



Blood metal levels linked with hematological, oxidative, and hepatic-renal function disruption in Swiss albino mice exposed to multi-metal mixture

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Abstract

This study evaluates the toxic effect of various doses of multi-metal mixtures leading to metal accumulation in the blood of exposed mice and alterations in the blood biomarkers. To explore the health consequence of multiple metal exposures, *Swiss Albino* mice were orally given different doses of metal mixtures via drinking water for 8 weeks. The mice were randomly divided into fourteen groups. Besides the control animals, each mouse received a corresponding dose of heavy metal mixture [MPL (maximum permissible limit), 1 ×, 5 ×, 10 ×, 50 × or 100 ×]. The mice were sacrificed, and blood was extracted. Significant increase in the blood metal concentration was observed after exposure to multi-metal mixtures. The amount of As and Hg in the blood of mice subjected to high concentration of metal mixture was found more than tenfold high, whereas other metals (Cd, Pb, Ni, Cr) were less than threefold high with respect to each element in the blood of control animals. There was a noteworthy decline in the RBC count (32.1% male; 30.3% female) and HGB (30.68% male; 29.20% female) in the 100 × male and female groups. The enzymatic antioxidant system, such as SOD, CAT, GSH, and MDA, also mediates the relationship between heavy metal mixtures and hematological parameters. Serum ALT, AST, ALP, CR, and BUN significantly increased ($p < 0.05$) in multi-metal exposed group (50 × and 100 ×) indicating hepatic-renal cellular injuries. The level of serum GLU, TC, and LDL, the three markers involving glucose and lipid metabolism, was also significantly ($p < 0.05$) higher in the multi-metal exposure group. A dose-dependent loading of each metal in the blood suggests significant relation between blood morphology, oxidative stress indices, and other serum biomarkers. The overall results revealed abnormalities in the hematological system, decreased renal function, hepatic injury, and disturbances in the blood metal concentration in the animals subjected to high dose of multi-metal solution. A comprehensive analysis of varying concentration of multi-metal mixture (low to high dose) on oxidative, hematological, and hepatorenal parameters signify that blood could be a sensitive toxicological indicator of multi-metal exposure *in vivo*.

Keywords Biomarkers · Blood · Hematology · Oxidative stress · Multi metal mixture · Toxicity

Introduction

Heavy metal pollution is currently a serious global environmental health concern. Arsenic (As), cadmium (Cd), lead (Pb), chromium (Cr), iron (Fe), manganese (Mn), nickel (Ni), and mercury (Hg) are widely used in anthropogenic activities (Butler et al. 2019; Balali-Mood et al. 2021; Bist and Choudhary 2022). These applications often lead to the

emission of heavy metal exhaust into the air, deposition of their particles on the soil surfaces, and discharge of waste water into rivers (Singh et al. 2017; Butler et al. 2019). Multiple heavy metals are released into the environment, gradually resulting in the heavy metal pollution (Butler et al. 2019). Metals with no known biological functions such as As, Cd, Pb, Cr, and Hg are highly toxic and often escape the control mechanism and binds to the various protein sites by displacing essential elements causing cell toxicity (Jaishankar et al. 2014). These metals are known to be systemic toxicants even at very low dose and affect multiple organ systems. Arsenic (As) is a toxic element, which when released into an ecological environment can be a danger to both terrestrial and aquatic organism (Chi et al. 2017). An

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impressive number of epidemiological studies have identified hematological and biochemical changes in participants exposed to Pb and Cd (Chen et al. 2019). Both of these elements are identified as environmental toxins and have been linked to neurotoxicity, hepatotoxicity, nephrotoxicity, and reproductive toxicity (Balali-Mood et al. 2021). Inhalation is the primary route of occupational exposure to metals (Breton et al. 2013). After absorption, lead distributes into three major compartments in the body: blood, soft tissues, and bones. About 95% of blood metal is accumulated in erythrocytes disturbing their function. Anemia is a well-known toxic effect of heavy metal action (Dobrakowski et al. 2016). A decrease in the hematocrit or hemoglobin level due to metal exposure may be caused by increased erythrophagocytosis, hemolysis, and splenic sequestration of red blood cells or by impaired erythropoiesis. It is well established that heavy metal impairs the biosynthesis of heme by inhibiting enzyme (Dobrakowski et al. 2016). In addition, heavy metal has been shown to have a direct positive effect on erythrocyte antioxidants and have highlighted the accumulation of heavy metals in the target organs along with the increase in pollution gradient (Tete et al. 2015). These toxic elements cause the excessive production of ROS, which results in toxic effects, namely, cytogenetic alterations, lipid peroxidation, DNA damage, and oxidative damage to tissue (Sall et al. 2020; Balali-Mood et al. 2021). Chromium (Cr) is an omnipresent hazardous contaminant, which in its hexavalent state, Cr(VI), can cross the cell membrane and affect the cellular function (Feng et al. 2019). Many epidemiological studies have also indicated that both environmental and occupational (Lacerda et al. 2019) exposure to Cr(VI) can cause hematological and biochemical changes and generate several reactive oxygen species (ROS) (Feng et al. 2019). The toxicity of Cr(VI) is attributable to its ability to increase oxidative stress. Specifically, the reduction of Cr(VI) to a lower oxidative state forms several reactive oxygen species (ROS) that induce oxidative stress (Lacerda et al. 2019). Mercury in all its forms is toxic to humans, and its exposure can lead to its accumulation in target organs culminating in ROS, oxidation of lipid peroxidation, and cell degeneration (Kanwal et al. 2020). Although Mn and Fe are considered as essential biological elements for the human body, they play a major role in the metabolic and intracellular process (Bresciani et al. 2015). However, excessive exposure to these elements can disrupt the antioxidant system, generating oxygen-derived free radicals (Nascimento et al. 2016). Iron plays a noticeable role in DNA synthesis and acts as prosthetic group constituent in various cellular enzymes like oxidases and cytochromes (Jaishankar et al. 2014). It is also an essential component of heme, within hemoglobin, the protein responsible for transporting oxygen throughout the body. Mn is of great environmental and public health significance due to its broad usage in the ferroalloy industry.

In particular, overexposure to these essential metals may disrupt the cellular metal hemostasis and have grave effect on human and animal health. Many studies have reported high concentration of Mn and Fe in blood, liver, kidney, pancreas, and the brain (Wang et al. 2018; Feng et al. 2019; Hu et al. 2021). Like other metals, high exposure to Ni impairs the homeostasis of other essential metal ions such as Ca, Mn, Zn, and Fe in blood and tissues (Abudayyak et al. 2017; Sule et al. 2020). It is noteworthy that Ni has similar chemical properties to the abovementioned metals and competes for metal-binding sites and transporters, as well as for the enzymatic proteins (Sule et al. 2020). The content of Ni in blood and tissue is vital for the function of many biological organisms, but it can also be toxic to organisms due to its omnipresence in the environment. This element has been implicated as the cause to induce hematological disturbances leading to cardiovascular, hepatic, and renal disorders (Genchi et al. 2020).

There are numerous reports on the toxicity of single metal, but few studies have encountered the role of multi-metal exposure as occurring in the natural environment (Butler et al. 2019). Epidemiological evidence indicates that co-exposure to multiple metals is associated with oxidative stress and hematological, hepatic, and renal biomarker alterations (Xu et al. 2020). It is important to consider how exposure to multiple metal results in fluctuation of different metallic elements in blood, and it subsequently deteriorates the health status of exposed subjects. The present study was designed to investigate the dose-dependent effects of multi-metal exposure and alterations in metal homeostasis inducing biotoxicity. This study was conducted for a period of 8 weeks, and the effect of multiple metal exposures in respect to alterations in metal concentration, hematological parameters, hepatorenal markers, and degree of oxidative damage in blood of Swiss albino mice was analyzed.

Materials and methods

Study design and animals

Six-week-old Swiss Albino mice were purchased from the LalaLajpatRai University of Veterinary & Animal Science, Hisar (Haryana, India). One hundred and twelve mice (weight 22 ± 2.3 to 24 ± 2.3 g) were kept in our animal facilities (22°C ; 12-h dark/12-h light) for 14 days prior to the experiments. The treatment doses were given to mice for a period of 8 weeks by spiking the drinking water with multi-metal solution. Details of metal mixture doses and experimental groups are presented in Table 1. Stock solutions of each metal salts ($\text{CdCl}_2 \cdot \text{H}_2\text{O}$, HgCl_2 , CrO_3 , $\text{Pb}(\text{NO}_3)_2$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, NaAsO_2 , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and FeCl_3) were prepared in deionized water and stored. Drinking water

Table 1 Grouping of mice and doses throughout period of 8 weeks

Group (<i>n</i> = 8)	Sex	Mode of administration	Metal solution exposure dose
1	Male	Oral (spiked in drinking water)	Control
2			1×
3			5×
4			10×
5			50×
6			100×
7			MPL
8	Female		Control
9			1×
10			5×
11			10×
12			50×
13			100×
14			MPL

Control group 1 (deionized drinking water), group 2 (1×metal mixture), group 3 (5×metal mixture), group 4 (10×metal mixture), group 5 (50×metal mixture), group 6 (100×metal mixture), group 7 (MPL) [MPL (maximum permissible limit) in accordance to WHO with modifications in accordance to Jadhav et al. 2007]. Control group 8 (deionized drinking water), group 9 (1×metal mixture), group 10 (5×metal mixture), group 11 (10×metal mixture), group 12 (50×metal mixture), group 13 (100×metal mixture), group 14 (MPL). 1× solution contained NaAsO₂ (0.380 ppm); CdCl₂ (0.098 ppm); Pb (NO₃)₂ (0.220 ppm); HgCl₂ (0.060 ppm); CrO₃ (0.346 ppm); NiCl₂·6H₂O (0.810 ppm); MnCl₂·4H₂O (2.026 ppm); and FeCl₃ (2.033 ppm)

supplemented with metal mixture was changed regularly for a period of 8 weeks. All animals were fed with a basal diet. Animals were observed once daily for any adverse physical signs of toxicity resulting from administration of metal doses. All the animals were fasted overnight prior to blood collection.

Blood collection

At the end of the experimental period, the control and exposed mice were weighed and sacrificed with cervical dislocation. The blood samples were collected through cardiac puncture. Blood samples were centrifuged at 3000 rpm for 10–15 min, and the supernatant was stored at 4 °C. The part of fresh blood samples and supernatant were used for blood analysis. The clear non-hemolyzed sera were stored at –20 °C for biochemical analyses.

Evaluation of hematological parameters

Whole blood samples from all the groups were tested shortly after collection to determine the hemoglobin (HBC), hematocrit (HCT), red blood cell (RBC) count, white blood cells

(WBCs), eosinophils (EOS), neutrophils (NEU), lymphocytes (LYM), basophils (BAS), monocytes (MON), and platelet (PLT) count by Sysmex automatic hematology analyzer. MCV (mean corpuscular volume), MCHC (mean corpuscular hemoglobin concentration), and MCH (mean corpuscular hemoglobin) were calculated.

Metal determination in mice blood

The procedure for digestion was carried out for whole blood samples after 8 weeks of study. Fifty microliters of whole blood was wet digested with 500 µl of optima grade nitric acid (3%) at 65 °C for 60 min in a plastic digestion vessel on a blocked heater. After partial evaporation, samples were cooled down and diluted to 10 ml with ultrapure water. The concentrations of As, Cd, Pb, Cr, Fe, Mn, Ni, and Hg in metal exposed blood were digested and measured by a quadrupole-based inductively coupled plasma mass spectrometer (ICP-MS Agilent 7500cs, Agilent technologies). All reagents were of analytical grade. Data were expressed as µg L⁻¹.

Evaluation of biochemical parameters

In the stored serum samples, MDA was assayed according to the method of Shafiq-Ur-Rehman (1984). One milliliter of sample was combined with equal volume of trichloroacetic acid. This mixture was centrifuged at 2000 rpm for 10 min, and the supernatant was collected and heated in a water bath for 10 min, to which 1 ml thiobarbituric acid was added. To the reaction mixture, 1 ml of distilled water was added, and the absorbance was read at 535 nm. The reduced glutathione (GSH) content in erythrocytes was estimated by the method of Prins and Loos (1969). Hemolysate of 200 µl was mixed with 4 ml H₂SO₄ and incubated for 10 min. Following incubation, 500 µl of tungstate solution was added and centrifuged again for 15 min at 2000 rpm. The supernatant (2 ml) was combined with 2.5 ml Tris-buffer and 0.2 ml 5,5-dithiobis-2-nitrobenzoic acid, and the absorbance was read at 412 nm. The activity of superoxide dismutase (SOD) was assessed by Madesh and Balasubramanian's method (1998). The reaction mixture included 650 µl PBS, 30 µl MTT (1.25 mM), 75 µl pyrogallol (100 mM), and 0.01 ml hemolysate. After 5 min of incubation, 750 µl dimethyl sulfoxide was added to stop the reaction, and the absorbance was read at 570 nm. The catalase (CAT) activity was measured according to Aebi (1983). To 2 ml phosphate buffer, pH 7.0, the hemolysate was added in a cuvette. After adding 1 ml H₂O₂ (10 mM), the absorbance was read at 240 nm every 10 s for 1 min. Stored serum samples were analyzed for the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), blood urea nitrogen

(BUN), creatinine (CR), and uric acid which were determined using kits in accordance with the manufacturer's instructions (BioVision, Abcam). Also, serum glucose (GLU), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and total cholesterol (TC) were determined using kits in accordance with the manufacturer's instructions (BioVision, Abcam).

Statistical analysis

All the significant analysis was performed using one-way ANOVA (SPSS 22.0 software program). All data were expressed as mean \pm standard error mean for each group. Multiple comparisons between groups were analyzed by Tukey's post hoc test for oxidative stress parameters. Statistical significance was set at $p < 0.05$ and $p < 0.01$.

Results

Hematological analysis

Hematological changes in the animals following treatment with the multi-metal mixture for 8 weeks are presented in Tables 2 and 3. In the 10 \times male group, there was a significant increase in the number of neutrophils (75.23%) but a decrease in the lymphocyte count (29.01%) in comparison to the control group. In the 100 \times male and female group, the percentage of neutrophils significantly declined (47.7% male; 61.6% female) while lymphocytes increased (53.66% male; 101.8% female) compared to control groups. The monocytes, eosinophil, and basophil count for the metal mixture treated groups (1 \times , 5 \times , 10 \times , 50 \times , and 100 \times)

showed no significant changes in comparison with the control groups (Table 2). The total WBC count increased significantly in 10 \times , 50 \times , and 100 \times metal-exposed groups as compared to control group. As shown in Table 2, there was a noteworthy decrease in the RBC count (32.1% male; 30.3% female) and HGB (30.68% male; 29.20% female) in the 100 \times male and female groups. Compared with the control group, a significant fall in the PLT count was recorded in both the genders in 50 \times and 100 \times ($P < 0.05$). While, MCV and MCHC showed significant changes in 50 \times and 100 \times exposed animals ($P < 0.05$). In the 10 \times female group, a significant increase in MCV was seen. The HCT value decreased marginally in the low dose group (1 \times , 5 \times , 10 \times); however, in high-dose group (100 \times) in comparison to control, 10.28% and 10.96% decline was recorded in male and female mice, respectively (Table 2). In consideration to the gender-specific changes in the hematological parameters, similar treatment-related biological effect of the metal mixtures was observed in low dose (1 \times , 5 \times , 10 \times) in both the sexes.

Analysis of metal concentrations in blood

To study the body burden of multi-metal mixtures in mice, the content of each metallic element in the blood was determined after treatment period of 8 weeks. In comparison to control, no significant changes in Cd, Pb, As, Hg, Ni, Mn, Fe, and Cr level were recorded in the blood derived from animals exposed to low dose of metal mixture (1 \times and 5 \times); however, obvious changes were observed in animals subjected to higher concentration of multi-metal mix (10 \times , 50 \times , and 100 \times). After 8 weeks of multi-metal mixture treatment (Fig. 1a–h), the Cd concentration in the blood

Table 2 Hematological parameters of male mice treated with various doses of multi-metal mixtures

Parameters	Unit	Control	MPL	1 \times	5 \times	10 \times	50 \times	100 \times
White blood cells								
WBC	10 ³ / μ L	6.1 \pm 1.8	6.37 \pm 0.9	5.24 \pm 1.1	4.29 \pm 1.4	9.00 \pm 1.8	11.2 \pm 1.4	13.50 \pm 1.8*
NEU	10 ³ / μ L	2.83 \pm 0.36	2.78 \pm 0.37	1.07 \pm 0.54	2.96 \pm 0.10	4.96 \pm 0.28*	1.69 \pm 0.47	1.48 \pm 0.33
EOS	10 ³ / μ L	0.28 \pm 0.21	0.21 \pm 0.66	0.26 \pm 0.89	0.17 \pm 0.36	0.87 \pm 0.17	0.61 \pm 0.65	0.23 \pm 0.52
LYM	10 ³ / μ L	8.48 \pm 0.69	8.42 \pm 0.94	8.59 \pm 0.58	8.43 \pm 0.83	6.02 \pm 0.92*	11.78 \pm 0.38*	13.03 \pm 0.37*
MON	10 ³ / μ L	0.31 \pm 0.47	0.26 \pm 0.13	0.17 \pm 0.05	0.39 \pm 0.63	0.20 \pm 0.11	0.28 \pm 0.72	0.32 \pm 0.49
BAS	10 ³ / μ L	0.02 \pm 0.14	0.03 \pm 0.14	0.01 \pm 0.14	0.01 \pm 0.14	0.02 \pm 0.14	0.04 \pm 0.14	0.02 \pm 0.14
Red blood cells								
HCT	%	43.20 \pm 3.81	44.03 \pm 2.41	42.73 \pm 0.58	41.79 \pm 1.38	40.67 \pm 2.84	40.22 \pm 1.49*	38.76 \pm 0.92*
MCV	fL	58.54 \pm 3.10	58.71 \pm 3.62	60.18 \pm 3.74	58.37 \pm 1.93	61.44 \pm 3.02	66.59 \pm 2.83*	77.37 \pm 3.47*
RBC	10 ⁶ / μ L	7.38 \pm 0.41	7.50 \pm 0.22	7.10 \pm 0.23	7.16 \pm 0.41	6.62 \pm 0.23	6.04 \pm 0.47	5.01 \pm 0.73*
HGB	g/dL	13.2 \pm 0.28	13.60 \pm 1.20	12.20 \pm 1.15	11.99 \pm 0.98	11.93 \pm 0.36	9.35 \pm 0.83*	9.15 \pm 0.55*
MCH	pg	17.99 \pm 1.30	18.13 \pm 1.12	17.20 \pm 1.68	16.75 \pm 1.49	18.02 \pm 1.44	15.50 \pm 0.49*	18.30 \pm 1.15
MCHC	%	30.56 \pm 1.22	30.89 \pm 2.12	28.55 \pm 1.76	28.70 \pm 1.51	29.33 \pm 2.72	23.24 \pm 4.26*	23.61 \pm 3.38*
PLT	10 ³ / μ L	0.448 \pm 0.08	0.412 \pm 0.03	0.595 \pm 0.12	0.501 \pm 0.03	0.692 \pm 0.33	0.268 \pm 0.42*	0.237 \pm 0.21*

Table 3 Hematological parameters of female mice treated with various doses of multi-metal mixtures

Parameters	Unit	Control	MPL	1×	5×	10×	50×	100×
White blood cells								
WBC	10 ³ /μL	4.7 ± 1.22	4.44 ± 1.12	5.37 ± 1.03	6.43 ± 1.21	6.06 ± 1.26	8.58 ± 1.71	10.22 ± 1.12*
NEU	10 ³ /μL	3.23 ± 0.23	2.01 ± 0.23	2.45 ± 0.36	2.11 ± 0.08	2.00 ± 0.31	1.95 ± 0.58	1.24 ± 0.22
EOS	10 ³ /μL	0.13 ± 0.11	0.11 ± 0.56	0.09 ± 0.83	0.12 ± 0.26	0.47 ± 0.11	0.21 ± 0.15	0.12 ± 0.27
LYM	10 ³ /μL	6.95 ± 0.34	4.77 ± 0.24	5.78 ± 0.47	5.43 ± 0.52	6.12 ± 0.22*	9.17 ± 0.35*	14.03 ± 0.51*
MON	10 ³ /μL	0.24 ± 0.27	0.16 ± 0.18	0.38 ± 0.01	0.47 ± 0.27	0.35 ± 0.10	0.18 ± 0.36	0.32 ± 0.46
BAS	10 ³ /μL	0.00 ± 0.14	0.01 ± 0.26	0.00 ± 0.13	0.02 ± 0.08	0.02 ± 0.11	0.04 ± 0.16	0.02 ± 0.28
Red blood cells								
HCT	%	46.28 ± 2.25	48.77 ± 1.36	47.09 ± 0.58	47.81 ± 1.72	44.67 ± 2.01	41.62 ± 1.17*	41.21 ± 0.62
MCV	fL	57.78 ± 2.82	64.00 ± 3.36	60.84 ± 3.94	66.31 ± 1.77	71.82 ± 3.07	72.51 ± 2.68	73.85 ± 2.03
RBC	10 ⁶ /μL	8.01 ± 0.21	7.62 ± 0.22	7.74 ± 0.23	7.21 ± 0.41	6.22 ± 0.23	5.74 ± 0.47	5.58 ± 0.73*
HGB	g/dL	14.42 ± 0.26	14.03 ± 1.27	15.01 ± 1.09	13.21 ± 0.71	13.13 ± 0.39	11.92 ± 0.79*	10.21 ± 0.91*
MCH	pg	18.00 ± 1.22	18.41 ± 1.72	19.40 ± 1.28	18.32 ± 1.55	21.11 ± 1.83	20.87 ± 0.96	22.30 ± 0.91*
MCHC	%	31.16 ± 1.05	28.77 ± 2.02	31.88 ± 1.92	27.63 ± 2.71*	29.40 ± 2.03	28.64 ± 2.78*	24.78 ± 2.06*
PLT	10 ³ /μL	0.768 ± 0.05	0.712 ± 0.06	0.711 ± 0.58	0.642 ± 0.07	0.894 ± 0.58	0.402 ± 0.22*	0.396 ± 0.09*

There is a significant difference between the exposure groups and control shown. Results shown as mean ± SEM

WBC white blood cells, EOS eosinophil, BAS basophils, MON monocytes, NEU neutrophil, LYM lymphocyte, RBC red blood cells, HCT hematocrit, MCV mean corpuscular volume, HGB hemoglobin, MCH mean corpuscular hemoglobin, MCHC mean corpuscular hemoglobin concentration, PLT platelet

* $P \leq 0.05$

obtained from 100× male and female was higher by 2.7- and 2.8-fold, respectively. Similarly, the accumulation of Pb in 100× group increased significantly ($p < 0.05$) being about 2.8- and 2.3-fold higher than control in male and female group, respectively. Higher accumulation of Hg was seen in the 10× (tenfold), 50× (12.1-fold), and 100× (15.3-fold) male groups with respect to the control group. In the female mice, Hg content in the blood increased by 16.2-fold in the 100× dosed group after 8 weeks (Fig. 1c). More noticeable dose-dependent increase in the As content was observed, in the male group exposed to 50× and 100× metal solution being about 24.2- and 25.8-fold increase with respect to the control. The Fe content in male mice blood showed no significant change with an increasing multi-metal mixture dose. Iron content slightly increased at 100× high concentration (2.4% male; 3.6% female) after 8 weeks. Blood Mn level increased significantly (15.6%) in the 100× male group during 8-week study as compared to the control group. In females, a non-significant 3–3.2% increase was noted in 50× and 100× group. One significant finding was that the Cr content in the 5× (1.1-fold male; 1.02-fold female) and 10× (0.92-fold male; 0.65-fold female) groups rapidly increased relative to the control mice. Interestingly, in the other two groups exposed to higher concentration of metal mix (50× and 100×), no obvious differences in the levels of Cr were seen relative to the control group. In addition, elevated Ni serum levels were also evident in animals exposed to high dose of metal mixture (Fig. 1h). The overall

results suggest that the metal load in the blood of exposed mice shows a dose-dependent response to multi-metal exposure ($p < 0.05$).

Effect of multi-metal mixtures on the level of oxidative stress

As depicted in Table 4, the level of MDA was significantly ($p < 0.05$) enhanced in the serum of metal-exposed mice. When multi-metal mixture treatment was given for 8 weeks, in male mice, the MDA level was elevated by 1.20- and 1.61-fold in 50× and 100× metal-exposed groups as compared to control. With the increase of multi-metal concentration in the blood, the activity of CAT and GSH was significantly ($p < 0.05$) reduced in the serum of animals treated with high of dose metal mixture (50× and 100×). Serum SOD activity significantly decreased in the 10×, 50×, and 100× group in both the sexes in comparison with the respective control group.

Effect of multi-metal mixtures on the liver, kidney, and lipid biomarkers

Serum ALT, AST, and ALP significantly increased ($p < 0.05$) in multi-metal exposed high group (50× and 100×) indicating hepatocellular injuries. Animals exposed to 100× concentration of multi-metal mixture responded with very high activity of ALT in comparison to control.

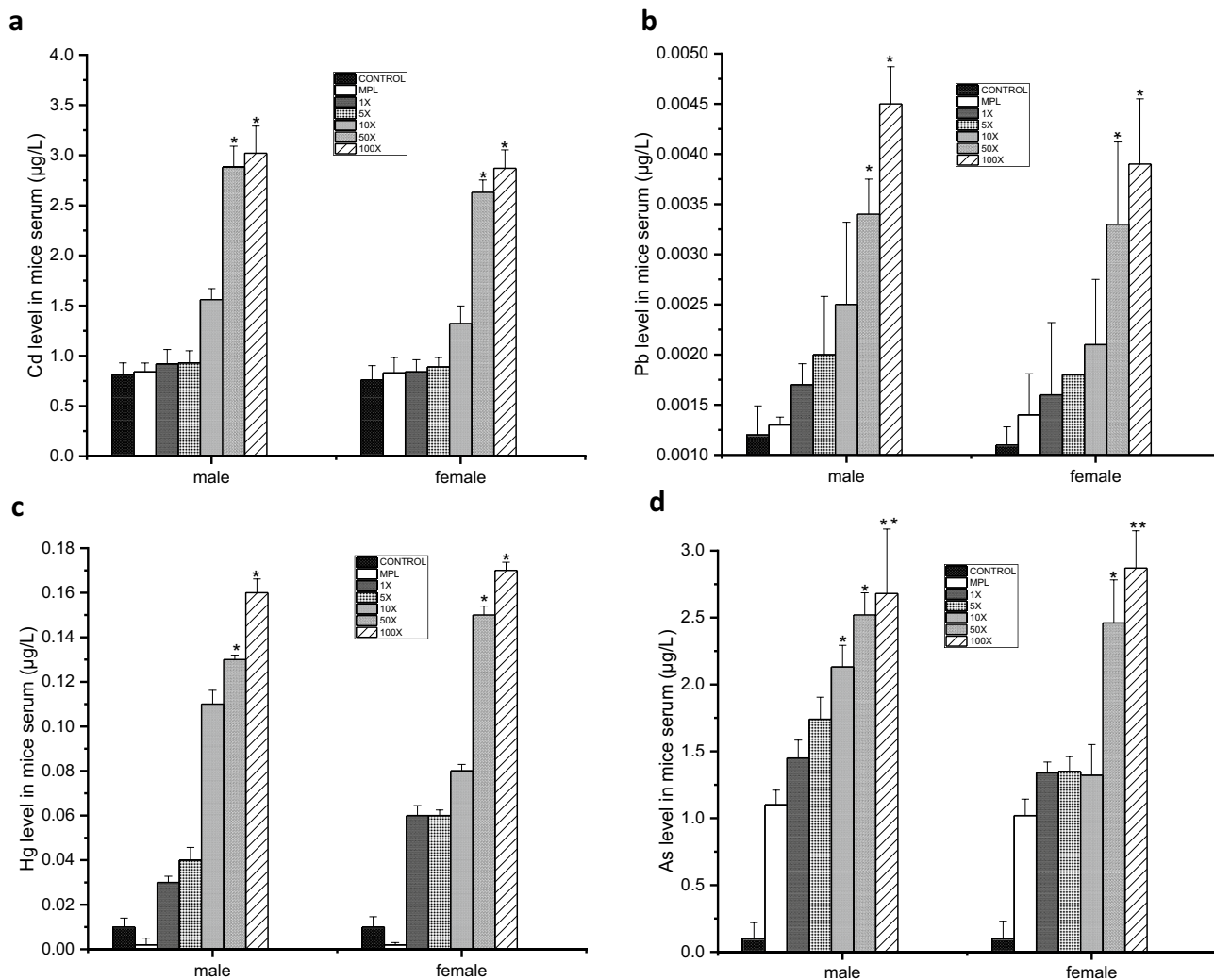


Fig. 1 Effects of various dose concentration of multi-metal mixtures on the change of **a** Cd, **b** Pb, **c** Hg, **d** As, **e** Mn, **f** Fe, **g** Cr, and **h** Ni levels in blood collected from male and female mice. The values are

presented as means \pm standard error mean (8 mice/sex/group). Compared with control group * $p < 0.05$; ** $p < 0.01$

Figure 2d, e show significant decrease in TP and albumin in 50 \times and 100 \times metal exposure groups, and non-significant change was evident in the low dosed groups. During the study, BUN levels were significantly increased ($p < 0.05$) in 50 \times (45.11% male; 58.54% female) and 100 \times (55.61% male; 65.30% female) after 8-week exposure (Fig. 3a). There was an estimated increase from 11 to 61% (lower dosed groups) to 88 to 121% (higher dosed groups) for CR levels in male mice serum, while in females an increase from 10 to 18% (lower dosed groups) to 43 to 65% (higher dosed groups). However, uric acid did not show any significant change with respect to control in any of the metal dosed groups (Fig. 3c). The overall results show that multi-metal mixtures were responsible for inducing liver-renal injury.

No significant difference in HDL was reported between any of the metal mix exposed groups (Fig. 4d). The serum

GLU levels increased after exposure to multi-metal mix in 10 \times (21.04% male; 22.83% female), 50 \times (25.89% male; 31.89% female), and 100 \times (33.3% male; 33.76% female) compared to the corresponding levels in their control. Significant increases ($p < 0.05$) in LDL level were recorded in 50 \times and 100 \times groups exposed to multi-metal mixtures compared to control groups (Fig. 4a). The concentration of TC in the mice serum was significantly higher at 50 \times (13.5% male; 10.53% female) and 100 \times (23.21% male; 23.89% female) dose.

Discussion

Human beings are generally exposed to multiple heavy metals at the same time either through contaminated water, air, food, inhalation, dermal contact, or fumes from industrial

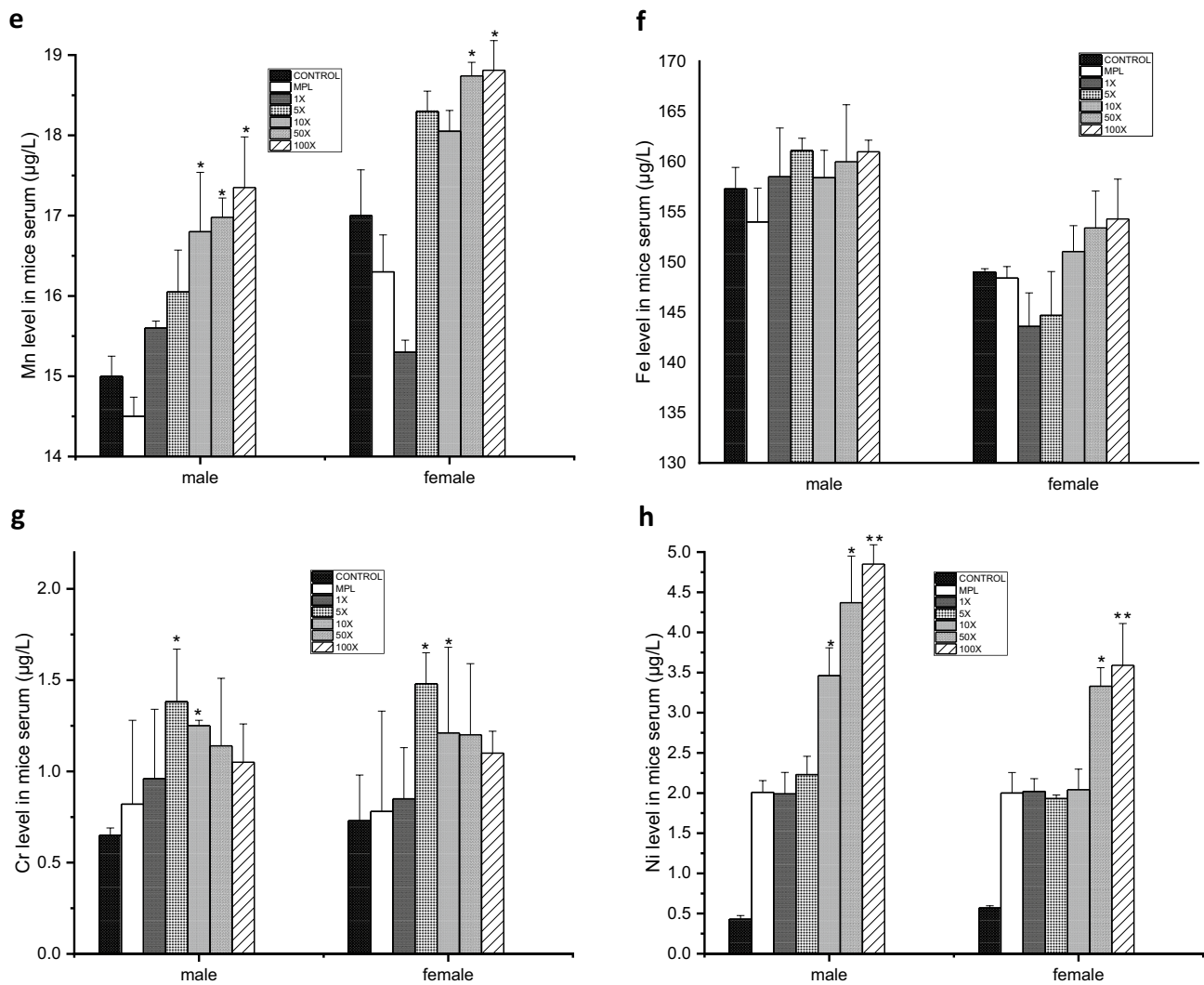


Fig. 1 (continued)

areas (Singh et al. 2017). Individuals residing in the exposed area may have high levels of heavy metal in their blood (Ni et al. 2014). Metals once ingested can be transported and absorbed by intestinal cells and are then released into the blood, where most of the metal is detected in red blood cells (Yu et al. 2020). Furthermore, some of the metals move from the blood to other tissues, such as the brain, bone, and liver. Thus, exposure to multiple heavy metals is a threat to the animal and human health with gradual accumulation in various tissues.

Various health implications like gastrointestinal disorder, hematological disorders, and hepatic and renal damage have been reported due to heavy metal exposure (Balali-Mood et al. 2021). Considering the wide distribution of health hazard caused by multiple heavy metals in the environment, assessment of multi-metal effect has more practical significance in comparison to single metal exposure. The

combined effect of co-exposure is also dependent on the competitive interaction which occurs among these essential and non-essential metallic elements. In this study following 8-week exposure to multi-metal mixture, no obvious physiological and clinical defects were observed in the low dosed groups; however, some animals in high-dose exposure groups responded to metal toxicity by showing changes in hair loss, scabbing, vocalization, reduced body weight, reduced feed efficiency, and water consumption. In response to high dose of metal mixture, significant amount of Cd, As, Hg, Pb, and Mn accumulation was detected in the mice blood. High metal concentration in blood indicated the effect of metal mixture was significantly more apparent in the high-dose group in comparison to the control. Similar observations were presented in a study, where mice were exposed to 20 and 100 mg ml⁻¹ Cd in drinking water (Breton et al. 2013). A study by Wang et al. (2020) reported the effect of chronic

Table 4 Antioxidant status of mice erythrocytes for 8 weeks

Group	LPO	GSH	CAT	SOD
Control male	4.68 ± 0.25	0.380 ± 0.014	138.00 ± 1.15	6.01 ± 0.17
MPL	4.71 ± 0.28	0.391 ± 0.023	138.23 ± 1.38	6.21 ± 0.37
1 × m	5.01 ± 0.18	0.380 ± 0.022	137.53 ± 1.03	5.28 ± 0.34
5 × m	5.19 ± 0.46	0.373 ± 0.067	137.08 ± 1.22	5.14 ± 0.29
10 × m	6.29 ± 0.31	0.358 ± 0.025	131.24 ± 4.04	3.78 ± 0.44*
50 × m	10.24 ± 0.52*	0.289 ± 0.034*	110.33 ± 3.24*	3.00 ± 0.15*
100 × m	12.24 ± 0.32*	0.268 ± 0.037*	105.90 ± 4.17*	3.05 ± 0.23*
Control female	4.59 ± 0.33	0.360 ± 0.026	136.50 ± 1.35	5.96 ± 0.29
MPL	4.73 ± 0.68	0.363 ± 0.073	138.00 ± 1.95	5.99 ± 0.49
1 × f	5.47 ± 0.43	0.357 ± 0.016	136.11 ± 1.57	5.90 ± 0.52
5 × f	5.69 ± 0.38	0.354 ± 0.066	132.01 ± 1.62	5.43 ± 0.31
10 × f	5.77 ± 0.52	0.320 ± 0.041	130.00 ± 1.03	4.02 ± 0.45*
50 × f	9.64 ± 0.61*	0.270 ± 0.052*	115.82 ± 2.03*	3.41 ± 0.52*
100 × f	11.52 ± 0.58*	0.254 ± 0.048*	111.02 ± 2.12*	3.11 ± 0.71*

Lipid peroxidation (LPO): nmol malondialdehyde/ml packed erythrocytes; reduced glutathione (GSH) mmol/ml packed erythrocytes; catalase (CAT) mmol H₂O₂ utilized/min/mg hemoglobin; superoxide dismutase (SOD): 1 unit is µg hemoglobin required to inhibit 3-(4–5 dimethyl thiazol 2-xl2, 5-diphenyl tetrazolium bromide reduction by 50%. Values represent mean ± SEM (*n* = 8)

* *p* < 0.05 compared to control

multi-metals. They observed differential accumulation of Hg, Cu, Cd, Zn, and Cr in the serum, liver, spleen, lungs, kidneys, and other organs. Hematocrit indicators are considered early and sensitive markers in checking adverse effects of heavy metals on rodents. Our results showed decreasing trend for HCT value along with increase in the metal concentration. We hypothesized that the elevated metallic content in blood in the 50 × and 100 × metal exposure group is due to high concentration of metal in the dosing solution. As described by Tete et al. (2015), the concentration of Pb and Cd significantly increased in blood along the pollution gradient, and along with this an increase in HCT value. In our study, exposure to the multi-metal mixture (50 × and 100 ×) resulted in a significant decrease of HGB, RBC, and MCHC and significant increase of MCV in serum pointing towards anemia. Elevated MCV and decreased MCHC could be the result of macrocytic anemia condition in mice exposed to high concentration of metal mixture (FiatiKenston et al. 2018). Dobrakowski et al. (2016) in their short-term study reported that occupational exposure to Pb in the laborers with a history of blast furnaces work resulted in decreased level of HGB and MCHC. Lymphocytes and neutrophils are WBC markers that reveal inflammation status (Kolaczowska and Kubes 2013). During the 8-week study, significant increase in WBC count (Table 2) in metal-exposed group may indicate a response of the immune system to combat infections (Lee et al. 2010). In the 50 × and 100 × male and female groups, the number of lymphocytes increased but the neutrophils decreased compared to the control group, but the completely opposite trend was seen in the 10 × male group. A high lymphocyte count along with a low neutrophil count

might be caused by high metal concentration in the blood resulting in inflammatory injury and compromised immune response. Regarding monocytes, eosinophils, and basophils, any significant change was not recorded; the same was also evident in a study by El-Boshy et al. (2015). Mice in the high-dose group (50 × and 100 ×) have a significantly high amount of Hg, As, and Mn load (Fig. 2a–h). These elements in the blood of high-exposure groups were tenfold higher than in control, whereas other metals (Cd, Pb, Ni, Cr) were only less than threefold higher than control (Fig. 2a–h). Results revealed that the burden of these metals in blood could significantly increase the lymphocyte count and decrease the neutrophils in mice sera. Mn-SOD also acts as the primary antioxidant that scavenges superoxide formed within the mitochondria and protects against oxidative stress and any damage in erythrocyte membrane (Li and Yang 2018). Significant increase in the Mn content in blood might suppress the activity of hematopoietic tissues leading to increased number of lymphocytes in blood. Heavy metal mixture-induced anemia may be due to the accumulation of non-essential toxic metal in the kidney, spleen, and liver. This metal overloading of tissue might suppress the activity of these hematopoietic tissues (Han et al. 2019; Tian et al. 2021). Moreover, the accumulation of metals in blood and tissues led to the formation of mucosal lesions which results in failure of intestinal uptake of Fe bringing obvious damage to erythrocyte and its membrane permeability (Gill and Epple 1993). This can be correlated with our result, where no significant change in Fe concentration in serum was noticed even with increase in dose concentration. There could be some relation between the failure of Fe uptake in the cells

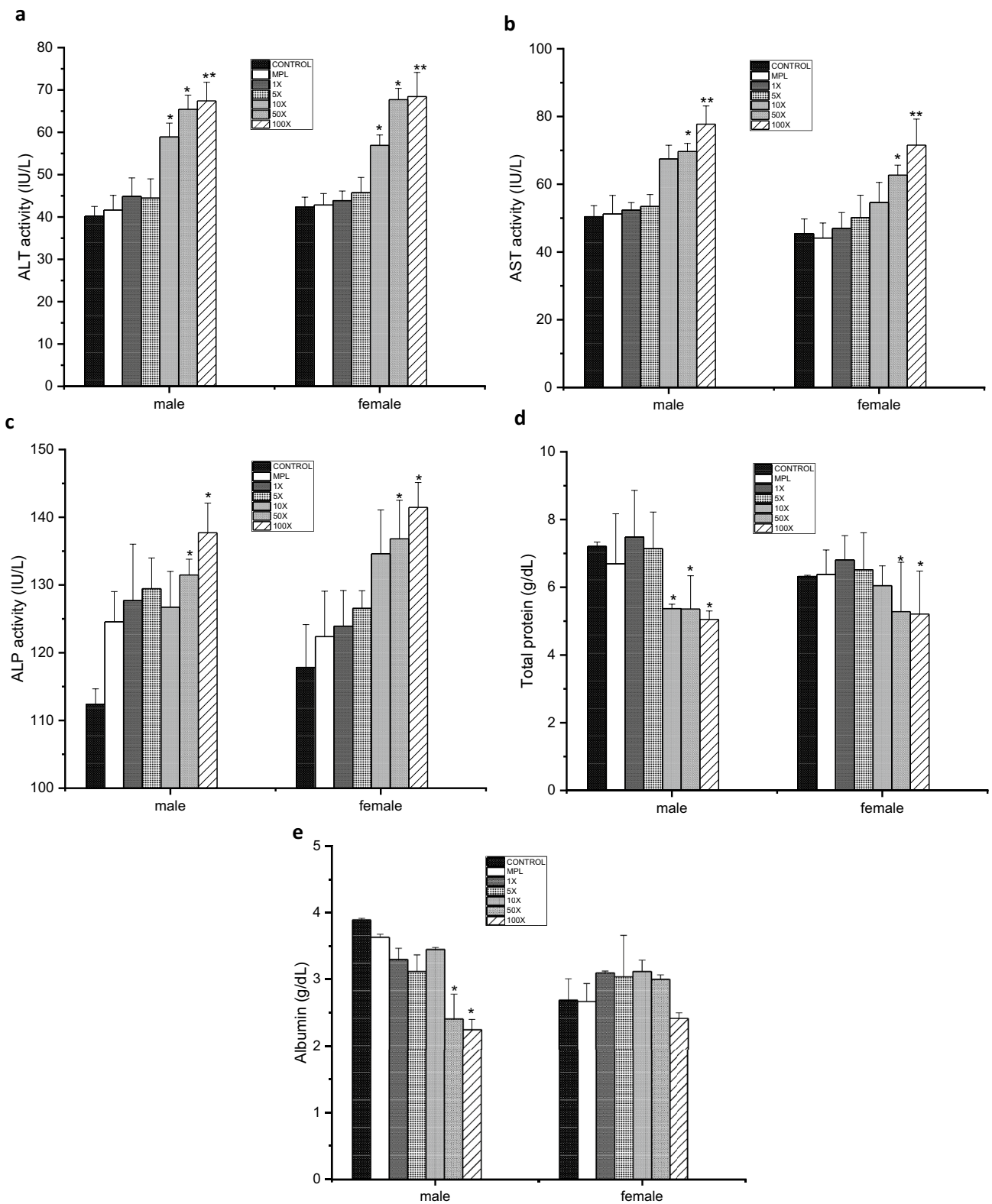


Fig. 2 Effects of various dose concentration of multi-metal mixtures on hepatic serum parameters **a** ALT, **b** AST, **c** ALP, **d** TP, and **e** albumin level in male and female mice. The values are presented

as means ± standard error mean (8 mice/sex/group). Compared with control group **p* < 0.05; ***p* < 0.01

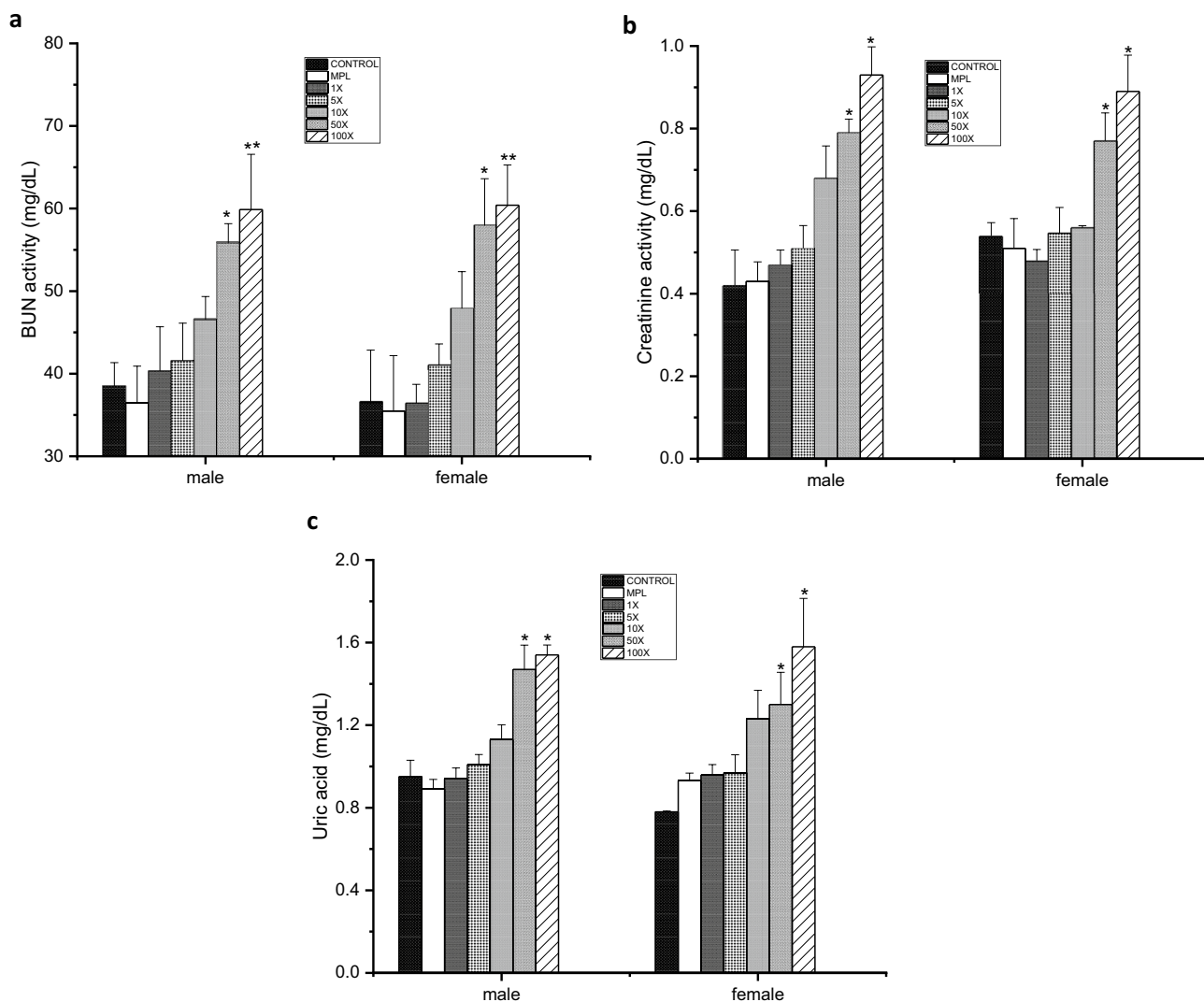


Fig. 3 Effects of various dose concentration of multi-metal mixtures on the renal serum parameters **a** BUN, **b** CR and **c** uric acid level in male and female mice. The values are presented as means \pm stand-

ard error mean (8 mice/sex/group). Compared with control group * $p < 0.05$ and ** $p < 0.01$

and serum Fe content. It was even verified by Lacerda et al. (2019) that there is a dose-dependent relationship between Cr levels in blood and enzymatic activity, suggesting that high Cr levels in blood will degrade the enzyme activity. In contrast to this, we found higher concentration of Cr in low-dose group. To a certain degree, in addition to anemia and other hematological disturbances, these results can affect the hematopoietic system.

In this study, the levels of AST and ALT were increased in the sera of high dose-exposed mice (50 \times and 100 \times). The elevated ALT and AST biomarkers are a consequence of hepatocyte membrane damage as reported by Honda et al. (2010). Any damage to liver cells results in elevations of ALT, AST, and ALP parameters in the blood (Tian et al. 2021). The detection of CR and BUN level in sera is one

of the methods to evaluate kidney dysfunction. Heavy metal accumulation in the kidney may damage nephrons and renal parenchyma and further reduce glomerular filtration rate resulting in high BUN and CR levels (Pallio et al. 2019). Curiously, serum biochemical indices in our results showed that BUN and CR increased significantly in the high dose-exposed mice whereas in low dose, increase was not significant (Fig. 3a, b). These results are consistent with the findings of Yuan et al. (2014) where a combined exposure of Pb and Cd significantly change the biochemical parameters in the blood of Sprague Dawley rats with dose response relationship. Liver is the main site for plasma protein synthesis; any changes in liver function markers, serum TP, and albumin might be associated with liver dysfunction (Chen et al. 2019). Administration of multi-metal mixture exerted

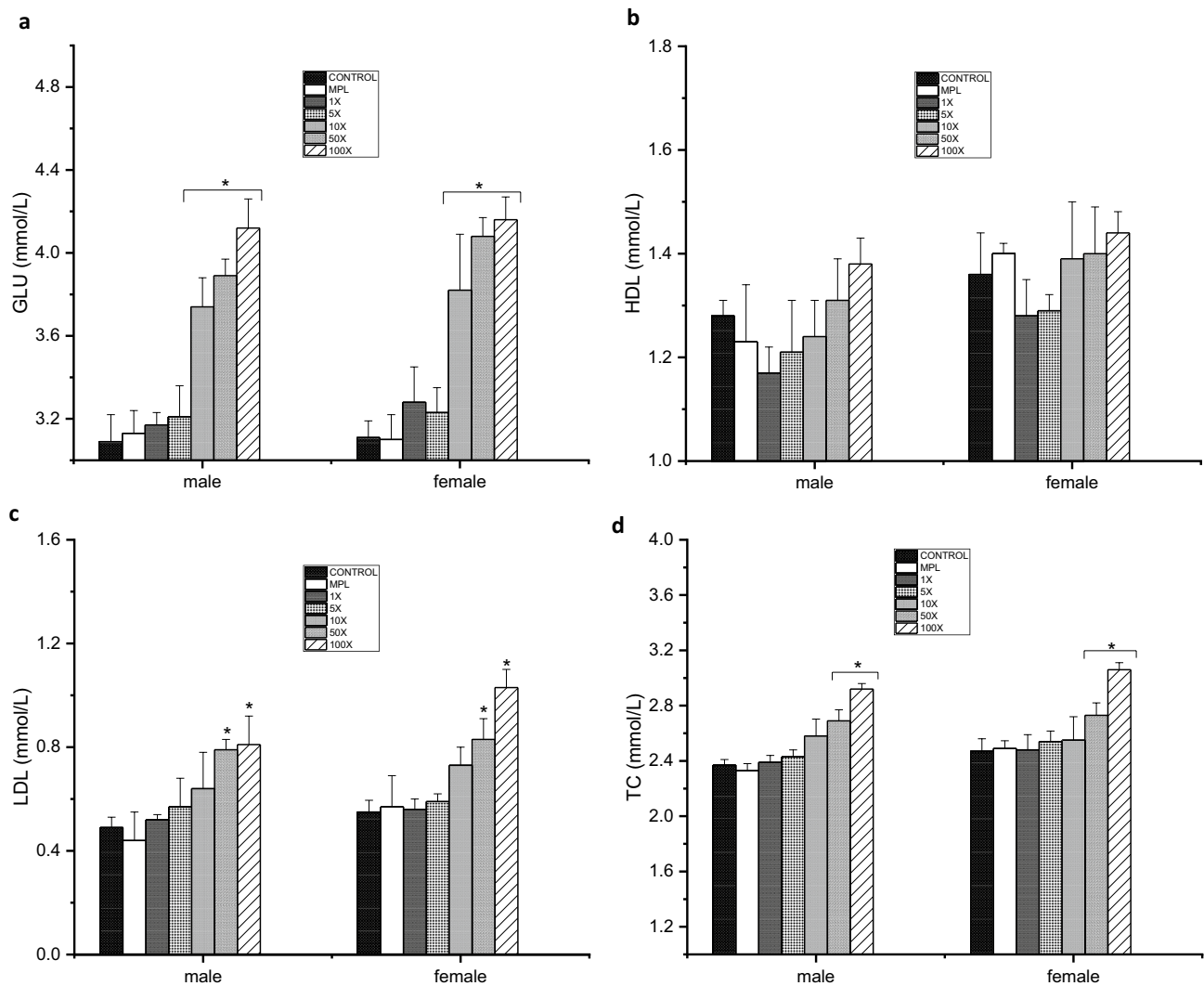


Fig. 4 Effects of various dose concentrations of multi-metal mixtures on **a** GLU level, and lipid metabolism **b** HDL, **c** LDL and **d** TC level in male and female mice. The values are presented as means \pm stand-

ard error mean (8 mice/sex/group). Compared with control group * $p < 0.05$; ** $p < 0.01$

hepatic injury as verified by the significant decline of TP and albumin in 50 \times and 100 \times group. The changes in liver markers due to metal exposure have been well documented in many studies (Renugadevi and Prabu 2010; Cobbina et al. 2015). The altered hepatorenal markers in the serum with increasing metal concentration in the blood suggest that the exposure to multi-metal mixtures in mice may induce liver and kidney toxicity.

Generally, heavy metal co-exposure to mice is involved in toxicity which is constantly producing imbalance in ROS levels subsequently leading to oxidative stress. Multi-metal mixture-induced ROS production was associated with significant alterations in LPO and antioxidants like SOD, GSH, and CAT (Table 4). The elevated oxidative stress levels in 50 \times and 100 \times group of both sexes observed in this study could be attributed to the increasing blood metal (Hg, Cd,

Pb, As, Ni, Cr, Mn) load. Xu et al. (2020) demonstrated how exposure to heavy metal mixtures has a dose response relationship with oxidative stress markers. Residents living near metal exposed area had elevated Cr and Pb concentrations in their blood and altered oxidative indices relative to non-exposed residents. Curiously, serum GLU, LDL, TC level increased significantly after 8-week exposure to high dose of multi-metal solution (Fig. 4a). Generally, elevated levels of GLU are tightly linked with liver-kidney function, which may lead to disruption in the lipid metabolism (Zhang et al. 2014). Tian et al. (2021) reported that administration of metal via drinking water for 8 weeks could cause the hyperlipidemia in normal mice with elevated level of serum TC and LDL. Elevated Cd, Pb, Hg, As, and Mn can induce oxidative stress and raise the level of liver-kidney markers in blood, which could be associated to liver-kidney

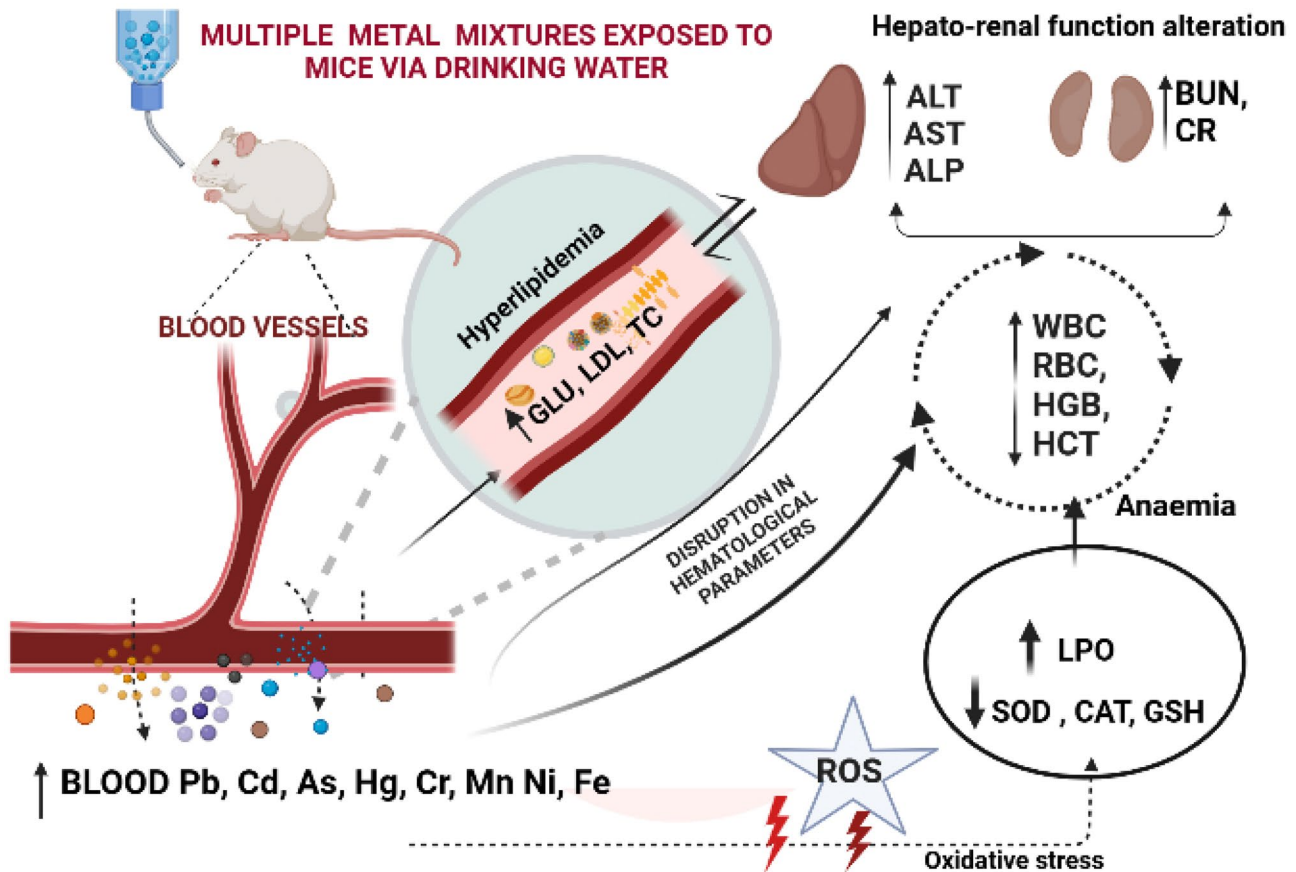


Fig. 5 Schematic abstract presenting the effect of multi-metal mixtures in mice blood

damage (Gonzalez Rendon et al. 2018). Additionally, Mn and Fe are considered essential metals important for occupational health, as these elements are required for normal physiological function. In this context, our results showed concentration of essential metal Fe was almost the same between exposed and non-exposed mice; however, Mn concentration was significantly high in experimental animals. The elevated levels of Mn in the animals subjected to high concentration of metal mix may be due to the compensatory effort of the enzymes to cope with excessive metal insults. Our body lacks a possible approach to eliminate detrimental heavy metals like Cd, As, Hg, or Pb unlike essential trace metals like Mn and Fe, where an effective biological mechanism is present to excrete excessive trace elements. As we failed to find any elevated blood Fe level in the metal mixture–exposed group, this relationship may be associated to the fact that Fe plays an important role in the antioxidant system. However, the effects and mechanism of multi-metal on various essential elements remain controversial. The above data suggest that the quantification of metals in blood plays an important role in unraveling the mechanism associated with hematological disorders, especially in higher dose multi-metal groups.

Conclusion

In conclusion, multiple metal administrations in mice resulted in variable load of each metal in the blood of animals exposed to different doses of multi-metal mix (Fig. 5). Dose–effect relationships exist between multi-metal exposure and blood biomarkers in mice. The concentration of some metals (Cd, Pb, Hg, As, Mn, Ni) showed significant changes in mice blood after exposure. Exposure to multi-metal mixture resulted in the impairment in hematological function and promoted the ROS generation with alterations in the oxidative indices, hepatorenal biomarkers, and lipid metabolism. The outcome of this research adds a sense of urgency to examine the potential public health risks associated with the consumption of drinking water contaminated with multiple metals.

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Author contribution Study design: Sangeeta Choudhary; conceptualization: Sangeeta Choudhary and Priyanka Bist; methodology and data collection: Priyanka Bist and Damini Singh; data interpretation:

Sangeeta Choudhary and Priyanka Bist; manuscript preparation: Sangeeta Choudhary, Priyanka Bist, Damini Singh; review and editing: Sangeeta Choudhary, Priyanka Bist.

Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Compliance with ethical standards

Funding This study was not supported by any funding.

Conflict of interest The authors declare no competing interests.

Ethical approval The study design, transportation, and care of the animals were approved and performed in compliance to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), government of India. Study procedure is approved by Institutional Animal Ethics Committee (IAEC), Banasthali Vidyapeeth, protocol no: BV/IAEC/2018/2.

Informed consent The authors declare that any information or data in regard to human subjects was not used for this study.

Consent for publication No use of human subjects for this study.

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