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Immunosuppressive Effect of Subchronic Exposure to a Mixture of Eight Heavy Metals, Found as Groundwater Contaminants in Different Areas of India, Through Drinking Water in Male Rats

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Abstract. Immunotoxicity is an important health hazard of heavy metal exposure. Because the risk of combined exposure in the population cannot be neglected, we examined whether subchronic exposure to a mixture of metals (arsenic, cadmium, lead, mercury, chromium, nickel, manganese, and iron) via drinking water at contemporary Indian groundwater contamination levels and at concentrations equivalent to the WHO maximum permissible limit (MPL) in drinking water can induce immunotoxicity in male rats. Data on groundwater contamination with metals in India were collected from literature and metals were selected on the basis of their frequency of occurrence and contamination level above the MPL. Male albino Wistar rats were exposed to the mixture at 0, 1, 10, and 100 times the mode concentrations (the most frequently occurring concentration) of the individual metals in drinking water for 90 days. In addition, one group was exposed to the mixture at a concentration equal to the MPL of the individual metal and another group was used as positive control for immune response studies. The end points assessed were weights of organs, hematological indices, humoral and cell-mediated immune responses, and histopathology of skin and spleen. The MPL and 1× doses did not significantly affect any of the parameters and none of the doses induced any significant changes after 30 days of exposure. The mixture at 10× and 100× doses increased the relative weight of the spleen, but that of thymus, adrenals, and popliteal lymphnodes were increased with the 100× dose. After 90 days, 10× and 100× doses decreased serum protein and globulin contents and increased the albumin:globulin ratio; the albumin level was decreased only with the 100× dose. After 60 days, the total erythrocyte count (TEC), hemoglobin (Hb) level, and packed cell volume (PCV) were decreased with the 100× dose, whereas after 90 days, 10× and 100× doses reduced the TEC, total leukocyte count, Hb level, PCV, mean corpuscular volume, and mean corpuscular hemoglobin. With the 100× dose, the lymphocyte count was decreased after 60 and 90 days, but the neutrophil number was increased after 90 days. Antibody titer was decreased after 75 days with the 100× dose, but after 90 days, it was decreased with both the 10× and 100× doses. In delayed-type hypersensitivity response, these two doses decreased ear thickness after 24 and 48 h and skin biopsies showed a dose-dependent decrease in inflammatory changes. Histologically, the spleen revealed depletion of lymphoid cells and atrophic follicles with reduced follicular activity with higher doses. The findings suggest that hematopoietic and immune systems are toxicologically sensitive to the mixture, which could lead to anemia and suppression of humoral and cell-mediated immune responses in male rats at 10 and 100 times the mode concentrations of the individual components in contaminated groundwater.

Heavy metals are nonbiodegradable environmental pollutants and their levels in different environmental compartments (air, water, and food) are gradually increasing due to industrial and agricultural practices. Growing pollution of the environment with metals contributes to various disorders, including cancer, hematotoxicity, allergic disease, and immunotoxicity. Anemia is a common finding in animals after exposure to certain heavy metals, such as lead (Goyer and Clarkson 2001), cadmium (Liu *et al.* 1999), arsenic (Lee *et al.* 2004), and mercury (Dieter *et al.* 1983; Lecavalier *et al.* 1994), and immunodeficiency is a consequence of long-term anemia and hypoxia (Radi and Thompson 2004). Conflicting results, mainly immunosuppression, have been published on the effects of metals on the immune responses. Arsenic (Arkusz *et al.* 2005), cadmium (Carey *et al.* 2006; Skoczynska *et al.* 2002), mercury (Via *et al.* 2003), lead (Carey *et al.* 2006; Dietert *et al.* 2004), chromium (Shrivastava *et al.* 2002), nickel (Harkin *et al.* 2003; Hostynek, 2002), manganese (Seth *et al.* 2003; Zelikoff *et al.* 2002), and iron (Omara *et al.* 1999; Zelikoff *et al.* 2002) are

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reported to produce immunosuppressive effects in animals and humans.

To prevent the adverse health effects from occupational and environmental chemicals, exposure limits have been established for many substances. The relevance of these exposure limits, however, seems to be questionable in the case of concurrent exposure to multiple chemicals. It is assumed that toxic interaction between chemical substances is minimal or that the degree of any additive or synergistic increase in toxicity will not exceed the safety factors applied, but relatively few studies have tested these assumptions. Because of the complex nature of the immune system with its multiple components of redundant and opposing effects, the interpretation of data from single-metal studies is mostly inconclusive, which makes it virtually impossible to predict the effect of a mixture of metals on humans. Although the relationship between the immune system and concurrent multimetal challenge is literally unknown, additive or synergistic effects on the immune system can be expected. Hence, there exists the possibility of immunosuppression, which might render the organism more susceptible to infection and cancer development. Bishayi and Sengupta (2006) reported synergism in immunotoxicological effects due to repeated combined administration of arsenic and lead in mice. Exposure to cobalt and lead in concentrations below or close to the threshold limit value leads to synergistic immunotoxic effects (Jung *et al.* 2003). Hengstler *et al.* (2003) reported that coexposure to cadmium, cobalt, and lead is more dangerous than expected. Mahaffey *et al*. (1981) and Fowler *et al*. (2004) demonstrated that interactions between toxic metals and alterations in tissue metal levels and toxicity do occur following concurrent exposure to lead, cadmium, and arsenic for 10–13 weeks in rats. Therefore, evaluation of the effects of subchronic simultaneous exposure to potentially toxic metals upon immune responses is important due to widespread pollution of water resources with metals and their persistence in the environment.

Contamination of groundwater is a global environmental concern. A survey of literature reveals that contamination of groundwater with toxic metals is widespread in India. According to an estimate, groundwater meets about 80% of India's drinking water requirement (Nagarathna 2001), which implies that daily exposure of Indian population to low doses of mixtures of toxic and persistent metals through drinking water is an undesirable reality. Current understanding of the toxicity of these metals is primarily based on the studies performed on animals with single metals. However, the effects of any interactions among these metals on their toxicity are virtually unknown. Because each of the hazardous sites and geologically contaminated water sources has a unique set of conditions, it is virtually impossible to find a representative sample of the different sites. This shows an uncertainty about the estimate of various metal intakes through drinking water by the general population. We, therefore, formulated a mixture of eight most frequently occurring metals (arsenic, lead, cadmium, mercury, chromium, nickel, manganese, and iron) in water sources in different parts of India to simulate a worst real-life scenario. This chemically defined mixture of metals does not represent any particular contaminated water resource. As earlier studies indicate that the heavy metals investigated in the present study can alter hematopoietic and immune systems and pose health risks, we investigated in the current study whether simultaneous repeated in vivo exposure to eight heavy metals via drinking water for 90 days at contemporary Indian water contamination levels and at concentrations equivalent to the maximum permissible limit (MPL) in drinking water (World Health Organization) can alter immune responses in male rats.

Materials and Methods

Selection of Metals, Mixture Formulation, and Experimental Design

Details regarding the selection of metals, preparation of metal mixture and animal management have been reported earlier (Jadhav *et al.* 2007a). Briefly, data on groundwater contamination with metals in different parts of India were collected through literature survey. Metals were selected primarily on the basis of frequency of their occurrence and contamination level above the MPL in drinking water. Mode concentration (the most frequently occurring concentration) of each metal was derived from the reported concentrations and considered as the baseline dose (1×). Male Wistar rats (100–120 g) were exposed daily to the mixture of metals through drinking water (deionized) for 90 days. Five groups (I–V) of 15 rats each were used, of which 7–8 rats were used for humoral immune response and another 7–8 rats were used for assessment of cell-mediated immunity and hematological studies. Table 1 shows the mixture of metals and its dose levels. Group I was given only deionized water and served as the control. Group II, the baseline dose group (1×), was given mode concentrations of individual metals. Groups III and IV were given 10- and 100-fold concentrations of the baseline group, respectively. Rats of Group V were given metals at concentrations equivalent to the MPL. One more group was used as the positive control (antigen/hapten) for evaluation of immune responses. During the course of the experiment, two to three (13–20%) rats died in each group, including the control group. However, to maintain the uniformity of group size, the data of six animals have been presented. None of the animals died or survived showed any visible signs of toxicity.

Blood and Tissue Collection

Blood was collected from the retro-orbital plexus with the help of a capillary tube after 30, 60, or 90 days of exposure in heparinized (2 mg/mL) tubes for hematological examinations and in tubes without anticoagulant for separation of serum. Serum was refrigerated at 4°C until the estimation of proteins. At the end of the experiment, rats were killed by cervical dislocation. Spleen, thymus, adrenal glands, and popliteal lymphnodes were removed, cleared of adhering tissues, and weighed. A portion of the spleen was fixed in 10% formalin for histopathological examination.

Hematological Evaluation

Total erythrocyte count (TEC), total leukocyte count (TLC), and differential leukocyte count (DLC; 10% Giemsa stain) were done by the standard hemocytometer methods and packed cell volume (PCV) was obtained by the Wintrobe method. The hemoglobin (Hb) level was estimated by a using diagnostic kit (Beacon, India). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated by the standard formulas.

Table 1. Mixture of metals and its dose levels for subchronic toxicity studies in male rats

Metal salt	Mode concentration (ppm)[a]	Dose levels (ppm)[b]				
		Control	1×	10×	100×	MPL
Sodium arsenite	0.200 (15)	0.0	0.380	3.80	38.0	0.096
Cadmium chloride	0.055 (30)	0.0	0.098	0.98	9.8	0.005
Lead acetate	0.120 (23)	0.0	0.220	2.20	22.0	0.018
Mercuric chloride	0.044 (17)	0.0	0.060	0.60	6.0	0.008
Chromium trioxide	0.180 (15)	0.0	0.346	3.46	34.6	0.141
Nickel chloride	0.100 (23)	0.0	0.810	8.10	81.0	0.081
Manganese chloride	0.560 (15)	0.0	2.026	20.26	202.6	1.800
Ferric chloride	0.700 (29)	0.0	2.033	20.33	203.3	0.870

[a] Figures in parentheses indicate the number of reports studied to derive the mode concentration of each metal.
[b] The baseline dose (1×) for each chemical was adjusted to give representative elemental concentration of mode concentration and then safety factors (*i.e.*, 10× and 100×) were incorporated for dose selection because of probable interspecies and interindividual variations as per the standard protocol (Yang and Rauckman 1987). The WHO MPL in drinking water was also adjusted to give a representative elemental concentration.

Serum Proteins

Total protein and albumin were estimated by using diagnostic kits (Span, India). Globulin content was obtained as the difference between total protein and albumin levels. The albumin and globulin (A:G) ratio was calculated by dividing the albumin level by the globulin concentration.

Heat-Extracted Bacterial Antigen

A live culture of *Salmonella typhimurium* was grown in 1 L of tryptose soya broth for 24 h at 37°C as static culture. The bacteria were pelleted at 3000*g* for 15 min, washed twice with pyrogen-free normal sterile saline (NSS), repelleted, and, finally, suspended in 50 mL of pyrogen-free NSS in 50-mL sterile disposable screw-capped plastic tubes. The bacteria were subjected to heat treatment in boiling water bath for 1 h with intermittent agitation. After cooling, the suspension was centrifuged at 1000*g* for 10 min. The supernatant, designated as heat-extracted antigen (HE-Ag), was collected and stored at 4°C until use. The protein content of HE-Ag was estimated by the method of Lowry *et al*. (1951), using bovine serum albumin as the standard.

Immunization of Rats with HE-Ag

Rats of positive control and mixture-treated groups were inoculated a dose of HE-Ag (250 μg protein in 0.2 mL of Freund's incomplete adjuvant [FIA] per rat, sc), whereas vehicle control rats received an equal volume of FIA. The second inoculation was done on day 30 (immediately after blood collection) of the first inoculation, following the same protocol. Blood samples were collected on day 0 (prior to the first inoculation) and days 15, 30, 45, 60, 75, and 90 after the first inoculation.

Indirect Enzyme-Linked Immunosorbent Assay

Indirect enzyme-linked immunosorbent assay (ELISA) was standardized by Checkerboard Analysis and performed as per the method of Law *et al.* (1996). Briefly, 96-well flat bottom polyvinyl plates (Greiner, Germany) were coated with HE-Ag at 1, 2.5, 5, and 10 μg of protein/mL of coating or carbonate buffer (0.1 M, pH 9.6, 100 μL/well), incubated at 4°C overnight and washed three times with sodium–potassium phosphate-buffered saline (PBS; 0.01 M, pH 7.2) containing 0.05% Tween-20 (PBS-T) to remove unabsorbed antigen. The unsaturated sites of the plate were blocked by 1% bovine serum albumin fraction V powder in PBS-T (150 μL/well). The plates were incubated overnight at 4°C and then washed with PBS-T. The positive and negative sera were diluted 10^{-2} to 10^{-6} in PBS, added 100 μL/well in respective wells in duplicate, and incubated at 37°C for 2 h. The plates were washed three times with PBS-T. Rabbit anti-rat IgG horseradish peroxidase conjugate (Genei, Bangalore) was added (100 μL/well, 1:1000 to 1:8000). The plates were again incubated at 37°C for 1 h and washed three times with PBS-T. Finally, the substrate solution (10 mg of *O*-phenylenediamine dihydrochloride in 10 mL of citrate buffer containing 1.2 mL of H_2O_2 [30% solution]) was added at 100 μL/well and the plates were incubated for 15 min in darkness. The color reaction was stopped by adding 2 N H_2SO_4 (100 μL/well) and the plates were read by an ELISA reader (EC, India) at 490 nm. A serum sample of 10^{-3} dilution with a positive-to-negative ratio of 2 was considered positive employing antigen (2.5 μg protein/well) and anti-rat horseradish peroxidase conjugate (1:8000 dilution). Antibody titer was calculated by multiplying the absorbance at 490 nm by 1000.

Delayed-Type Hypersensitivity Response

The delayed-type hypersensitivity (DTH) response was assessed as described by Kataranovski *et al*. (2003). One day before the application of 1-chloro-2,4-dinitrochlorobenzene (DNCB) at the end of experiment, the dorsal skin (neck) of rats was shaved. Positive control and mixture-treated rats received 100 μL of DNCB (2% [w/v] in acetone:olive oil [4:1]), whereas control rats received an equal volume of vehicle (acetone and olive oil) on the shaved area for 2 consecutive days. Three days after sensitization, the rats were challenged on the dorsum of one ear with 50 μL of three times lesser concentration of DNCB than that used in the sensitization phase. The DTH reaction was assessed by measuring the thickness of the challenged ear after 24 and 48 h of challenge. The response was quantified as the difference in the thickness between challenged and nontreated ears of the same animal, according to the formula $(C - N)/N \times 100$, where C is thickness of the challenged ear and N is thickness of the nontreated ear and is expressed as percent increase in ear thickness.

Histology of Skin and Spleen

Ear skin samples were taken from DNCB application sites of rats after 48 h of challenge and fixed in 4% buffered formalin (pH 6.9). These skin samples and the formalin-fixed portion of the spleen were embedded in paraffin blocks. Sections of about 5 μm were cut, stained with hematoxylin and eosin by the standard method, and examined under light microscope.

Data Analysis

Results are expressed as mean ± SEM. The data were analyzed by one-way analysis of variance and the difference between the means was compared by Dunnett's multiple comparison test at $p < 0.05$.

Results

Organ Weight

No significant changes were observed in absolute weights of the spleen, thymus, adrenal glands, and popliteal lymphnodes (Table 2). Relative weights of the spleen were increased with 10× and 100× doses, whereas those of the thymus, adrenal glands, and popliteal lymphnodes were increased with the 100× dose in comparison to controls (Table 2).

Serum Proteins

Total protein, albumin, and globulin levels were not significantly affected by the mixture given for 30 and 60 days (Table 3). After 90 days of exposure, the mixture at 10× and 100× doses significantly decreased the total protein and globulin contents and increased the A:G ratio compared to controls, but the albumin level was significantly decreased with the 100× dose only (Table 3).

Hematology

Effects of the metal mixture on different hematological attributes are presented in Tables 4 and 5. No significant changes were observed after 30 days of exposure. After 60 days, the 100× dose significantly decreased the TEC, Hb level, and PCV compared to controls. MCH was decreased compared to the MPL group only. In addition to these, after 90 days of exposure, the mixture at 10× and 100× doses caused a significant reduction in TLC and MCV (Table 4). For the DLC, the mixture at the 100× dose significantly decreased the percentage of lymphocytes after 60 days as well as 90 days of exposure. The reduction in lymphocyte count (%) was associated with the increase in neutrophil percentage only after 90 days (Table 5).

Humoral Immune Response

The effect of the mixture on humoral immune response to HE-Ag antigen is depicted in Figure 1. No significant alterations in antibody titer were observed up to 60 days compared to the positive control. Antibody titer was significantly decreased after 75 days with the 100× dose (20.4%) and after 90 days with the 10× (25.1%) as well as 100× (39.5%) doses in comparison to the positive control.

Cell-Mediated Immune Response

The MPL and 1× dose levels did not significantly affect the ear thickness compared to the positive control (Table 6). At the 10× and 100× doses, the mixture significantly and dose-dependently decreased the ear thickness after 24 and 48 h (Table 6). Skin biopsies from positive-control rats revealed acute inflammatory changes characterized by vascular congestion, hemorrhages, focal areas of necrosis, extensive edema, and mononuclear cell infiltration. The changes observed in the MPL and 1× dose groups were almost similar to the positive-control rats. Biopsies from the 10× group showed moderate inflammatory responses, whereas rats exposed to the 100× dose exhibited mild congestion and edema with a marked decrease in mononuclear cell infiltration.

Histopathology of Spleen

Histological changes in the spleen were dose dependent in nature. Rats given the MPL and 1× doses revealed almost the normal architecture of the control rats (Fig. 2). Rats exposed to the 10× dose showed depletion of the lymphoid cell population, atrophic splenic follicles with less follicular activity, and degeneration and necrosis of the lymphoid cells. At the 100× exposure level, animals showed similar changes with more severity (Figs. 3 and 4).

Discussion

In the current study, the effects of daily exposure to a mixture of eight heavy metals (found as contaminants of various water bodies in India) for 90 days via drinking water were investigated on immune competence of male rats. Results of the present study demonstrate that the mixture caused hematotoxicity and impairment of immune function in higher doses, which indicate that chronic consumption of water contaminated with these metals in above their mode concentrations could interfere with the immnunocompetence of consumers. It is known that the xenobiotic-induced decrease in immunocompetence might result in repeated, more severe, or prolonged infections as well as the development of cancer in human beings. The relative weight of the spleen was significantly increased and similar results were reported with cadmium (Liu *et al*. 1999) and lead (Kim *et al*. 2000); however, unlike our finding, they reported a decrease in relative thymus weight. On the contrary, Dieter *et al*. (1983) reported decreases in absolute weight, but no change in the relative weight of the thymus and spleen with mercuric chloride. The spleen index is an indicator of immune dysfunction (Kim *et al.* 2000). The present results suggest greater responsiveness of the spleen to the toxic effect of the mixture than other organs and the spleen

Table 2. Weights of organs of male rats exposed to the mixture of metals for 90 days through drinking water

Treatment	Spleen	Thymus	Adrenal glands	PLN
Absolute weight (mg)				
Control	669 ± 10.8	267 ± 8.7	68.2 ± 3.7	26.2 ± 1.3
MPL	675 ± 18.2	272 ± 8.4	71.3 ± 3.0	26.5 ± 1.5
1×	670 ± 17.8	271 ± 10.4	70.3 ± 4.2	24.3 ± 1.8
10×	652 ± 19.4	260 ± 11.2	67.7 ± 2.7	27.1 ± 1.3
100×	636 ± 14.5	253 ± 17.0	62.0 ± 2.1	25.2 ± 2.1
Relative weight (mg/100 g body weight)				
Control	209 ± 3.3[a]	84 ± 2.7[a]	20.4 ± 1.1[a]	7.6 ± 0.6[a]
MPL	215 ± 5.2[a]	81 ± 4.5[a]	21.2 ± 1.1[a]	7.9 ± 0.4[a]
1×	205 ± 6.6[a]	83 ± 7.2[a]	21.5 ± 1.1[a]	8.0 ± 0.4[a]
10×	226 ± 5.0[b]	88 ± 5.1[a]	22.8 ± 0.9[ab]	9.2 ± 0.4[ab]
100×	268 ± 7.6[c]	104 ± 6.1[b]	25.4 ± 0.9[b]	10.3 ± 0.9[b]

PLN: Popliteal lymphnodes.
Note: Values (mean ± SEM; $n = 6$) in the same column bearing no superscript (a, b, c) in common vary significantly ($p < 0.05$).

Table 3. Levels of serum proteins in male rats exposed to the mixture of metals for 90 days through drinking water

Treatment	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Albumin:globulin ratio
After 30 days of exposure				
Control	7.92 ± 0.14	4.10 ± 0.09	3.77 ± 0.07	1.08 ± 0.05
MPL	8.34 ± 0.16	4.21 ± 0.08	4.06 ± 0.08	1.05 ± 0.06
1×	7.83 ± 0.21	4.15 ± 0.10	3.69 ± 0.12	1.12 ± 0.04
10×	8.17 ± 0.14	4.21 ± 0.08	3.95 ± 0.07	1.07 ± 0.06
100×	8.05 ± 0.21	4.20 ± 0.15	3.80 ± 0.06	1.10 ± 0.04
After 60 days of exposure				
Control	8.21 ± 0.11	4.29 ± 0.09	3.92 ± 0.06	1.09 ± 0.02
MPL	8.23 ± 0.09	4.26 ± 0.06	3.97 ± 0.09	1.07 ± 0.04
1×	8.14 ± 0.10	4.28 ± 0.12	3.86 ± 0.04	1.10 ± 0.06
10×	8.06 ± 0.07	4.17 ± 0.07	3.89 ± 0.07	1.07 ± 0.06
100×	7.89 ± 0.13	4.19 ± 0.15	3.70 ± 0.09	1.13 ± 0.04
After 90 days of exposure				
Control	7.87 ± 0.08[a]	4.12 ± 0.06[a]	3.67 ± 0.08[a]	1.12 ± 0.02[a]
MPL	7.97 ± 0.06[a]	4.32 ± 0.07[a]	3.80 ± 0.05[a]	1.13 ± 0.02[a]
1×	7.71 ± 0.08[a]	4.08 ± 0.05[a]	3.55 ± 0.07[a]	1.15 ± 0.01[ab]
10×	7.45 ± 0.11[b]	4.04 ± 0.07[a]	3.38 ± 0.07[b]	1.19 ± 0.01[b]
100×	6.89 ± 0.12[c]	3.81 ± 0.09[b]	3.05 ± 0.06[c]	1.24 ± 0.02[b]

Note: Values (mean ± SEM; $n = 6$) in the same column bearing no superscript (a, b, c) in common vary significantly ($p < 0.05$).

weight might have increased for the degradation of damaged erythrocytes induced by the mixture (Kim *et al.* 2000; Tanaka *et al.* 1987), whereas decreased hematocrit might be an early signal for a negative impact of the mixture on the oxygen-transport capacity of blood in rats.

Humoral immune function, as measured by the antibody response to HE-Ag in rats, is a sensitive indicator of immunotoxicity. The metal mixture decreased antibody titer to HE-Ag in rats with higher doses, suggesting suppression of humoral immunity. Prior studies have demonstrated that subacute to subchronic exposures to arsenic, cadmium, mercury, lead, chromium, nickel, and manganese (Blakely *et al.* 1980; Graham *et al.* 1975, 1978; Koller 1980; Ohi *et al.* 1976; Srisuchart *et al.* 1987) can impair antibody responses in rodents. A synergistic trend of immunotoxicity during simultaneous exposure to arsenic and lead was reported by Bishayi and Sengupta (2006). Exposure to cobalt and lead in concentrations below or close to the threshold limit value led to synergistic immunotoxic effects (Jung *et al.* 2003). The metal mixture in higher doses also caused a decrease in DTH response, a T-cell-mediated inflammatory response (Kataranovski *et al.* 2003), indicating that the mixture affects the cell-mediated immunity. Arsenic (Savabieasfahani *et al.* 1998), cadmium (Institoris *et al.* 2002), and lead (McCabe *et al.* 1999) decreased the DTH response in rodents. Histologically, the observed dose-related decrease in inflammatory response with a marked decrease in infiltration of mononuclear cells suggests that the mixture might have decreased the migration of the inflammatory cells and/or inhibited the activation of macrophages.

The immunosuppressive effect of the mixture might lie at different levels, but the present findings suggest that the mixture causes immunotoxic effects by mechanisms that are dose dependent and time dependent in nature. Currently, no data are available in the literature that are directly comparable to the results of the present study. It is known that the spleen comprises functionally distinct lymphocyte subpopulations of about 53% B-cells and 21% T-cells and these cells play a central role in humoral and cell-mediated immunity (Hathcock *et al.* 1981). Histological changes in the spleen revealed depletion of lymphoid cell population and diminution of lymphoid follicular

Table 4. Hematological profile of male rats exposed to the mixture of metals for 90 days through drinking water

Treatment	TEC (million/μL)	TLC (thousand/μL)	Hb (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (%)
After 30 days of exposure							
Control	7.67 ± 0.16	14.33 ± 0.57	14.61 ± 0.61	47.36 ± 1.69	59.72 ± 3.52	17.84 ± 1.70	30.42 ± 1.94
MPL	8.13 ± 0.19	15.81 ± 0.49	16.13 ± 0.43	52.07 ± 1.03	66.61 ± 3.53	22.87 ± 2.06	36.00 ± 1.39
1×	7.81 ± 0.16	14.69 ± 0.40	14.86 ± 0.67	48.04 ± 2.46	60.42 ± 3.52	20.16 ± 1.51	31.43 ± 2.27
10×	7.94 ± 0.15	15.39 ± 0.74	15.38 ± 0.49	50.24 ± 1.68	63.74 ± 3.52	18.81 ± 1.42	33.36 ± 2.53
100×	7.86 ± 0.15	15.02 ± 0.64	14.96 ± 0.60	49.70 ± 1.97	62.04 ± 3.52	20.76 ± 2.09	32.92 ± 1.44
After 60 days of exposure							
Control	7.34 ± 0.21[a]	15.86 ± 0.63	13.95 ± 0.48[a]	42.56 ± 1.05[a]	57.48 ± 1.74	18.33 ± 0.85[ab]	31.84 ± 0.75
MPL	7.54 ± 0.17[a]	16.22 ± 0.82	14.34 ± 0.53[a]	43.47 ± 1.06[a]	58.36 ± 2.79	19.27 ± 0.96 [b]	33.31 ± 0.87
1×	7.22 ± 0.24[a]	15.53 ± 0.70	13.54 ± 0.55[a]	41.34 ± 0.82[a]	57.55 ± 2.36	18.07 ± 0.91[ab]	32.57 ± 1.36
10×	7.12 ± 0.27[a]	15.04 ± 0.82	13.01 ± 0.38[a]	40.38 ± 1.09[ab]	55.84 ± 1.82	17.18 ± 1.05[ab]	32.52 ± 0.52
100×	6.71 ± 0.29[b]	14.17 ± 0.99	12.02 ± 0.51[b]	36.60 ± 1.14[b]	55.09 ± 2.65	15.68 ± 1.50[a]	30.75 ± 0.98
After 90 days of exposure							
Control	7.96 ± 0.23[a]	14.23 ± 0.60[a]	15.37 ± 0.64[a]	45.33 ± 1.92[a]	56.82 ± 1.87[a]	20.47 ± 0.60[a]	31.10 ± 0.78
MPL	8.18 ± 0.31[a]	15.12 ± 0.46[a]	16.36 ± 0.76[a]	47.60 ± 2.30[a]	58.25 ± 2.74[a]	21.40 ± 0.54[a]	32.06 ± 0.74
1×	7.64 ± 0.24[ab]	13.71 ± 0.74[a]	14.39 ± 0.57[a]	43.17 ± 1.70[a]	51.04 ± 2.97[ab]	20.43 ± 0.68[a]	30.74 ± 0.68
10×	7.20 ± 0.21[b]	11.54 ± 0.62[b]	12.21 ± 0.39[b]	36.63 ± 1.18[b]	48.07 ± 1.65[b]	15.75 ± 0.47[b]	30.81 ± 0.87
100×	6.58 ± 0.29[c]	8.16 ± 0.53[c]	9.47 ± 0.26[c]	28.42 ± 0.80[c]	43.70 ± 2.76[b]	13.25 ± 0.56[c]	29.98 ± 0.43

Note: Values (mean ± SEM; $n = 6$) in the same column bearing no superscript (a, b, c) in common vary significantly ($p < 0.05$).

Table 5. Differential leukocyte count in male rats exposed to the mixture of metals for 90 days through drinking water

Treatment	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)	Eosinophil (%)
After 30 days of exposure				
Control	78.66 ± 2.36	14.67 ± 2.07	5.33 ± 0.33	1.33 ± 0.21
MPL	81.17 ± 1.98	13.00 ± 1.75	5.00 ± 0.36	0.83 ± 0.30
1×	75.83 ± 2.13	17.50 ± 2.23	5.67 ± 0.33	1.00 ± 0.26
10×	78.67 ± 2.51	14.83 ± 2.24	5.50 ± 0.43	1.00 ± 0.36
100×	81.67 ± 1.76	12.67 ± 1.35	4.66 ± 0.50	1.00 ± 0.26
After 60 days of exposure				
Control	81.50 ± 2.12[a]	12.67 ± 2.05	5.17 ± 0.60	0.67 ± 0.20
MPL	81.17 ± 2.14[a]	12.54 ± 2.14	5.50 ± 0.43	0.67 ± 0.17
1×	80.17 ± 1.83[a]	13.33 ± 1.68	5.66 ± 0.42	0.83 ± 0.66
10×	78.33 ± 1.72[a]	14.50 ± 1.40	6.17 ± 0.48	1.00 ± 0.26
100×	74.83 ± 1.51[b]	17.50 ± 1.49	6.50 ± 0.43	1.17 ± 0.31
After 90 days of exposure				
Control	71.83 ± 2.70[a]	20.66 ± 2.65[a]	5.50 ± 0.67	1.33 ± 0.33
MPL	73.83 ± 3.48[a]	20.83 ± 2.95[a]	5.67 ± 0.42	1.16 ± 0.30
1×	69.17 ± 3.81[a]	24.66 ± 3.64[a]	5.33 ± 0.42	1.67 ± 0.21
10×	64.83 ± 3.70[ab]	27.50 ± 3.63[a]	6.00 ± 0.45	1.83 ± 0.17
100×	57.83 ± 4.05[b]	33.83 ± 4.03[b]	6.67 ± 0.33	1.83 ± 0.31

Note: Values (mean ± SEM; $n = 6$) in the same column bearing no superscript (a, b, c) in common vary significantly ($p < 0.05$).

activity. Thus, the mixture-induced toxicity to subpopulations of splenic lymphoid cells might relate to impairment of immune functions in male rats. The decrease in DTH response might partly be attributed to increased production of adrenal corticosteroids. The observed increase in the relative weight of the adrenal glands suggests a possible increase in glucocorticoid production and induction of stress. Arsenic (Jana *et al*. 2006), cadmium (Lall and Dan, 1999), lead (Kishikawa *et al*. 1997), and mercury (Dieter *et al*. 1983) increase the production of corticosteroids. Lall and Dan (1999) reported involvement of adrenal hormones in cadmium-induced immunosuppression and suggested that cadmium activates the corticosteroid-associated immunoregulatory circuit. The immunosuppressive and anti-inflammatory effects of glucocorticoids are linked, as both involve inhibition of leukocyte functions. Glucocorticoids lead to a decreased number of circulating lymphocytes; in contrast, they increase circulating polymorphonuclear leukocytes (Schimmer and Parker 2001). Similar findings on circulating leukocytes were observed in the current study. The percentage of neutrophils was increased, whereas the percentage of lymphocytes decreased. Liu *et al* (1999) also reported a similar phenomenon in which repeated administration of cadmium chloride for 10 weeks resulted in a dose- and time-dependent alteration of the blood leukocyte profile; the neutrophil count increased from 20% to 60%, whereas the lymphocyte number decreased from 80% to 40% in mice.

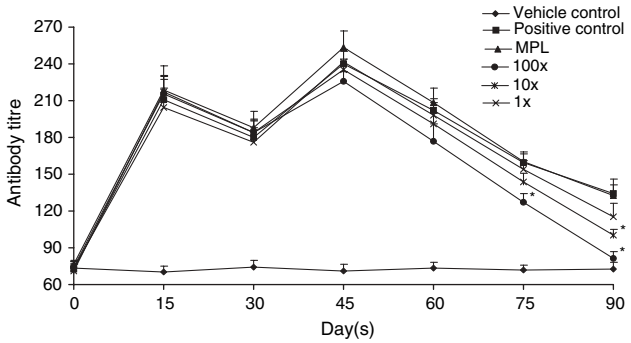

Fig. 1. Changes in *S. typhimurium* heat-extracted antigen-induced serum antibody titer of rats exposed to the mixture of metals through drinking water for 90 days. Results are expressed as mean ± SEM ($n = 6$). *$p < 0.05$ compared to positive control

Table 6. Delayed-type hypersensitivity response in rats exposed to the mixture of metals for 90 days through drinking water

	DTH response (% increase in ear thickness)	
Treatment	24 h	48 h
Positive control	47.65 ± 4.55[a]	40.53 ± 2.85[a]
MPL	44.99 ± 3.79[a]	37.04 ± 3.46[a]
1×	41.85 ± 4.11 [a]	33.22 ± 3.00[a]
10×	34.27 ± 2.52[b]	27.13 ± 2.98[b]
100×	22.52 ± 3.03[c]	14.34 ± 1.96[c]
Vehicle control	NDC	NDC

DNCB = 1-chloro-2,4-dinitrochlorobenzene; NDC = no detectable change.
Note: Values (mean ± SEM; $n = 6$) in the same column bearing no superscript (a, b, c) in common vary significantly ($p < 0.05$).

Anemia is a common finding in animals after exposure to certain heavy metals, including lead (Goyer and Clarkson 2001), cadmium (Liu *et al.* 1999), arsenic (Lee *et al.* 2004), and mercury (Dieter *et al.* 1983, Lecavalier *et al.* 1994). In our study, microcytic and hypochromic anemia was evident with a high dose, as evidenced by decreases in erythrocyte count, Hb concentration, hematocrit, MCH, and MCV. The mechanism of the mixture-induced anemia might involve several factors and could be the consequence of the composite action of all the metals. However, contribution of iron deficiency could be ruled out, as the dosing solution of the mixture *per se* contained a high level of iron. Additionally, a decrease in MCV and no change in MCHC do not support that iron deficiency contributed to development of anemia (Liu *et al.* 1999). Lead, an important component of the mixture, induces microcytic and hypochromic anemia, which results from two basic defects: shortened erythrocyte life span and impaired heme synthesis. The former is attributed to increased erythrocyte membrane fragility accompanied by inhibition of Na^+ -K^+ -ATPase (Goyer and Clarkson 2001). Peroxidation of membrane lipids not only alters the lipid milieu and structural as well as functional integrity of cell membrane but also affects the activity of various membrane-bound enzymes, including Na^+ -K^+-ATPase (Gurel *et al.* 2004; Rauchova *et al.* 1995). Recently, we reported that subchronic exposure to this mixture of metals through drinking water induces oxidative stress in rat

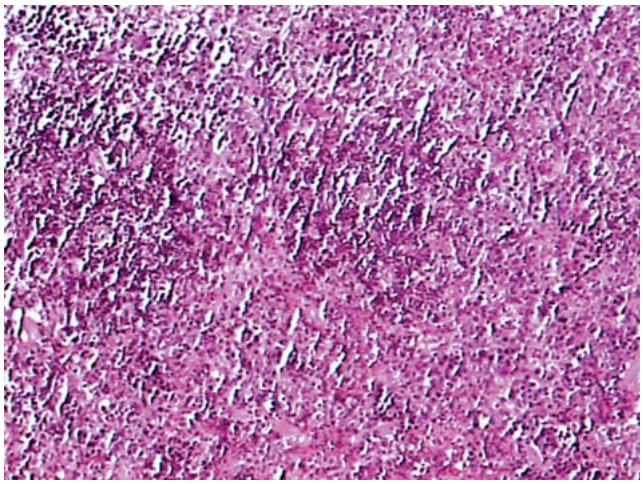

Fig. 2. Microphotograph of spleen (control group) showing normal architecture with centrally placed splenic vessels and normal follicular activity in the cortical region (H&E; magnification = 160×)

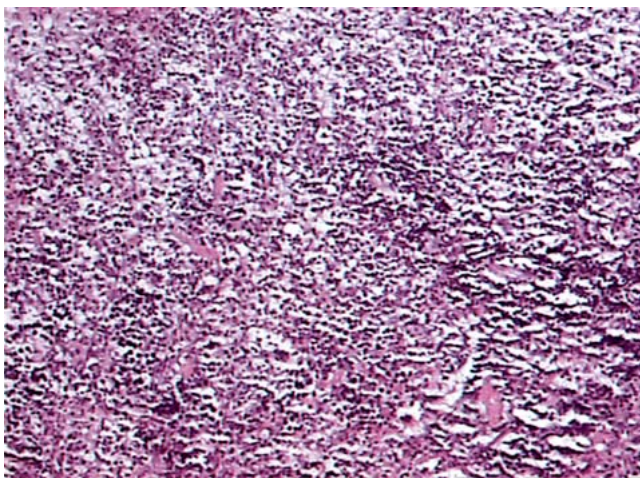

Fig. 3. Microphotograph of spleen (group: 100×) showing severe depletion of lymphoid population with extensive degeneration of lymphocytes in the cortex (H&E; magnification = 160×)

erythrocytes through increased lipid peroxidation (Jadhav *et al.* 2007a). Hemolytic anemia due to erythrocyte sequestration in the spleen resulting in a shorter life span and increased destruction of erythrocytes in the spleen and liver (Tanaka et al. 1987) and hypoproduction of erythropoietin due to renal injury (Horiguchi *et al.* 1994) might be the major causes for the observed anemia. The current study is a part of a larger study in which the oxidative-stress-inducing potential of the metal mixture in various tissues was evaluated. Induction of oxidative stress was evident, as lipid peroxidation, a biomarker of oxidative stress, was increased with decrease in enzymatic and nonenzymatic antioxidative defense systems in erythrocytes (Jadhav *et al.* 2007a), kidney (Jadhav *et al.* 2007b), and spleen (Jadhav *et al.* 2006); the kidney was the most affected among them. Blood chemistry and histopathology also support the maximum damage to the kidney (unpublished data), which indicates a possible reduction of

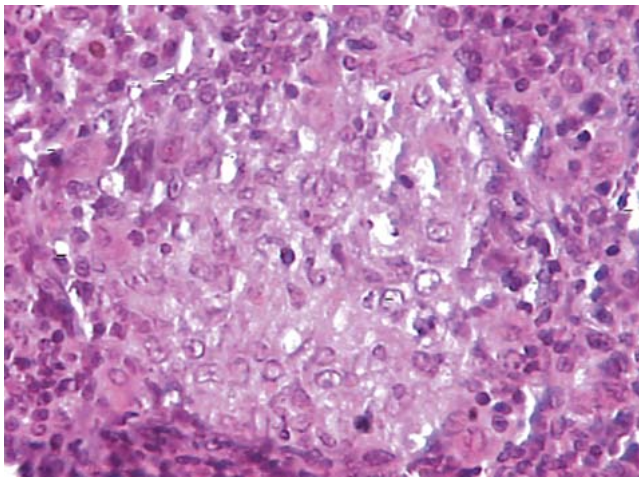

Fig. 4. Microphotograph of spleen (group: 100×) showing focal necrosis of splenocytes and lymphoid elements in the cortical region (H&E; magnification = 360×)

erythropoietin production due to mixture-mediated nephrotoxicity (Hiratsuka *et al.* 1996; Horiguchi *et al.* 1994). Hence, the contribution of the factors to the mixture-induced anemia might not be mutually exclusive.

In conclusion, the findings reveal that hematopoietic and immune systems could be the potential targets for the mixture, as it causes anemia and suppresses both humoral and cell-mediated immune responses in male rats at 10 and 100 times the mode concentrations of the individual metal components in contaminated water sources. Because the mixture at 10× concentrations induced some immunohematological changes in rats, the mode concentrations of the metals in Indian drinking water appear to be quite high, which might be a matter of concern for a susceptible part of human and animal populations considering lifetime exposure. Although the mixture induced adverse effects reflecting a potential hazard, it is too early to translate these immunotoxic outcomes into a direct measurable decrease of resistance to infections and diseases. Hence, further investigations, including studies on the mechanism of action, are required before appropriate immunotoxicological interpretations could be drawn for the alterations induced by the simultaneous multimetal exposure.

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