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ARTICLE

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Cadmium exposure is associated with overexpression of the metallothionein gene and heightened urinary deoxy-guanosine and protein carbonylation status in an exposed population of West Bengal, India

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BACKGROUND: Cadmium (Cd), being a heavy metal, causes myriad clinical problems if exposed to the environment through food or drinking water or through inhalation. The river Churni of West Bengal is highly contaminated with heavy metals, particularly Cd, due to the disposal of waste materials from paint, sugar, and textile industries. People residing in the catchment area of the river are using the river water for drinking, cooking, and household work, and, therefore, are exposed to high levels of Cd throughout the years.

OBJECTIVE: Evaluation of biomarkers in chronic Cd-induced health hazards.

METHOD: A total of 105 study participants were recruited to evaluate chronic Cd-exposure-induced biomarkers. Among them, 50 were from the Cd-exposed population, and 55 were from the unexposed control. Cadmium concentration was measured by Graphite Furnace AAS in drinking and cooking water samples, and the biological samples (blood, urine) collected from every participant.

RESULT: The extent of oxidative DNA damage, protein carbonylation, plasma nitrate, and malondialdehyde (MDA) concentration was measured in people and found to be significantly higher in the Cd-exposed population in comparison to the unexposed control. Expression of the metallothionein (MT) gene and protein has also been studied in both populations, with significantly higher MT gene expression observed in the Cd-exposed population. Evaluation of cell death using the comet assay and acridine orange/propidium iodide staining revealed significantly higher comet tail moment and apoptosis in isolated lymphocytes from exposed participants.

SIGNIFICANCE: Thus, it can be concluded that Cd exposure is associated with oxidative damage and health risk. Cadmium-induced oxidative damage and MT gene expression in the exposed population can serve as a biomarker.

IMPACT STATEMENT: This cross-sectional study demonstrates that Cd-induced oxidative damage and MT gene expression can serve as biomarkers in a chronically exposed population. In the future, protein carbonylation, oxidative DNA damage, and MT gene expression may serve as biomarkers of Cd exposure-induced health hazards for diagnostic purposes.

Keywords: Cadmium exposure; Food chain contamination; Protein carbonylation; DNA oxidation; Metallothionein Gene expression.

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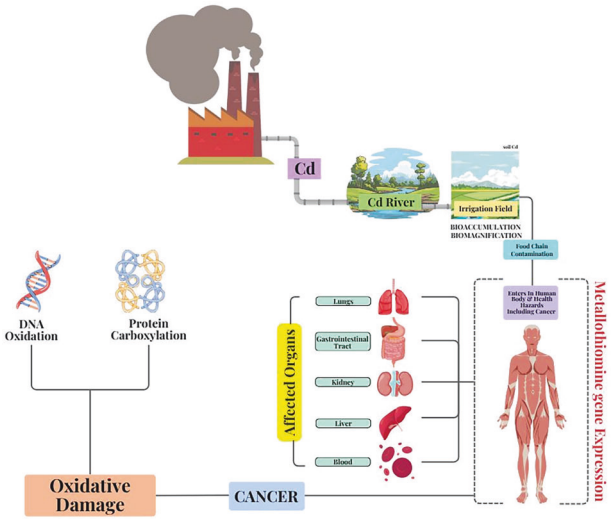
Graphical Abstract

Graphical abstract showing the effects of Cd on the human body by food chain contamination.



INTRODUCTION

Cadmium (Cd) is a naturally occurring heavy metal generally found in the earth's crust at an average concentration of 0.1–0.2 ppm. The presence of Cd in nature is due to geogenic or anthropogenic causes, such as volcanic eruptions or anthropogenic combustion of natural biomass. Although environmental levels of Cd are low, chronic exposure in humans is associated with kidney, liver, cardiovascular and nervous system toxicity and with cancer risk [1, 2]. The effect of Cd on cardiovascular diseases has been studied, with urinary Cd concentration linearly correlated with cardiovascular disease [3]. Metals can affect the overall homeostasis of the body by generating reactive oxygen species (ROS) to cause DNA damage, and may also cause the generation of neurodegenerative diseases, including Parkinson's and Alzheimer's [4, 5]. Heavy metals, including Cd induces cancer; however, the mechanism is not known clearly [6]. Some anthropogenic activities, like burning of fossil fuels and disposal of industrial waste materials, increase environmental Cd concentration beyond the permissible limit, which is 0.005 mg L^{-1} or 5 µg L^{-1} recommended by the World Health Organization and Environmental Protection Agency [7, 8]. It may occasionally reach levels as high as 500 µg L^{-1} due to anthropogenic activities, causing a range of clinical symptoms in humans and other animals chronically exposed to it [2]. Surprisingly, Cd is not involved in any biological functions in the human body. So far, no Cd biotransforming enzyme has been identified in the human system to form a bioavailable Cd compound. It has been shown that there is a potential increase in the expression level of the metallothionein (MT) gene in animal systems [9, 10]. Human activities like combustion of fossil fuels or burning of wastes, the process of mining of metal ores, and continuous industrial emissions lead to the environmental load of Cd, which ultimately accumulates in humans and other higher animals through food chain contamination and causes a great public health threat [7, 8].

Cd^{2+} forms coordination complexes with proteins and nucleic acids, competing with essential divalent cations (Zn^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}) for binding sites [11]. Depending on the protein to which Cd is bound, clusters can also be generated, like Cd- MT complex, in which cysteine-rich regions of the proteins are able to cluster with Cd^{2+} and, even at low Cd concentrations, mRNA for MTs is up-regulated [12, 13]. Metallothioneins are a family of ubiquitous small cysteine-rich proteins that are specific to bind with and regulate the metabolism of Zn [9, 10]. Metallothioneins also bind Cd and other heavy metals, protecting biological systems against heavy metal toxicity by inhibiting DNA damage and oxidative stress. Most of the Cd^{2+}-MTs complexes are excreted via urine, but within the kidney threshold. The cysteine content of MTs may be up to 30%, which is actually helpful for complex formation with Cd (9,10). When the amount of Cd exposure exceeds the limit for its biotransformation or metabolism, including binding to MTs, and exceeds the kidney threshold for excretion in urine, there will be accumulation of Cd in the body's soft tissues, increasing cellular Cd load. This Cd accumulation mainly occurs in the kidneys (30%) and liver (30%), with the rest dispersed throughout the other organs and having an extremely long half-life of 10–30 years (9,10). The biological half-life of Cd in the blood has been estimated to be in the range of 75 to 128 days, which actually refers to the half-life by its deposition in the organs, not the body's clearance rate [10, 14]. Cadmium bioaccumulation in various tissues affects cellular homeostasis and physiological functions, such as proliferation and differentiation, by inducing oxidative stress and the formation of high levels of ROS, thereby inducing cellular damage and apoptosis [15, 16]. The binding of Cd with the mitochondrial enzymes and mt-DNA leads to inhibition of cellular respiration and oxidative phosphorylation [15, 17]. Cd inhibits the nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) systems, and thereby induces single and double-stranded DNA breaks and mutation [18]. Cadmium biotransformation and metabolism in living systems require thiol-containing enzymes and proteins, including glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) [18, 19]. Therefore, in the human system, individual Glutathione-S-Transferase (GST) genotype status may also contribute to the overall biotransformation and excretion of Cd [19–21]. Although in this work we have not worked out the individual GST genotype status and the individual's thiol-containing enzyme status to rule out the involvement of genotypic variations in Cd-induced oxidative stress. The genotypic variation of GST and other thiol-containing enzyme genes is beyond the scope of our study. The inhibition of antioxidant defence enzymes, such as superoxide dismutase (SOD), lactate dehydrogenase (LDH), catalase (CAT), thioredoxin-reductase (TrxR), and glutathione peroxidase (GPx) leads to the further dysregulation of the cellular redox state [19–21].

Environmental Cd load accumulates in plants from the soil. From edible plants, it is transmitted into primary and secondary consumers and ultimately to humans. This is called food chain contamination[22]. This accumulation increases as pH decreases in

the biological system. In particular, the food items that mainly contribute to the daily Cd intake include cereals and bread (34%), leafy vegetables, particularly spinach (20%), potatoes (11%), legumes and nuts (7%), stem/root vegetables (6%), and fruits (5%). In Eastern countries, including India and Bangladesh, fish and shellfish are major sources of Cd, alongside crops, particularly rice (Kim et al. 2018). It is estimated that the extent of Cd exposure through food grains are 44% in Japan [23] and 56% in China [23], though the limit of gastrointestinal absorption of Cd through oral food grain is relatively low and it is only about 10% of the orally ingested dose [24]. In Japan, people living in the Jinzu and Kakehashi river basins experience a rare but typical disease called itai itai disease due to consuming highly Cd-contaminated water from these rivers. The soil of these river basins is heavily contaminated with Cd derived from industrial waste, and local people who frequently consumed rice cooked with Cd-contaminated water developed a disease characterized by severe bone deformation and multiple fractures, especially in women [25]. The radiographs showed osteomalacia, bone decalcification, and osteoporosis due to the replacement of Ca^{2+} by Cd^{2+} [25].

In India, the distributaries and tributaries of the Ganga River are highly contaminated by industrial waste containing high levels of Cd. For example, we can point out the current scenario of the river Churni, where there is a high level of Cd due to the presence of industrial effluents in the river bed [26]. We have conducted a survey of local residents on the banks of the Churni River in the Nadia district of West Bengal, near Shatipur and Fulia. We have seen that people are experiencing myriad clinical symptoms, including gastrointestinal, bone, and blood-related disorders, and their food sources, especially rice, river fish, and cooking water, are highly contaminated with Cd. The Cd concentrations in drinking and cooking water, rice, and fish have been estimated, along with their blood and urine Cd concentrations, reflecting both chronic and current exposure. Although several reports document contamination of river water with heavy metals, including Cd, in various states in India, including West Bengal, there are few or no reports on the health effects of chronic Cd exposure, with biomarker assessment in exposed populations.

In this study, we will therefore evaluate the overall health risk assessment, oxidative damage, and the identification of potential biomarkers for Cd exposure in people chronically exposed to Cd. In a preliminary study, it was shown that Cd exposure, along with other heavy metals, induces the expression of microparticles such as CD14, CD62, and CD62E in a statistically significant manner [27]. Statistically significant high levels of vascular endothelial macroparticles (EMP) and platelet microparticles (PMP) are also demonstrated in this work, which are actually released by cells during apoptosis. In the present study, we will evaluate the cellular dysfunction and disruption of the cellular microenvironment, including oxidative damage, cell death, and altered gene expression, following chronic Cd exposure in humans.

MATERIALS AND METHODS

Selection of the study population

Initially, from the Cd-exposed and unexposed areas, a total of 187 study subjects were selected (exposed $N = 90$, unexposed $N = 97$). From those selected subjects, 20 exposed subjects and 10 unexposed subjects were rejected for their age, which was slightly beyond our selected range, and also due to their unwillingness. Thereafter, we started with 70 exposed and 87 unexposed study subjects. During the procedure of subject incorporation, 20 Cd-exposed subjects and 32 Cd-unexposed subjects were excluded from the experimental procedure due to their prior and current medications for hypertension, anemia, hypothyroidism, etc. Finally, we recruited a total of 105 study subjects for the exposed and control groups. The exposed subjects ($N = 50$) were selected from two block areas of Nadia district (Shantipur, Fulia), through which the river Churni flows. All of the exposed subjects resided along the banks of the River Churni and were engaged in the paint and textile industries. All exposed subjects had current and past exposure to Cd as a constituent of factory raw materials

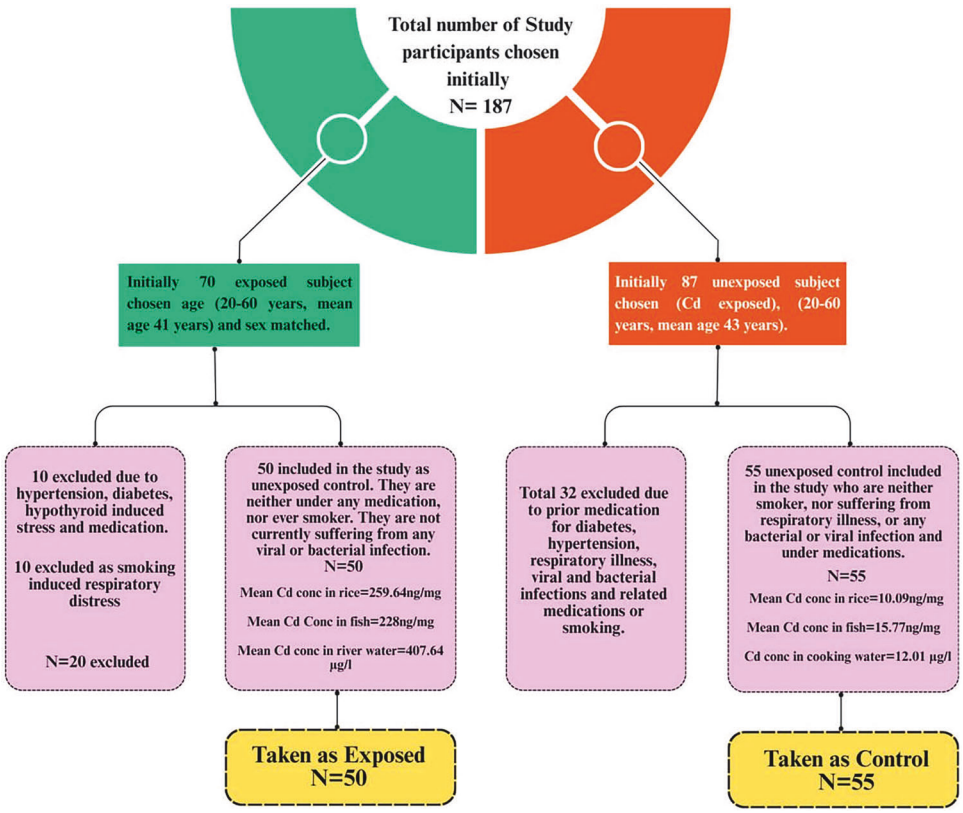


Fig. 1 Flow chart. Flow chart showing the criteria of sampling and the number of samples in each group.

Table 1. Demographic distribution of the study population.

Group name	Age (yrs)	Sex	Average, Ht, Wt	Smoking habit	Occupation	Average duration of exposure
Group I Control	Age group (i) 20–40 yrs	(i) M = 18, F = 12	M= Ht=162 cm	M=all ex-smoker	Farmers =26 small traders=15 hawker=09 primary school teacher=05	0 y.
	$N = 30$		Wt=67 kg	F=all		
	(ii) 41–60 yrs $N = 25$	(ii) M = 15, F = 5	Ht=159 cm, Wt=57 kg	Non smoker		
$N = 55$						
Group II	(i) 20–40 yrs	(i) M = 26,	M=Ht=	M=	farmers=17	6.31 y
					Small	
	$N = 30$	F = 6	163 cm		Traders=10	
Exposed $N = 50$	(ii) 41–60yrs $N = 20$	(ii) M = 17 F = 8	Wt=66 kg	36 males, ex-smoker Females are non smoker	Service holder=04 Service holder at sugar industry=7	
			F=Ht=154 cm,		daily labour at industries=12	
			Wt=61 kg			

*Considered significant.

and factory waste coming from textile painting and sugar industry, as they were employed in the paint and textile industry at the time of subject selection, and for the last 6–10 years. Evidence of current Cd exposure is reflected in urinary Cd concentrations among the study subjects, and all of them use Cd-contaminated river water for their industrial and household work, including cooking. They also use local vegetables and fish. The inclusion and exclusion criteria for study participants (exposed and unexposed) are summarized in the flowchart (Fig. 1). Age and sex-matched control subjects ($N = 55$) have been selected from South 24 Parganas, Baruipur sub-divisional area, who are not exposed to Cd and whose drinking and cooking water Cd concentrations, as well as urinary Cd concentration, lie within the permissible limits. 24-h food samples were collected from Cd-exposed and unexposed subjects for microwave digestion and assay to determine food Cd concentrations. This 24-h sample for each type of consumed food material is actually representative of their usual food chart for the day.

Also, drinking and cooking water samples, as well as morning void urine samples, were collected from each participant to determine Cd concentrations in water and urine. Average concentration of Cd in raw rice and fish in Cd-exposed population was 259.64 ng mg^{-1} and 228.15 ^{ng} mg^{-1}. The range of exposure to Cd through food was 138–376 ng mg^{-1}, as measured in our laboratory from 24-h food samples collected from participants. Morning void urine samples had been collected from every participant in a sterilized

polypropylene collection vial. Blood was also collected from every participant in an Ethylenediaminetetraacetic acid (EDTA) vial. Urinary Cd concentration was 117.11 µg L^{-1} on average. Average blood Cd concentration in exposed participants was 203.74 µg L^{-1}, and in river water the Cd concentration was 407.64 µg L^{-1} (Table 2).

Demographic distribution for Cd-exposed and unexposed study subjects is tabulated in Table 1, whereas Cd concentration in river water, drinking and cooking water, raw rice, and fish is tabulated in Table 2. Written informed consent was obtained from all participants before drawing their blood and taking their urine samples. The institute's ethical principles are guided by rules formulated by the Indian Council of Medical Research, which align with the Helsinki rules. This study was performed in line with the principles of the Declaration of Helsinki. Approval and ethical clearance were granted by the Ethics Committee of Rammohan College (Date 09/12/2021./Approval No. REC/ENV005/0021). Collected blood and urine samples were carried in an ice box to the Rammohan College for further analysis.

All chemicals (analytical grade and molecular grade) were purchased from Merck Millipore to ensure quality control, and Millipore deionised water was used for reagent preparation.

Collection of water, urine, blood and food samples

River water used as a cooking and household water source, and drinking water samples, were collected from each participant in sterile polypropylene containers and stored at −20 °C until Cd measurement. Morning void urine samples were collected in sterile polypropylene containers using a protease inhibitor and placed in an ice bucket for transport and storage at −20 °C until analysis. Blood was collected in an EDTA container from each participant by peripheral venepuncture with the help of a professional phlebotomist and transported in an ice bucket to the laboratory, where it was stored at −20 °C until further analysis. 24-hr food samples were collected in sterile ziplock bags and transported in an ice bucket to the laboratory, where they were stored at −20 °C until Cd measurement by GFAAS.

Drinking water and cooking water were the same for the exposed population, and it was river water. The river water had been treated with simple filtration or boiling to make it bacteriologically safe before being used as drinking water.

Determination of Cd concentration in water, urine, blood, and food

The concentration of Cd in drinking water, blood, and urine was determined by Graphite Furnace Atomic Absorption

Spectrophotometry (GFAAS) according to the manufacturer's instructions (AAS—with Graphite Furnace, Perkin Elmer PinAAcle 900 T, Singapore) in the University Science Instrumentation Centre, University of Calcutta. For Cd measurement, the liquid samples were acidified with 1 mL of 1.6 M nitric acid (HNO_3), then stabilized for 10 min, and centrifuged for 5 min at 3000 rpm to collect the supernatant (urine, water). It was subjected to aspiration into an air-acetylene flame, and then the Cd concentration was measured at 228.8 nm [28]. Biological samples, such as blood and urine, were preserved at -20 °C in a freezer until analysis by GFAAS for Cd concentration measurement. Solid food samples were dried and digested by microwave-assisted digestion (PerkinElmer MPS 320), then subjected to GFAAS as dry ash, according to the manufacturer's instructions [29]. University Science Instrumentation Center (USIC), Chemical Technology laboratory, the University of Calcutta, is a NABL-accredited laboratory that ensures quality control in every step. Graphite Furnace Atomic Absorption Spectrophotometry analytical conditions remained constant throughout the process of measurement of Cd. For whole blood, HNO_3-digested wet ash was taken, and the sample detection limit was 0.4 µg L^{-1} according to the manufacturer's instructions.

A standard was prepared, and a standard curve was obtained to measure Cd concentration. Both the standard and spike samples were analyzed using a blank. Spike recovery was used to accurately measure Cd concentration. An accurate amount of Cd was added to the blank for spectroscopy, and the blank was then normalized to determine the Cd concentrations in the given samples according to the manufacturer's instructions. A reference standard solution of Cadmium (Cd) was prepared at a concentration of 1000 ± 2 mg L^{-1} in 2% v/v HNO_3, purchased from Merck. The recovery percentage was calculated in every measurement. The

Table 2. Mean Cadmium concentration in food, water, urine and blood of exposed and unexposed study participants.

Conc. of Cd in mean ± SD	Exposed group (group II) $N = 50$	Control group (group I) $N = 55$	P value (<0.05 taken to consider)
Cd concentration in food ng mg^{-1} (in rice)	259.64 ± 21.34	10.09 ± 3.57	$P < 0.001$*
Cd concentration in fish ng mg^{-1}	228. 15 ± 27.31	15.77 ± 2.89	$P < 0.001$*
Cd concentration in urine μg L^{-1}	117.11 ± 18.57	14.43 ± 2.65	$P < 0.001$*
Cd concentration in blood serum μg L^{-1}	203.74 ± 19.77	11.56 ± 3.43	$P < 0.001$*
Cd concentration in cooking water μg L^{-1}(directly comes from the river)	407.64 ± 32.54	12.08 ± 3.71	$P < 0.001$*
Cd concentration in factory waste sludge μgL^{-1} (sample collected from drainage) (collected from 9 factories)	733.543 ± 87.64	----------	

limit of detection was determined at a 90% confidence level, with a limit as low as 20 ng mg^{-1} for solid food materials. All the measurements were made thrice.

Concentrations of heavy metals (HMs) in water and biological matrices were determined following a standardized protocol [28]. Cadmium concentrations were calculated using the following equations:

$$\text{Cd concentration in liquid samples}(\mu g/mL) = \frac{\text{GFAAS reading} \times V}{\text{sample volume}(mL)}$$

$$\text{Cdconcentrationinfoodorbloodsamples}(\mu g/g) = \frac{\text{GFAAS reading} \times V}{\text{sample weight}(g)}$$

where V denotes the volume of the dilution solution used for sample preparation.

Recovery assessment

Method accuracy was evaluated through recovery experiments. The recovery percentage (%) was computed as:

$$\text{Recovery}(\%) = \left(\frac{\text{amount obtained}}{\text{amount spiked}}\right) \times 100$$

Recoveries ranged from 95–98%, confirming the analytical reliability and precision of the method.

Determination of lipid peroxidation

Malondialdehyde (MDA) measurement: MDA was measured as an index of lipid peroxidation. Briefly, plasma samples from each participant were reacted with the TCA/TBA/HCl reaction solution (5:2:1 by vol), heated for 60 min in a boiling water bath, then cooled in tap water and centrifuged. The absorbance of the supernatant was then measured spectrophotometrically at 532 nm using 1,1,3,3-tetraethoxypropane as the standard [30].

NO measurement

Nitrate was measured in the deproteinised plasma samples according to the manufacturer's instructions (colorimetric assay kit from Abcam: ab 65328). In the reaction, the generated nitrate was converted to nitrite using nitrate reductase, provided in the kit. Then Griess reagent was used to convert nitrite to an azo compound with a deep blue-purple colour, which was detected spectrophotometrically at 540 nm [31]. Briefly, the deproteinised samples were mixed with enzymes and a cofactor, then incubated at room temperature for 60 min. Then, the enhancer (diluted in assay buffer) and Griess reagent were added, and the mixture was incubated at room temperature for 10 min, after which the optical density was measured at 540 nm.

Protein carbonylation assay

Protein carbonylation was assayed in plasma from exposed and unexposed participants by non-competitive ELISA based on a previously described method with further detailing and implementation in other published protocols [32–34].

Study of DNA oxidation: deoxyguanosine (8-OHdG) assay

Oxidative DNA damage was measured by the concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in urine samples collected from each participant using the 8-OHdG check ELISA kit (Genox Corporation, Baltimore, MD, USA) according to the manufacturer's instructions, as previously reported [34]. In this assay, the urine samples were centrifuged at 6000 rpm for 5 min at 6 °C to remove particulate matter, and the supernatant was collected. It was then centrifuged again at 3000 rpm for 5 min at 6 °C to remove cellular debris, and the supernatant was collected for the assay, which was performed according to the manufacturer's instructions.

Study of the expression of the metallothionein gene

Total RNA was extracted from the blood samples taken from every study participant using Trizol reagent (Sigma–Aldrich, St. Louis, MO, USA). Purity and consistency were assessed by the absorption ratio at 260/280. A total of 1.5–2.5 μg of RNA was reverse transcribed using reverse transcriptase in a 25 μL reaction mixture containing the respective forward and reverse primers, dNTPs, and buffer. The thermal cycle was set as denaturation at 70 °C for 5 min, annealing at 42 °C and elongation at 60 °C. For MT gene expression, MT1 and MT2 gene primers were used with acc. nos. RATMETI and XM_001062488, respectively [35], and the β-actin gene was co-expressed as a positive control in each sample.

Metallothionein protein measurement

Metallothionein protein level in the plasma was determined by spectrophotometric assay using Ellman's reagent as per standardized protocol [35]. The Ellman's reagent or DNTB in 100 mM potassium dihydrogen phosphate (KH_2PO_4) at pH 8.4 was used. Plasma was deproteinized and extracted with ethanol/chloroform according to a previous standardized protocol [36].

Study of cell death by acridine orange/propidium iodide

Hundred μL of whole blood from the exposed persons and unexposed persons were diluted with Phosphate-Buffered Saline (PBS) (1:1) and incubated for 10 min at 37 °C. Lymphocytes were isolated using Ficoll and Histopaque by the conventional method. The cells were suspended in ice-cold PBS and then stained with Acrydine-Orange and Propidium Iodide [37]. Ten microliters of each Acridine Orange (AO) and Propidium Iodide (PI) were used for staining, and samples were observed under a fluorescence microscope (Axiolab, Zeiss, Germany) using a blue filter and compared with the control. Cadmium-induced cellular death was recorded in the exposed population.

Comet assay and study of apoptosis

A single-cell suspension was prepared by isolating lymphocytes from fresh, exposed and unexposed blood. Cells were then placed on an agarose-coated frosted slide by mixing them with low-melting agarose. An additional layer of low-melting agarose was then coated over the slide to immobilize the cells, which were then placed in the lysis buffer and electrophoresed to perform the alkaline comet assay [38]. The slides were then seen under a fluorescence microscope (Zeiss Fluorescence Microscope: Zeiss Axio). Comet tail DNA and tail length were studied by the Zeiss fluorescence microscope using the comet assay software.

RESULT

Demographic data for the Cd-exposed and unexposed study population were tabulated in Table 1. It was found that the

Table 3. Mean level of Oxidative markers, Comet assay including Olive tail moment and MT gene expression in Cd-exposed and unexposed population (data presented as mean ± SD).

Sample	MDA nmol/g protein	NO nmol/µl	Protein carbonylation µg L^{-1}	DNA (8OHdG) µg L^{-1}	Comet assay % apoptotic cell	Olive tail moment (arbitrary unit)	MT expression	% of live cells (lymphocyte)
Control $N = 55$	1.77 ± 0.08	36 ± 4.34	169.68 ± 7.34	9.63 ± 1.79	19 ± 7.64	0.54± 0.034	1 fold	75%
Cd exposed group $N = 50$	5.46 ± 0.54 $P < 0.05$*	77 ± 6.73 $P < 0.01$*	274.41 ± 9.61 $P < 0.01$*	15.89 ± 3.16 $P < 0.05$*	43 ± 8.87 $P < 0.05$*	3.23 ± 0.078 $P < 0.05$*	3 fold $P < 0.05$*	35% >2fold decrease

*Considered significant.

concentration of Cd in the river water (River Churni) is much beyond the permissible limit. The mean Cd concentration in river water is 407. 64 µg L^{-1}. Table 2 shows the concentration of Cd in cooking water, which directly comes from the river, and the mean concentration of Cd in cooked rice and fresh fish. The sample sizes in the exposed and unexposed groups were 50 and 55, respectively. Criteria and procedures for subject selection are described in the materials and methods section, including a flowchart (Fig. 1). All the numerical data presented here are expressed as mean ± SD. After assessing the data's normality, parametric statistics were performed to assess the significance of differences between group means. Mean Cd concentration in blood, urine, drinking water, and food was presented in Table 2 for both exposed and unexposed groups, which shows a significant increase in Cd concentration in blood and urine samples taken from the exposed group ($p < 0.001$).

This increase in blood and urine Cd concentrations indicates current exposure among the study participants. An increase in drinking and cooking water Cd concentrations, with high Cd accumulation in food, increases the risk of clinical symptoms, such as gastrointestinal disorders, from chronic Cd exposure.

People chronically exposed to Cd in the catchment area of river Churni experiences a significant level ($p < 0.01$, $p < 0.05$) of oxidative damage reflected by significantly high level of serum MDA concentration, serum Nitric Oxide (NO) concentration, significant increase of protein carbonylation and urinary 8-OHdG status ($p < 0.05$) in comparison to unexposed counterpart of the population (Table 3).

Metallothionein gene expression is significantly increased in the exposed group compared with the unexposed group by 3-fold, but it is not positively correlated with the degree of protein carbonylation or DNA oxidation in individual exposed subjects (Table 3). Though protein carbonylation and DNA oxidation are both significantly higher in the Cd-exposed group ($p < 0.01$, $p < 0.05$, respectively). However, serum MT protein level is not correlated with the degree of MT gene expression in the Cd-exposed group, and it is not at all significantly higher in the exposed population.

A study of the comet assay on isolated lymphocytes from Cd-exposed and unexposed subjects reveals a higher percentage of cell death in the exposed group ($p < 0.05$) compared to the unexposed control (Table 3), indicating only 35% live cells in the Cd-exposed group. Olive tail moment and the mean tail moment are increased by almost 3-fold ($p < 0.05$) in comparison to the unexposed counterpart (Table 3), which is indicative of DNA damage and cell death. Whereas in the Cd-unexposed subject group, the percentage of live cells is about 75% ($p < 0.05$). The results show no correlation between male and female counterparts within the exposed group, nor between the exposed and unexposed groups, although MDA levels and NO concentrations are higher in male subjects within the exposed population (vide Table 3). A comparative analysis of comet assay done with peripheral blood lymphocytes isolated from Cr exposed and unexposed persons to show the proportion of live and dead cells when stained with acrydine orange and propidium iodide (Fig. 2a, b).

DISCUSSION

Waste materials from the paint, sugar, and textile industries largely contain hazardous chemicals, including HMs such as Cd, chromium (Cr), and lead (Pb), as well as other organic and inorganic materials that pose potential risks to human health [39, 40]. Cadmium is present in quite considerable concentrations in the factory waste of the painting and textile industries due to its use as a dye in these industries. In contrast, the presence of Cd in sugar industry waste is considerably low [40]. The participants we have chosen for the Cd-exposed group were mostly the workers

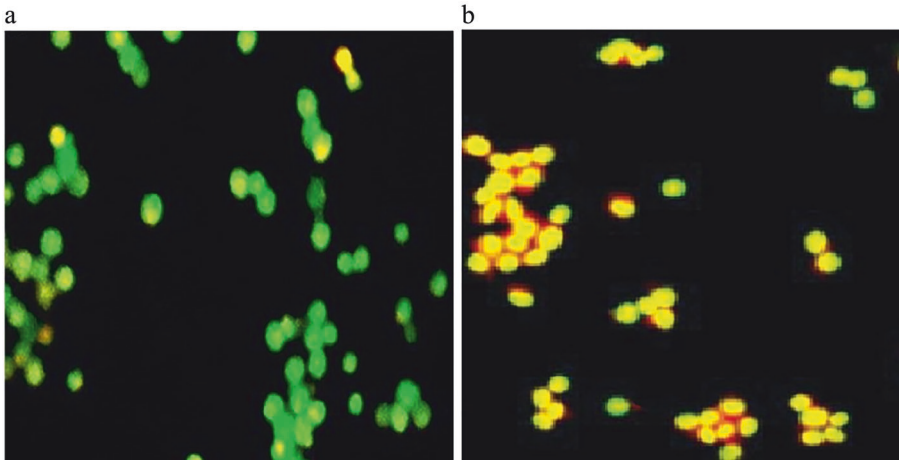


Fig. 2 Peripheral blood lymphocytes taken from Cr exposed and unexposed persons and stained with Acrydine Orange and Propidium Iodide to show proportion of live and dead cells. a Lymphocytes from Cr unexposed human blood, showing live cells (green in colour), **b** Lymphocytes from Cr exposed persons. Showing dead cells (orange in colour).

of textile and paint factories, and they were the local inhabitants of the catchment area of the river Churni, near Shantipur and Fulia, which is highly contaminated with factory waste materials containing a high level of Cd [26, 27]. We have studied the concentration of Cd in the sludge from factory waste, and it is high, far beyond the WHO-recommended permissible limit (vide Table 2). The sludge is deposited directly into river water without further treatment, resulting in high Cd concentrations in the water, which is used by local people for cooking, other household work, and irrigation. This actually increases the probability of food chain contamination and bioaccumulation in local people. We have studied the food materials, taken as samples for 24 h consumed food, to assay the Cd concentration, which was also far beyond the permissible limit (vide Table 2). The urinary Cd concentration in exposed participants was also above the WHO-prescribed permissible limit, indicating current Cd exposure. Though we haven't assayed the sludge and river water for other HMs or metalloids in every factory under our study, primary assessment for HMs in the factory waste, taken from nine textile and paint factories, revealed the highest concentration of Cd in the sludge over Cr and Pb. We therefore focused on assessing Cd concentrations in blood, urine, and food materials collected from all exposed and unexposed participants to evaluate the association between chronic Cd exposure and MT gene expression and oxidative damage. We have found a significant increase in protein carbonylation and 8-OHdG in Cd-exposed participants compared with normal, unexposed controls, and a significant overexpression of the MT gene. Both of these findings can be used as biomarkers of Cd exposure. Cadmium, after entering the human body, is taken up by the liver for detoxification and induces expression of the MT gene [41]. High levels of MT expression after Cd exposure result in Cd protein complex formation and ensure 80–90% of absorbed Cd forms a complex. The unbound Cd is excreted from the kidney [41]. High concentration of Cd absorption in the body causes loss of renal tubular function and renal failure [42, 43] along with bronchial infection, dyspnoea, chest pain, chemical pneumonitis and interstitial fibrosis in case of inhalation exposure [42]. Cadmium enters the cell through divalent cationic channel protein by non-specific binding and also uses Zn-specific transporters like ZIP8 and ZIP14 [42]. Cadmium ingestion causes gastrointestinal irritation, vomiting, and abdominal pain, and in higher doses, it crosses the blood-brain barrier and affects the brain parenchyma [44]. In humans, Cd also causes testicular damage, osteomalacia, osteoporosis, and marked damage to the hematopoietic system and adrenal glands [45], but how it promotes these diverse health hazards remains unclear. There is very limited information on the biotransformation and bioavailability of inorganic Cd. Our present work does not have the scope to explore the routes of biotransformation and the bioavailability of Cd in biological systems. However, the chronic Cd exposure associated with oxidative damage is explored here.

Our study reveals a positive association between chronic Cd exposure and protein carbonylation and associated DNA oxidation, but Cd cannot directly produce ROS because it does not undergo Fenton-like reactions; however, it can replace redox-active metal ions and downregulate antioxidant enzymes, leading to lipid peroxidation and DNA damage [43, 46]. The major enzymatic antioxidants against Cd-induced oxidative damage are SOD, catalase, and GSH, which scavenge superoxide radicals, thereby inactivating hydrogen peroxide (H_2O_2) and hydroperoxides. Chronic and acute exposure to Cd beyond its tolerable limit increases the expression of these enzymes, facilitating the scavenging of ROS [47]. In our exposed study population, chronic Cd exposure also increases the production of MDA and NO, markers of oxidative damage. Cadmium has been shown to impair the global DNA repair system by downregulating the activities of DNA repair enzymes, and thereby interferes with the removal of damaged DNA from the cell [48]. The organs most affected by Cd overexposure are the kidneys [49]. As much as about 30% of body Cd is deposited in the kidney tubular region, causing tubular damage and renal necrosis proportionally to the quantity of Cd deposited as cellular load, by escaping the MTs [50]. Therefore, MT expression and binding of Cd with MT is simply a biotransformation procedure that increases the potentiality of Cd release from the body.

Cadmium is classified as a genotoxic and mutagenic agent by the EPA [8]. It binds to DNA, forms DNA adducts, and thereby causes immunological alterations [51]. Cadmium-induced clastogenic actions, which are studied by a number of researchers, though a study directly on the exposed population is not available. We are the first to evaluate the clastogenic action and oxidative stress induced by Cd in an exposed population. A number of toxicogenomic studies confirm the involvement of Cd in mutations in the following genes: immediate early response genes (IEGs), stress response genes, transcription factors, and translational factors [20]. One mechanism by which Cd influences gene expression involves not only altered Ca homeostasis but also replacement of Ca. Cadmium is such an element whose concentration inside the cell can be influenced by intracellular Ca concentration [20]. In addition to Ca, Cd also replaces Zn from a variety of cellular reactions and thereby inhibits a number of reactions, and ultimately causes breakdown of cellular

homeostasis [20]. Cadmium competitively replaces Zn and Ca in the cellular microenvironment, thereby disrupting normal immunological homeostasis. Zinc is an essential micronutrient for innate and adaptive immune responses. The Zn-to-Cd ratio within the cell actually determines the cellular destiny of the cell cycle [42]. Cadmium induces nephrotoxicity, hepatotoxicity, cardiotoxicity, neurotoxicity, and haematotoxicity, and disrupts the normal metabolic processes of the gut. It replaces Ca from bones to induce osteoporotic activity. Cd also disrupts transcriptomic balance in the brain and liver, with more prominent effects observed in the liver, where it is biotransformed. Cadmium load in blood and target cells reduces transporter activity and has also been shown to down-regulate transcriptomics of transporters [51]. Dysregulation of brain and hepatic transporters, as well as drug-metabolizing enzyme activity in the liver, reduces Cd elimination via the kidney and promotes various genotoxic and mutagenic effects. It is important to note that most differentially regulated transporters are involved in Cd exposure, which may cause immune-modulation [51].

Cadmium treatment in rodents has been shown to be associated with an increased expression rate of MT isoforms and leads to binding of Cd with MT, resulting in immune suppression [52]. Although protein levels of MT are not significantly higher in the exposed group, mRNA levels of the MT gene are significantly higher after Cd exposure compared with the unexposed control population. In support of our finding, we can also refer to the study of Genchi et al., which showed that Cd exposure is associated with overexpression of MT isoforms in liver and kidney [45]. Cells expressing a good amount of MTs are resistant to Cd-induced clastogenic actions, while cells that underexpress MTs are sensitive to Cd-induced toxicity [45]. Metallothionein expression regulates the cell's mitotic potential and fate, determining whether it follows the apoptotic or necrotic pathway. Cadmium is associated with altered cellular redox potential, thereby affecting the mitochondrial electron transport chain and inducing ROS generation [45] in the cellular microenvironment, which may finally cause cell death. We have also found increased NO, MDA levels, and increased cell death in Cd-exposed population, which are indicative of oxidative damage and a potential cause of cell death. Cadmium tolerance is also associated with MT gene expression in aquatic ecosystems, and the tissue-specific plasticity of the MT gene is associated with metal homeostasis in exposed populations [53]. MT gene expression is increased in species under Cd-induced stress and drives thiol-mediated detoxification [54]. Cadmium exposure with other metals also increases the expression of the MT gene in *Clethrionomys glareolus* [55]. To date, only three reports are available to highlight the differential expression of MT gene isoforms in human populations occupationally exposed to Cd [56–58]. But the lack of a human population-based study restricts the display of MT gene expression as a Cd-induced biomarker. More such studies are required to establish that as a biomarker. This is one of the major drives to explore this area of research.

Epidemiological data showed that chronic Cd exposure may induce various types of cancers, though the underlying mechanism is not clear. Oxidative stress, macromolecular breakdown, and subsequent cell death may be causative factors. MT is a Zn sequestrating protein that acts as a free radical scavenger [59]. Therefore, the degree of MT expression after Cd exposure and its dose-response analysis are important. We have found marked overexpression of the MT gene in humans chronically exposed to Cd via contaminated water and food, without reflecting a dose-response pattern. All of these Cd-associated toxicities and clastogenic actions are mediated by intracellular Cd load that escapes elimination. The intracellular Cd load increases ROS production and damages macromolecules, including DNA. This ultimately promotes cell death. Our present work, therefore, focuses on evaluating the extent of ROS-induced cellular death in the chronic Cd-exposed population of West Bengal. Protein carbonylation, NO and 8-OHdG are important markers for ROS generation in addition to MDA and antioxidant enzyme profile. We have found a significant increase in MDA levels, protein carbonylation, urinary 8-OHdG levels, and NO in the exposed population compared with the unexposed control. High levels of ROS are associated with protein carbonylation, mitophagy, DNA fragmentation, and lipid peroxidation in cells and eventually cause cell death [60, 61]. Protein damage is estimated by measuring protein carbonylation, a marker of oxidative stress. Heavy metals, including Cd, can induce protein carbonylation in different systems, including plants and fish [62–64]. The formation of carbonyl derivatives of proteins is irreversible and increases the susceptibility of proteins and thus causes protein breakdown [62]. Protein carbonylation is associated with abnormal protein folding and causes precipitation of several diseases, including neurodegenerative diseases in humans and animals [65–67]. Heavy metal exposure has been reported as a cause of oxidative damage among municipal waste workers in China and has been shown to significantly increase DNA oxidation levels (Yang et al. [68]). Significantly elevated levels of protein carbonylation and urinary 8-OHdG in persons chronically exposed to Cd through water and food, as observed in our study, are quite similar to previously reported levels (Yang et al. [68]). DNA damage is reported from the inhabitants of Lahore, Pakistan, who are exposed chronically to high levels of HMs, including Cd [69]. Comet is also an important biomarker for HM-induced genotoxicity [70]. Persons chronically exposed to Pb showed a significant level of DNA damage estimated by the comet assay [71]. Although no report is available in favour of the Cd-induced comet assay in exposed humans. A very few reports are available to point out the incidence of DNA damage by the comet assay on microorganisms [72]. NO may be a biomarker alongside other established biomarkers of tissue damage in HM exposure in living organisms. Conflicting reports are available for HM-induced NO generation in plant tissues. NO enhances Pb-induced cytotoxicity [73] in one report, whereas, in other reports, it can ameliorate HM-induced toxicity in plants by increasing antioxidant capacity. In Cd exposure, reduces Cd translocation [74] and also blocks the translocation of metals in plant tissue. In humans, it has been reported that Cd induces nitric oxide synthase (NOS) gene transcription, which is further dependent on NOS gene polymorphisms [75]. Nitric oxide replaces Cd in MT, thereby increasing the cellular Cd load. NO is an important intracellular messenger in inflammatory responses. It may reduce Cd-induced cell viability and decrease colony-forming ability. NO is reported to cause an over-expression of the C-Myc gene and promote cancer progression [59]. Our data is quite similar to some of the reports available regarding Cd-induced cytotoxicity studied on a cell culture system. This is the first report of a Cd-exposed population in West Bengal, India, experiencing chronic Cd exposure resulting from contamination of the River Churni by industrial effluents. This report is a cross-sectional study to demonstrate the association between oxidative stress and cell death after Cd exposure with MT gene overexpression in a population chronically exposed to it. However, the study has some limitations. The study was conducted with a small group of 110 participants. The sample group recruited in the study is representative of a large population and reflects the health hazards of chronic Cd exposure. All participants selected for our work were randomly chosen without bias. Though initially 187 study participants were recruited, some of the participants were excluded due to their past medical history and current medications for hypertension, hypothyroidism, respiratory illness, and anaemia. Local people also showed reluctance to participate in this study. We haven't extensively examined the concentrations of other metals or metalloids in all the factories studied for their sludge, and therefore failed to find any associations with our parameters.

Another limitation of the study is the loss of scope to study the speciation of Cd in the body. The absence of scope to study the bioavailability, bioaccumulation, and biotransformation of Cd in the human body, which may further potentiate the impact of Cd exposure through MT gene overexpression and oxidative damage. Speciation of Cd can provide more precise information on the ratio of body retention to urinary excretion, which is the factor controlling the health hazards associated with its chronic exposure. Further, the assay of Cd concentration in water and all biological samples is a single-point exposure assay. It was not repeated across different areas, times, or seasons to justify the constant exposure to river water. The samples were collected from April to June, which reflects the summer in West Bengal. We have not conducted the survey and sample collection to assess any seasonal variation in Cd deposition in river water or in food chain contamination. However, the health hazards associated with chronic exposure remained the same throughout the year, as evidenced by historical data obtained during biological sample collection from study participants.

Therefore, to establish the degree of MT gene expression as a protective event and biomarker for Cd exposure, the study should be continued in a large sample group with factual corrections of all the limitations, if possible. In the near future, we would like to share our findings on the biotransformation and bioaccumulation of Cd and their health hazards in an exposed population in a dose-response pattern.

CONCLUSION

Cadmium contamination of the river Churni may be one of the factors contributing to oxidative stress and cellular damage in people chronically exposed to it through drinking water and food, though it may not be the only one. Maybe other contributing factors, like water pollution from sewage discharge and the leaching of other metals and metalloids from factory wastes, poor nutrition, also have added contributions. But continuous water surveys revealed a marked increase in Cd in the river, particularly at the point of Cd-induced damage. People showed significant DNA oxidation, protein carbonylation, overexpression of the MT gene, and apoptosis in peripheral blood leukocytes, as assessed by the comet assay. Furthermore, the degree of oxidative damage (8-OHdG, protein carbonylation, NO, MDA) and apoptosis is not correlated with MT gene expression in the exposed population, although MT gene expression increases significantly after exposure. Therefore, the study's conclusion is a positive correlation between the degree of oxidative damage and MT gene expression in response to Cd exposure.

DATA AVAILABILITY

Raw data was collected from a field survey on the selected blocks at the catchment area of the River Churni. It is not publicly available at any site or library. Individual participant raw data would be available from Rammohon College Central Library as well as from the corresponding author.

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AUTHOR CONTRIBUTIONS

SG and SC performed all wet-lab work, and SC, SG and AS collected the samples and completed the survey work before sampling. SC and RM wrote the full manuscript. SG and DS had done the statistical analysis and final polishing. SM has done the formatting, figure, and table preparation for the manuscript. AKS, RM and SM contributed to sampling and cadmium concentration measurements in water and body fluids.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

Ethical approval was taken from the Rammohan College Human Ethical Committee (No: REC/ENV005/0021, dated 09/12/2021) as well as the Kalyani University Human Ethical Committee (No: 139H-Phy-2019, dated 19/11/2021), which are designed and framed according to the Helsinki Declaration. Research meets ethical guidelines.

CONSENT TO PARTICIPATE

All of the study subjects from Cd-exposed as well as unexposed counterparts have given their written informed consent before drawing their blood, taking urine samples and food samples. A representative copy of written informed consent in English and Bengali is uploaded as supplementary materials.

CONSENT TO PUBLISH

Written informed consent was taken from all the study subjects before drawing their blood and taking their urine samples. All of the study subjects have given their consent for the publication of their data in the present format (hiding personal data and pictures).

ADDITIONAL INFORMATION

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