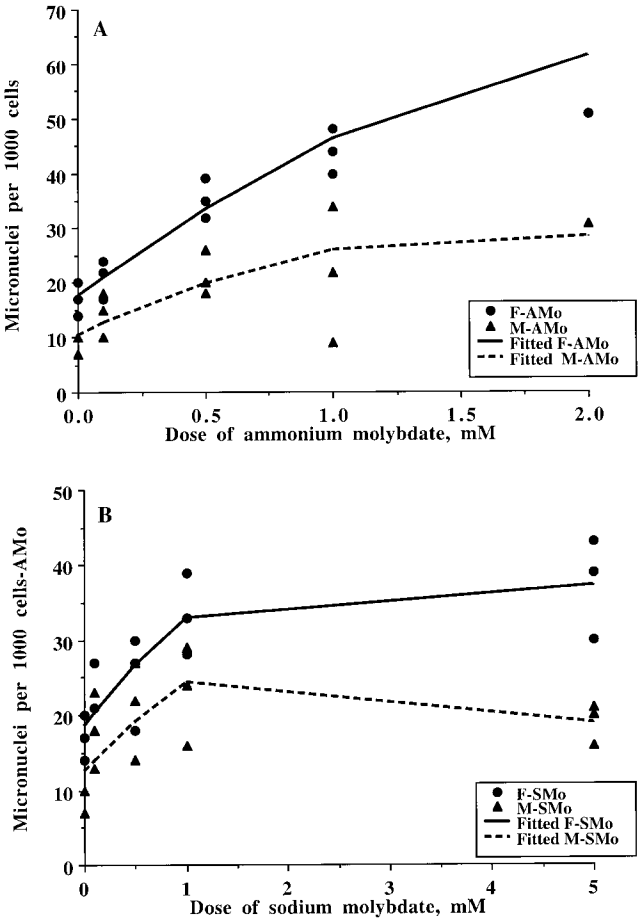

Fig. 1. Dose–response of the micronuclei induced by ammonium molybdate (**A**) and sodium molybdate (**B**) in lymphocytes of two donors: female, original datapoints shown with circles; male, data shown with triangles. One thousand cells were scored for each of the three experiments for both salts. Dose–response curves of best-fit present results of statistical modeling, which included assessment variability between donors and experiments and interaction between dose and donor. The difference between experiments was negligible, while donors differed significantly ($P < 0.001$). Effect of the dose was highly significant for both salts, $P < 0.001$.

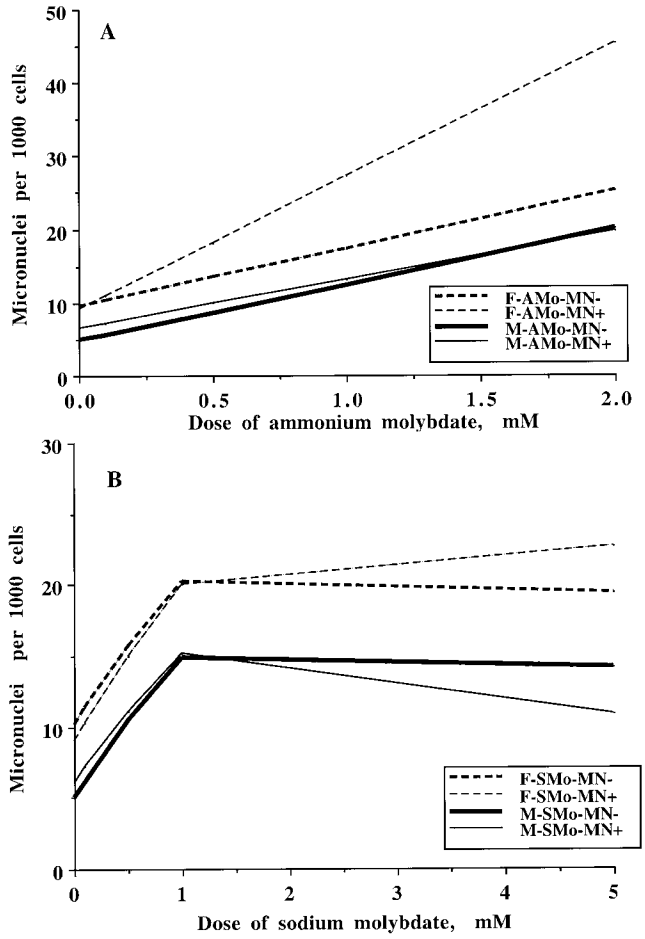

Fig. 2. Kinetochore-positive (MN+) and kinetochore-negative micronuclei (MN-) in human lymphocytes treated with molybdenum salts. MN+ and MN− were differentiated by the presence of bright yellow-green signal after antikinetochore-antibody staining. Cells were counterstained with DAPI. Data from two donors (female and male) and three experiments for both sodium molybdate and ammonium molybdate were included in this analysis. Fitted plots present results of statistical modeling of dose effect taking into account variability between experiments and between donors, as well as interaction between donor and dose. MN frequency in two donors differed significantly ($P < 0.001$), while variability between experiments did not ($P > 0.05$). The increase in MN+ as well as MN- was dose-dependent and statistically significant for both ammonium molybdate (**A**) and sodium molybdate (**B**) ($P < 0.001$).

This preliminary data suggest that sodium molybdate was weakly genotoxic in mouse bone marrow, though it did not affect cell proliferation at the two concentrations studied.

Dominant Lethal Assay in Mice

The mating activity of C57BL/6J male mice was not affected by treatment with sodium molybdate (Table IV). No difference in the weight of males (26.0–26.8 g) or females (23.1–23.5 g) between treated and control groups was observed. Slightly fewer corpus lutea were found in females mated with treated males (8.0–8.7 per female) compared with control females (9.6 per female), but this difference was not significant ($P > 0.05$). Pregnancy rate was estimated as the percent of females with vaginal plugs which had at least one implant at the 15/16th day of pregnancy. There was a 10% decrease in the pregnancy rate in the 400 mg/kg group, but the difference was not statistically significant ($P > 0.05$) except in the first week. However, an overall dose-dependent increase was observed in total postimplantation loss (Table IV). From 6.7% in the control group, postimplantation loss was increased to 10.6% in the 200 mg/kg group and 16.3% in the 400 mg/kg group ($P = 0.001$).

This increase was most noticeable at the first week

TABLE II. Effect of Sodium Molybdate (Na_2MoO_4) on Mortality in Mice

Dose of Mo (mg/kg)	Number of animals injected	Number of mice dead*	Survival (%)
0	11	0	100
50	3	0	100
100	3	0	100
200	14	0	100
400	27	4	85.2
500	14	8	42.8**
750	12	10	16.7***
1,500	12	10	16.7***

*All deaths occurred during first 12 hr after i.p. injection of (Na_2MoO_4) in PBS. Statistically significant by Fisher's exact test, $^{**}P < 0.01$, $^{***}P < 0.001$.

(reflecting an effect on postmeiotic male germ cells, $P = 0.003$). Both early and late postimplantation loss were affected by molybdenum (Table V). A statistically significant and dose-dependent increase in the number of moles (early resorptions) was found at the first week (0.33 per female in control vs. 0.94 in 400 mg/kg group, $P = 0.013$). The average size of moles, though, in control and treated groups was similar (3.4 ± 1.1 mm in control, 3.8 ± 0.9 mm in the 200 mg/kg group, and 3.3 ± 0.6 mm in the 400 mg/kg group). Late postimplantation loss (dead embryos) were found in only one control female at the third week, and were comparatively rare in the treated group. A significant increase was observed only for the first week after treatment ($P = 0.036$). Since we observed a gradual increase in postimplantation loss in both control and treated groups at 3–7 weeks after treatment compared to the first week (Table V), we used a generalized linear model to assess which covariates were likely responsible for this trend. This analysis showed that the rise in postimplantation loss was associated with the increasing body weight of females at later dates of mating ($P < 0.001$) rather than with different stages of spermatogenesis affected. An appropriate correction for the difference in the weight of the females was made, after which a significant postmeiotic dose-dependent effect of sodium molybdate on postimplantation loss in the treated group of mice was confirmed. These preliminary results indicate a mild but statistically significant postmeiotic effect of sodium molybdate on dominant lethality in mice.

DISCUSSION

All three assays used in the present study yielded positive evidence of modest molybdenum salt genotoxicity both in vitro and in vivo. Ammonium molybdate was slightly more cytotoxic and genotoxic in human lymphocytes than sodium molybdate. However, each salt induced both kinetochore-positive MN, which suggests aneuploidy as at least one mechanism for their genotoxicity, and kinetochore-negative MN, which was most likely caused by chromosome breakage. Appreciable differences between the two donors were observed in both background and molybdenum-induced MN frequency. The female donor had higher MN frequencies, and also had a noticeably higher level of induction of kinetochore-positive MN. This difference may be explained by the known predominant elimination of the inactive X-chromosome in the cells of females [Tucker and Preston, 1996]. Our findings on MN induction by molybdenum salts correlate well with earlier studies published in the Russian literature in which chromosome aberrations and sister-chromatid exchanges in human lymphocytes were analyzed [Babaian et al., 1980; Bobyleva et al., 1991]. In these studies, workers occupationally exposed to molybdenum had a lower RI and increased frequency of single fragments and exchange types of chromosome aberrations in their peripheral blood lymphocytes. Moreover, when lymphocytes from these workers were treated with molybdenum salts in vitro an increase in cytogenetic damage was observed, suggesting a sensitization due to previous contact with molybdenum. Together these data show that molybdenum salts may cause chromosome damage in human lymphocytes at relatively high concentrations by at least two mechanisms, chromosome breakage and chromosome lagging which leads to aneuploidy.

On the other hand, in a study of molybdenum trioxide performed in Chinese hamster ovary (CHO) cells, no increase in either SCEs or chromosome aberrations was observed with or without metabolic activation [NTP, 1997]. There are several possible explanations for this discrepancy. Since molybdenum trioxide is more cytotoxic than molybdenum salts, cytogenetic analysis was limited only to concentrations allowing cell growth in the NTP study. These concentrations were 4–10 times lower than equimolar concentrations used in our study with molybdenum salts. No significant increase was seen in human lymphocytes at the range of concentrations comparable to those used for molybdenum trioxide. Other possible explanations are interspecies differences in sensitivity to molybdenum and/or differences in sensitivity of short-term human lymphocyte culture versus the transformed hamster cell line.

There are limited data available on the possible molecular mechanism of molybdenum genotoxicity. It is known that it may impair purine metabolism through an enhancement of xanthine oxidase activity [NTP, 1997]. Molybdenum also has a variety of oxidative states, and one can speculate that molybdenum could cause oxidative damage through the formation of free radicals, as was previously shown for the closely related element chromium [Itoh and Shimada, 1996]. Interference with protein metabolism and enzyme impairment described in animals fed a diet with a high level of molybdenum [Mills et al., 1958; NTP,

TABLE III. Effect of Sodium Molybdate (Na_2MoO_4) on Bone Marrow Micronuclei in Mice

Dose (mg/kg)	Number of cells scored	MN cells per 1,000 PCE	MN per 1,000 PCE	PCE/NCE[a]
0	9,000	2.8	2.8	0.97
200	9,000	4.8*	4.9*	1.06
400	8,000	4.6*	5.8*	0.83
Positive control (colchicine, 0.8)	4,600	9.6**	11.3**	0.13***

[a]400 cells were scored for the ratio of PCE/NCE, and at least 1,000 PCE were scored for the micronucleus (MN) frequency from each slide.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (one-tailed Fisher's exact test).

TABLE IV. Descriptive Values for Male and Female Mice in the Dominant Lethal Assay With Sodium Molybdate

	Treatment (mg/kg body-weight)		
	0	200	400
Males			
Number	10	10	12
Body weight[1]	26.4 ± 2.2	26.8 ± 2.4	26.0 ± 1.5
Mating activity[2]	45.2%	41.8%	38.1%
Females			
Number	57	55	54
Body weight[1] at day of conception	23.1 ± 2.0	23.5 ± 2.5	23.4 ± 2.4
Body weight[1] at 16th day of pregnancy	32.8 ± 5.3	32.8 ± 5.1	31.8 ± 5.8
Number of corpus lutea[1]	9.6 ± 1.5	8.7 ± 3.1	8.0 ± 2.7
Pregnancy rate (%)[3]	84.2	87.3	74.1
Postimplantation loss (%)[4]	6.7	10.6	16.3

[1]Mean ± SD.
[2]Mating activity was calculated as a frequency of successful mating (female/s found with vaginal plugs) for all males in the treatment group at all times they were placed with females.
[3]Rate of pregnancy was calculated as a portion of females found with implants out of a total number of females with vaginal plugs.
[4]Postimplantation loss was calculated as follows: 1- (number of dead implants/total number of implants per female). Dose-dependent increase was caused by sodium molybdate ($P = 0.001$).

1997] may also explain the aneuploidy induction observed as kinetochore-positive micronuclei in our experiments. More studies are warranted in order to understand the mechanism of molybdenum genotoxicity.

The comparatively low toxicity of sodium molybdate in vivo allowed concentrations for treatment of mice used for the bone marrow MN and dominant lethality assays to be within the range of concentrations used in vitro for human lymphocytes. The effect of sodium molybdate on bone marrow MN was statistically significant but relatively mild compared to the effects of the positive control colchicine. In a study of chromosome aberrations in mouse bone marrow, molybdenum trioxide, but not ammonium molybdate, induced chromosome damage [Chopikashvili et al., 1991]. These data suggest that molybdenum compounds may be genotoxic for mouse bone marrow in vivo.

Prior to this study, there were only two reports on the reproductive toxicity of molybdenum in mice and rats [Jeter and Davis, 1954; Schroeder and Mitchener, 1971], but to our knowledge no data were available on germ cell genotoxicity in mammals. The increase in postimplantation loss induced by sodium molybdate shown here is the first indication of possible reproductive genotoxicity of molybdenum in mice. The only other available reference reported that ammonium molybdate induced an increase in dominant lethality in Drosophila [Chopikashvili et al., 1991]. A postmeiotic effect of sodium molybdate in mice mostly affected spermatozoa and resulted in an increase in both early and late postimplantation death. A similar slight increase in postimplantation loss was induced in spermatids and spermatocytes, but only the effect on spermatozoa was statistically significant. Thus, like a vast majority of other mutagens studied [Russell et al., 1990], molybdenum appears to have a predominantly postmeiotic effect on male germ cells. However, we cannot reach

TABLE V. Postimplantation Loss in Mice After Treatment With Sodium Molybdate

	Treatment (mg/kg body weight)		
	0	200	400
Spermatozoa (week 1)			
Females	20	18	21
Early loss (moles/female)	0.33 ± 0.62[a]	0.44 ± 0.62	0.94 ± 1.35[b]
Dead embryos/female	0	0.11 ± 0.47	0.06 ± 0.24[c]
Total loss (%)	5.4	7.8	13.8[d]
Spermatids (week 3)			
Females	29	23	16
Early loss (moles/female)	0.55 ± 0.91	0.63 ± 0.83	0.62 ± 0.77
Dead embryos/female	0.03 ± 0.19	0.05 ± 0.23	0.31 ± 0.48
Total loss (%)	8.0	9.3	12.1
Spermatocytes (week 5)			
Females	7	7	10
Early loss (moles/female)	1.0 ± 1.0	0.5 ± 0.58	2.0 ± 1.63
Dead embryos/female	0	0	0.14 ± 0.38
Total loss (%)	12.5	12.5	24.6
Spermatogonia (week 7)			
Females	5	8	8
Early loss (moles/female)	0.8 ± 1.1	1.2	1.5
Dead embryos/female	0	0	0
Total loss (%)	26.4	14.1	21.4

[a]Mean ± SD.
[b]Dose-dependent increase, $P = 0.013$.
[c]Dose-dependent increase, $P = 0.036$.
[d]Postimplantation loss was increased, $P = 0.003$.

a definite conclusion on the genotoxicity of molybdenum on the meiotic and premeiotic stages of spermatogenesis based on the preliminary data available from our pilot experiment. More studies are needed to confirm our findings, to expand them to different salts of molybdenum, and possibly to use different treatment protocols.

Thus, evidence is accumulating that different compounds of molybdenum are moderately genotoxic in vitro and in vivo, both in somatic and germ cells. Since these compounds are often present in complex mixtures with other metals and chemicals during mining and metal processing, or are found in food and drinking water, one concern about molybdenum is its possible interaction with other mutagens. In a study of chromosome aberrations in the bone marrow of mice treated with ammonium molybdate in combination with the antibacterial drug dioxidine, a synergistic effect between the two agents was observed [Chopikashvili et al., 1991]. On the other hand, in experiments on survival and dominant lethality in Drosophila treated with ammonium molybdate or molybdenum trioxide in combination with other metals and/or vitamin C, no additional increase was produced by combinations of molybdenum, wolframite, and cadmium [Chopikashvili et al., 1991]. Moreover, vitamin C had a protective effect on the survival of Drosophila treated with ammonium molybdate. In its moderate genotoxicity and possible interaction with other agents, molybdenum is similar to other metals, such as lead [Kristensen et al., 1993], chromium and selenium [Itoh and Shimada, 1996], and mercury and cadmium [Bérces et al., 1993]. Further studies are warranted on the interaction of molybdenum compounds with other mutagens and antimutagens in vivo and in vitro.

In summary, molybdenum salts yielded moderately positive results at relatively high doses in three experimental systems. In light of its significant environmental presence in complex mixtures, particularly related to mining and metallurgy, and its increasing use in health care products, the possible adverse effects of molybdenum in humans deserve more attention.

ACKNOWLDGMENTS

Jinhua Shao was supported by a WHO fellowship. The views expressed here are solely those of the authors and not of the funding agencies. The authors are grateful to Barton Bowers for his help with preparation of the manuscript.

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Accepted by—
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