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Research Paper:

Impact of Multi-metal Mixture Administration Through Drinking Water on the Gut Bacterial Microflora and Oxidative Stress Parameters in Male and Female Mice

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ABSTRACT

Background: Heavy metal containing wastes reaches to the food chain either directly or indirectly. These ingested toxic elements manifest direct impact on the gut ecosystem and its overall functioning. The present study explores the alteration in mice gut bacteria on exposure to mixture of toxic heavy metals through drinking water.

Methods: Twelve experimental groups of Swiss albino male and female mice were exposed to the metal mixture of varying concentrations. Profiling of gut bacterial flora was done by periodical collection of fecal samples via culture-based technique. Redox status of all experimental animals was analyzed in blood samples collected on the day 30.

Results: In comparison to the controls, nearly a 10-fold decline in colony forming units/ml was observed at higher modal concentrations (50× & 100×) at the end of 15 days, but 100-fold reduced bacterial count was recorded following 30 days of dosing. Sex specific significant alteration in the bacteria count and diversity was also observed. Overall experimental results showed a heavy metal dose-dependent decline in bacterial count and loss in diversity. Disturbance in the oxidative stress markers was recorded in response to high dose of metal mixture. In group receiving 100× dose, malondialdehyde levels were increased in the erythrocytes ($P<0.05$), and all of the other antioxidant parameters were decreased ($P<0.05$), except for reduced glutathione in both male and female mice.

Conclusion: The present work is the first report on the multiple heavy metals induced gut microbiota alterations and its correlation to oxidative stress.

Keywords: Gut bacteria, Heavy metals, Intestinal microbiota, Oxidative stress, Reactive oxygen species

Introduction

Continuous release of heavy metals in the environment occurs due to various geochemical and anthropogenic activities. Intake of heavy metal ions by the living organisms via food chain is an important health concern. Based on various reports on the quality of water in different regions of India, it has been suggested that there is prevalent heavy metal contamination in groundwater. Fair amount of drinking water requirement is fulfilled from groundwater sources [1], thus routine exposure to toxic metals through polluted water appears to be a basic part of current life. The accumulation of heavy metal ions in animal system can cause varied degree of adverse effects on vital organs

and systems [2]. These ingested toxic elements remain in high amounts in the gut microenvironment as non-absorbed form and has direct impact on the gut environment and its overall functioning [3].

Microbes present in the Gastrointestinal (GI) tract, better known as the gut microbiota is considered to be a hidden organ having critical control over intestinal homeostasis [4]. Beneficial bacteria in the gut have valuable role in the fermentation of various compounds, immune system regulation, and also provide protection against pathogenic bacteria [5]. It is apparent that bacteria indigenous to the host gut, control the metabolism of contaminants and affect the toxicity and mobility. On the other hand, the contaminants do affect and modify the structural and functional diversity of the gut microflora. Gut bacteria and their metabolites impact numerous host factors, such as pH, oxidative balance, and detoxifying enzymatic systems [6]. Several types of environmental contaminants, nutritional intake, antibiotics and other drugs reaching the intestine have specific modes of interaction with the existing microbes. Thus, the complex interaction of host-gut microflora controls the mobility, toxicity and bioavailability of environmental pollutant, such as heavy metals inside the gut [7, 8].

As the inhabiting microbes experience the exposure to these toxicants prior to the host, their response to the exposure has great influence on the host reaction [9]. The intestinal flora contains 7.0×10^3 to 4.0×10^4 diverse bacterial strains belonging to 1800 genera [10]. Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria are the most prevalent bacterial phyla in the mammalian gut [11]. These dominant bacterial groups cohabit in the gut system during their entire life span. The influence of gender on the gut microbial diversity has been studied in rodent and human models [12]. Gender based differences in gut microbiome composition do lead to a sex specific variation in the immune system. Heavy metals cause cytotoxicity leading to Reactive Oxygen Species (ROS), viz., generation of superoxide and hydroxyl radicals [13]. Reactive oxygen species oxidize cellular thiol proteins, nucleic acids, and/or lipids [14]. Thus, metal-induced oxidative stress in cells could be an important cause for the toxic effects of heavy metals. Most of the reports on the alterations in structural and functional diversity of gut bacteria are on single metals or in bimetallic combinations as treatment dose [2, 8, 9].

Currently, there is a growing concern about the health hazards posed by heterogeneous metal mixtures mostly present in drinking water. Exposure of environmental chemicals to animals and human beings is not only limited to a single compound, thus it is essential to understand the hazard associated with several metals on simultaneous exposure. This study aimed to characterize the potential effect of mixture of environmental metals on the gut microbial ecology in mice model. Further, the redox status parameters were analyzed to understand the interaction between the heavy metals induced oxidative stress and microbiota functional changes. In order to replicate a situation in reality to which common mass is exposed via groundwater usage, a solution mixture of frequently found heavy metals (As, Cd, Pb, Hg, Cr, Mn, Fe, and Ni) was prepared for this study. Male and female Swiss albino mice were subjected to five different oral doses of multi metal mix solution for a period of 30 days. The information obtained from this study could provide an improved understanding of fundamental mechanisms behind gut microbiome and heavy metals interaction, and the significance to the host health and disease.

Materials and Methods

Animals: Male and female Swiss albino mice, aged six weeks, weighing 15-20g, were used in this study. Animals were housed in controlled environment (22°C, 12 h light/dark cycle) and provided with standard pellet diet *ad libitum* and water.

Experimental design: The treatment doses were given continuously for 30 days by spiking the mice drinking water with multi-metals solution. Stock solution of respective metal salts were prepared in deionized water and stored for further use. The concentrations of each metal in dosing solution mixture in graded concentration (1X, 5X, 10×, 50× or 100×) where 1X solution contains, Sodium arsenite (0.380 ppm); Cadmium chloride (0.098 ppm); Lead nitrate (0.220 ppm); Mercuric chloride (0.060 ppm); Chromium trioxide (0.346 ppm); Nickel chloride (0.810 ppm); Manganese chloride (2.026 ppm); and Ferric chloride (2.033 ppm) according to Jadhav, et al. [15] with required modifications. Metal salts were weighed in appropriate amount to prepare a stock of 100× solution of the mode concentration. Ten-fold serial dilution of 100× solution was done to prepare the lower doses (1X, 5X,10× or 50×) of the dosing solution. Periodical collection (15 and 30 days) of fecal samples was done to study the gut microflora. Blood samples were collected from retro-orbital plexus with the help of capillary tubes after 30 days. The amount of water consumption in each group of mice (n=8) was recorded daily and expressed as the average water consumption rate (ml/mouse/day). The body weights of mice were monitored weekly in the experimental group.

Administration of heavy metals mixture: Mice were divided into 12 groups at random consisting of 8 animals in each group. Groups 2 to 6 (male experimental) and groups 8 to 12 (female experimental) received 1X, 5X, 10×, 50× or 100× of heavy metals mixture in drinking water, respectively. Group 1 (male control) and group 7 (female control) were given deionized distilled water. The mode concentration was selected on the basis of previous reports and prevalence of respective metals as ground water contaminants above World Health Organization (WHO) Maximum Permissible Limit (MPL) [15].

Isolation and identification of mice fecal bacteria: The gut bacteria were studied using fecal pellets collected periodically and kept in test tubes. Approximately, 0.1g of fecal pellets were homogenized by vortexing for 30 sec. at maximal speed in 0.9 ml of phosphate buffer saline [16]. Enumeration of aerobic bacteria was done on blood agar, MacConkey agar and nutrient agar by serial dilution technique. For culturing anaerobes, fecal samples were spread on brain heart infusion agar plates, Tryptone-Peptone-Yeast Extract (TPY) agar with 2µg/L dicloxacillin. Anaerobic conditions were maintained, using Anaero gas Packs (Himedia). The plates were incubated at 37°C for 24hr, the morphologies of Colony Forming Units (CFU) were observed and their numbers counted. Biochemical characterization of the bacteria was done in accordance with the methodology in Bergey's Manual [2, 17].

Determination of oxidative stress parameters in erythrocytes: The levels of Lipid Peroxidation (LPO) were assessed by measuring malondialdehyde levels via the method of Shafiq-Ur-Rehman [18]. One ml of sample was combined with equal volume of trichloroacetic acid. This mixture was centrifuged at 2000 rpm for 10 minutes, and the supernatant was collected and heated in a water bath for 10 minutes, 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 [19]. Hemolysate of 200 µl was mixed with 4 ml H_2SO_4, and incubated for 10 min. Following incubation, 500 µl of tungstate solution was added and centrifuged again for 15 minutes at 2000 rpm. The 2ml supernatant was combined with 2.5 ml Tris buffer and 0.2 ml 5,5-dithiobis-2nitrobenzoic acid, and the absorbance was read at 412 nm.

The activity of Superoxide Dismutase (SOD) was assessed by Madesh and Balasubramanian method [20]. The reaction mixture included 650 µl PBS, 30 µl MTT (1.25 mM), 75 µl pyrogallol (100 mM), and 0.01ml 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 [21]. A 2 ml phosphate buffer aliquot at pH 7.0 and 0.01 ml hemolysate were added to a cuvette. After adding 1ml H_2O_2 (10 mM), the absorbance was read at 240 nm every 10 seconds for 1 minute.

Statistical analyses: All of the data were expressed as the mean standard error for each group. Differences between each group were analysed by one-way Analysis of Variance (ANOVA) test followed by Tukey's post hoc test. The $P<0.05$ was considered as statistically significant.

Results

Effect of heavy metal mixture on the gut bacterial profile: The toxic effect of the heavy metals' mixtures on bacterial population of mice GI tract was studied with pooled fecal samples collected periodically over 15 and 30 days of treatment, respectively.

In the control mice, nearly a constant number of total aerobes was observed ($1.15\pm0.147\times10^7$) throughout the period of 30 days. In general, a dose-dependent decline in the aerobe counts was observed during the exposure time in all of the experimental groups. At lower doses (1X and 5X), a slight decline in bacterial counts was observed compared to that of the control group ($P=0.0007$ & $P=0.0009$).

In comparison to the control group, a nearly 10-fold decline was seen in CFU /ml at higher modal concentrations (50× and 100×) at the end of 15 days. However, further decline to 100-fold was observed at the end of 30 days. In female groups, a10-fold higher bacterial count was recorded as compared to the male mice at 100× dose at the end of 30 days. The genus level identification of aerobes and anaerobes was performed in appropriate agar-based medium, revealing interesting results (Figures 1A and 1B).

Bacteria of diverse genus ranged from ~ 10^5 to 10^6 CFU/ml of sample in control mice. Among the seven genera identified, Proteus spp. was found to be the most sensitive, a sharp reduction in viable count by two orders of magnitude was observed in both sexes at 10× concentration. This genus could not be detected at higher doses following 15 days of exposure. Enterococcus was found to be quite resilient up to 15-day treatment with either slight increase or decrease in cell counts ($P>0.05$) with no changes observed in order of magnitude up to 50× dose.

Table 1. Lipid peroxidation and antioxidative systems in erythrocytes of male and female mice exposed to heavy metal mixture (n=8)

Groups	Mean±SEM			
	LPO	GSH	CAT	SOD
Control male	4.77±0.13	0.400±0.004	141.00±1.20	6.03±0.05
1x	5.06±0.08	0.381±0.003	138.53±1.23	5.88±0.05
5x	5.11±0.11	0.380±0.003	137.28±1.82	5.84±0.05
10×	5.18±0.14	0.381±0.003	135.24±3.03	5.82±0.13
50×	5.13±0.18	0.379±0.007	133.33±1.79	5.76±0.15
100×	6.04±0.32*	0.378±0.009	114.90±1.47*	5.05±0.03*
Control female	4.37±0.13	0.380±0.006	137.50±1.20	5.93±0.09
1x	4.67±0.07	0.381±0.006	135.28±1.23	5.88±0.19
5x	4.70±0.11	0.374±0.006	133.91±1.79	5.53±0.13
10×	4.97 ± 0.14	0.363±0.008	131.70±3.03	5.45±0.12
50×	5.04±0.17	0.358±0.007	130.45±1.84	5.42±0.19
100×	5.87±0.32*	0.354±0.007	112.28±1.92*	4.91±0.16*

LPO: Lipid Peroxidation=3-(4-5 dimethyl thiazol 2-xl) 2, 5-diphenyl tetrazolium bromide in table foot note by (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide). Asterisk (*) indicates a significant difference (*P<0.05) between control and treated groups.

After 15 days of exposure, the *E. coli* counts for both male and female mice showed a slight decline in CFU at high concentration (P=0.0046), i.e., there was no change in order of magnitude. However, only a 10-fold decline was recorded in both male and female mice groups at the end of 30 days (P<0.05). A dose-dependent decline in viable cell count for Clostridium spp. was not observed for this genus after 15 days of exposure. A slight increase in CFU count of this genus was observed in male and female groups. Sex specific significant alterations in bacterial counts were observed for the genus Bacillus and Lactobacillus. In the female groups that were treated with 10×-100× doses, the two genera declined by only 10-fold. Whereas they were not detected in male groups following 30 days of exposure.

The first colonizers of gastrointestinal tract, the probiotic Bifidobacterium spp. Count, were maintained up to a short exposure time of 15 days with minimal variation in numbers. This was true for the controls, nearly zero-fold change at highest concentration for both male and female groups. Among the gut bacteria, Lactobacillus, Proteus and Bacillus spp. were found more vulnerable to the toxic effect of the heavy metals' mixture as only few or no viable cells could be detected at 100-fold concentration of the baseline group. Whereas, *E. coli* and Clostridium were more resistant to the heavy metals in both sexes; Bifidobacterium count was maintained in male mice even at highest concentration. Thus, the overall data showed a dose-dependent change with decrease in bacterial diversity at higher modal concentration with the loss of some bacterial genus.

Alterations in oxidative stress parameters in erythrocytes: The data presented in Table 1 demonstrate that the 1X doses of the mixture of eight heavy metals administered to the male and female mice daily through drinking water failed to induce significant alterations in any of the oxidative stress-related endpoints in the erythrocytes after 30 days of exposure. In general, with increases in metal doses in the treatment groups, a decreasing trend was observed for CAT, SOD and GSH activities. Similarly, the male and female mice in the treatment groups of 5X, 10× and 50× dose led to slight elevation in the level of LPO, whereas, a marginal decline was observed in GSH, SOD and CAT activities compared to the controls.

A significant difference was observed for the increase in LPO (26.6%; 34.3%) in erythrocytes with 100× dose (groups 6 & 12; P<0.05). The exposure of male and female mice to the 100× dose also caused a significant decrease in the activities of SOD (16.25%; 17.2%) and

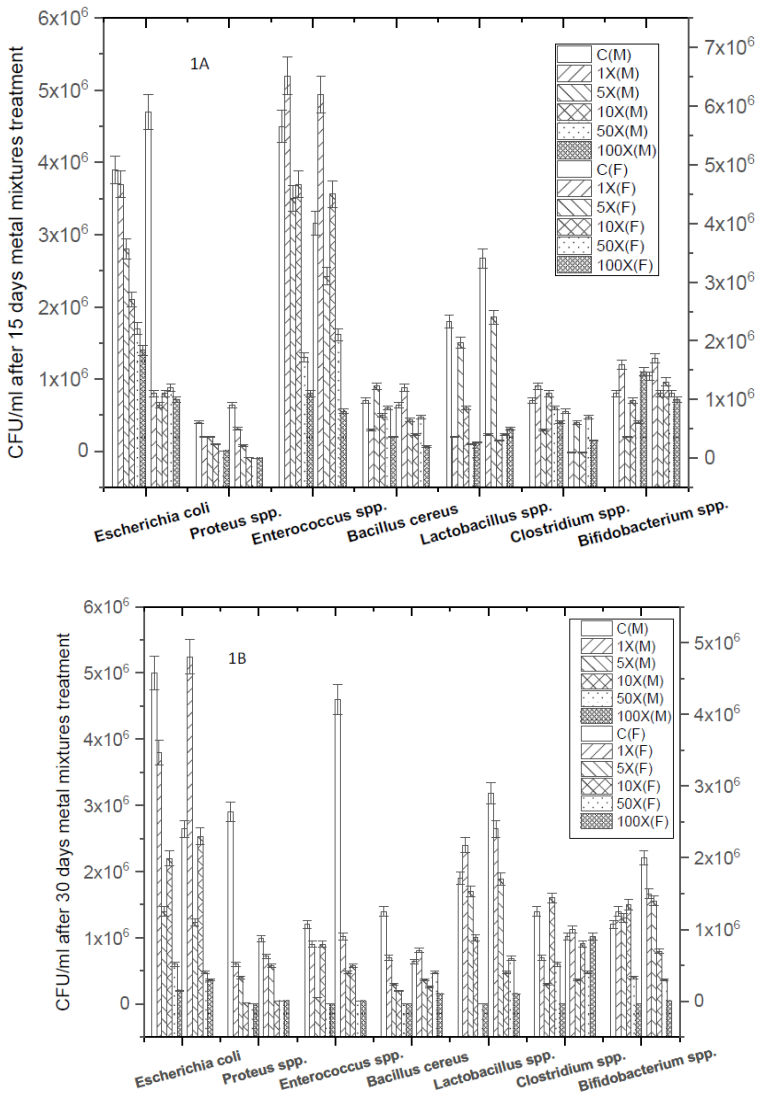

Figure 1. Genus specific count of bacterial population in fecal samples obtained from male and female mice following treatment with heavy metal mixtures

A: On day 15; B: On day 30; C: Control; M: Male; F: Female.

CAT (18.5%; 18.34%) ($P<0.05$). However, the decline in the level of GSH in 100× treatment group was not statistically significant for both male and female mice.

Discussion

There are numerous reports on the potential toxic effects of metals (Cd, Cu, Pb, Zn, As, Al, Ni etc.) on host organs such as liver, kidney and gonads [13, 22]. Similarly, alterations in the intestinal microbiota have been characterized in response to these toxic metals [23, 24]. However, the effect of multi-metal contamination on the gut microbiome has not been reported for drinking water.

This study investigated the variations in the gut microflora by analyzing periodical fecal samples obtained from the control and experimental groups for a period of 30 days following multi metals' exposure. The components of the mixture were selected on the basis of commonly found heavy metals in groundwater with modifications according to Jadhav, et al. [15]. The treatment condition in this study depicts sub-chronic exposure and is relevant to which common people are subjected via contaminated drinking water.

During the exposure period continuous change in gut bacteria (aerobes and anaerobes) was observed in each treatment group. In response to higher doses of 50× and

100× a time and dose-dependent decline of total cultivable bacterial count was observed. Such alteration in the gut bacteria profile indicates that prolonged exposure to the metal mixture at high doses through drinking water leads to a significant decrease in the gut microflora. Previous studies on single metal exposure also presented a concentration dependent in gut microbial count and diversity [2, 25]. In mice exposed to Cd-containing drinking water, a significant drop in abundance of most of bacteria was observed by Fazeli, et al. [2].

Similar changes in the gut bacteria generic composition were reported after chronic treatment with Cd and Pb in mice [8]. Variation in specific genus of aerobes and anaerobes following multi metal treatment was also observed in our study. Among the microflora, firmicutes such as Lactobacillus spp., Bacillus appeared sensitive with significant decline in the cell count in metal treated groups. On the other hand, the genus Clostridium, was less affected by heavy metal toxicity and an increase in their number was observed at low dose of metal treatment. Similarly, Enterococcus was also found to be moderately tolerant to heavy metal toxicity. The genus-specific response in gut bacterial abundance and diversity has been reported by many research groups.

Similar trends have been observed in animals exposed to heavy metals, causing reductions in the number of anaerobes and even larger numbers of firmicutes as compared to unexposed animals [8]. A study by Zhai, et al. [24] also evaluated the protective effects of Lactobacillus plantarum, a probiotic bacterium having good Cd accumulation potential, against acute cadmium toxicity in mice. Bifidobacterium is a major genus that colonize the gut microflora in mammals and has been found to be moderately susceptible to heavy metal treatment in the current study. Similar dysbiosis has been reported for human, rat and mice gut bacteria in response to low doses of environmental chemicals and heavy metals [23]. In this study, *E. coli* was prevalent even at higher dose groups following the 30-day exposure.

Similar findings of increased count of *E. coli* have been presented in broiler chicken fed with Ni supplemented feed [25]. Overall, it can be suggested that a high dose of multi metals solution leads to a significant decline in the bacterial count and diversity, whereas no significant change was recorded in response to low doses.

Our results revealed a sex specific variation in the total cultivable bacteria counts. For the male mice gut, the total bacteria declined nearly 10-fold higher compared to that in female mice following the 30-day exposure. Sex specific, significant alterations in the bacteria counts were observed for the genus Bacillus, Lactobacillus and Bifidobacterium. In female mice treated with metals, Bacillus and Lactobacillus were more prevalent even at high doses. On the other hand, they were undetected in the male groups. Bifidobacterium was detected in male mice even at the highest concentration, whereas Clostridium was prevalent at the highest concentration throughout the treatment period in female mice.

Based on our findings, a strong sex-specific response has been previously reported for the bacteria from *phylum Firmicutes* [26]. Besides the microbial composition and diversity, a recent study showed that sex was correlated with the functional gene richness of the colon [27]. The effect of sex difference on the gut microbiota and the relationship with other factors warrant further research.

The gut is believed to have a significant preventive mechanism against oxidative reactions derived from heavy metals, and is also the target of damages by ROS [28]. The interaction between the gut epithelia and some commensal bacteria induces the rapid generation of ROS. Several metals are known to cause LPO and perturbation of antioxidant systems in human and animal erythrocytes [29]. In corroboration with earlier studies, our results suggest that the disturbance of gut microbiota results in the generation of large amounts of ROS, which contribute to significant oxidative stress in that organ system.

The disturbance in the redox status was more apparent at highest treatment doses compared to those observed for lower metal doses. In this study, the increase in malondialdehyde levels noted in the 100× group corroborates with previous reports on metal induced contaminations. Reductions in such enzyme activities, as SOD, CAT, GSH, at higher doses of the metal mixture may be related to the higher roles of the enzymes against oxidative stress. A previous study on the exposure of rats to heavy metals mixture also demonstrated disturbances in oxidative stress enzymatic activity and LPO parameters [15].

The increased oxidative stress induced by the metals' mixture leads to the generation of ROS and depletion of erythrocyte sulfhydryl content, which weakens the enzymatic antioxidative defence [13, 14]. The intestinal microbiota disturbance contributes to the morphological and functional damages in the gut lining cells which in turn affects the intestinal barrier, thus favouring the transit of endotoxins into the gut vessels.

The subsequent production of ROS and increase in malondialdehyde content in the blood and other tissues

further lead to oxidative stress [30]. It may be suggested that oxidative stress induction in response to high concentrations of heavy metals' mixture has direct correlation with a reduction in overall gut bacteria counts along with alterations in the prevalent bacterial genus. Lastly, our findings suggest that metals' mixture modulates the gut microbiota in male and female mice which is associated with oxidative stress.

Conclusions

The findings of this study shed light on how the combination of various toxic metals influences the fecal bacteria and the oxidative stress parameters in both male and female mice. This work suggests that the dysbiosis in gut microbiome modulates oxidative stress in male and female mice upon exposure to higher doses of multi-metal solutions. Gender associated differences in the gut bacteria alterations following exposure to metals indicate the importance of both males and females being included in animal studies. Our results may aid in the assessment of potential risk of intestinal dysbiosis and diseases associated with heavy metals ingested via contaminated food and water.

Ethical Considerations

Compliance with ethical guidelines

All the experimental protocols were in accordance to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSCEA), government of India. Guidelines and objectives and study procedure is approved by Institutional Animal Ethics Committee (IAEC) (Protocol no: BV/IAEC/2018/2).

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Author's contributions

Both authors equally contributed to preparing this article.

Conflict of interest

The authors declare no conflict of interest.

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