Journal of
Cancer Science & Therapy

Sinha et al., J Cancer Sci Ther 2014, 6.3
DOI: 10.4172/1948-5956.1000250

Research Article Open Access

Chronic Low Level Arsenic Exposure Inflicts Pulmonary and Systemic Inflammation

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Abstract

Objective: To examine whether chronic low level arsenic (As) exposure (11-50 µg/L) from drinking water elicits inflammation and oxidative stress.

Methods: Never-smoking pre-menopausal women (n=267) from Nadia district, West Bengal, India, were enrolled into two groups (i) control (n=122, median age 39 yr) from villages with <10 µg/L of As in groundwater, and (ii) exposed (n=145, median age 38 yr) from the same district where the groundwater As was 11-50 µg/L. As in water was measured by atomic absorption spectrophtometry with vapour generation assembly. Sputum cytology and hematology were done by standard procedures. Enzyme-linked immunosorbent assays were used to measure tumor necrosis factor-alpha (TNF-α), interleukin-6, 8, 10, 12 (IL-6, IL-8, IL-10, IL-12) and C-reactive protein (CRP) in plasma and cortisol in serum. Serum nitric oxide (NO) was measured colorimetrically, myleperoxidase (MPO) and neutrophil elastase by spectrophotometry, reactive oxygen species (ROS) by flow cytometry, and inducible nitric oxide synthase (iNOS) by immunocytochemistry.

Results: As level in groundwater was higher in endemic areas (28.32 ± 13.51 vs. 2.72 ± 1.18, $p<0.05$), and exposed women had lower hemoglobin, leukocyte and erythrocyte levels but elevated platelet count than control and their sputum contained increased number of alveolar macrophages and inflammatory cells. In addition, they had elevated levels of TNF-α, IL-8, IL-6, IL-12, CRP, cortisol and NO but depleted level of IL-10 with excess generation of ROS and increased expression of iNOS in the airways. Neutrophils of As-exposed subjects had elevated levels of MPO and elastase. After controlling education and family income as potential confounders, the rise in pro-inflammatory mediators in blood and excess generation of ROS in the airways were positively associated with As levels in ground water.

Conclusion: Drinking of water contaminated with low level of As for long causes pulmonary and systemic inflammation and generates excess ROS in the airways.

Keywords: Airway inflammation; Arsenic; Women; India

Introduction

Groundwater arsenic (As) contamination has been one of the prime public health concerns due to the devastating toxic effects of the metalloid. In the Indian state of West Bengal and adjoining Bangladesh, an estimated 100 million people are at risk of exposure to groundwater As above the World Health Organization (WHO) guideline value of 10 µg/L [1,2]. On an average, more than 50% of the aquifers in the Ganga-Meghna-Brahamaputra plain of India and Bangladesh have As level above the WHO recommended limit [3].

Prolonged ingestion of As leads to its accumulation in the liver, kidneys, heart and the lungs and in smaller amounts in the muscles, nervous system, gastrointestinal tract and the spleen [4]. As a consequence, almost all the organ systems of the body could be the target of its toxic insults, resulting in multi-organ damage [5]. Chronic exposure to As causes a wide range of adverse health effects, including increased risk of carcinogenesis [6,7], cardiovascular disease [8] diabetes mellitus [9], neuropathies [10], liver disease [11], and skin lesions such as rain drop pigmentation, hypopigmentation, hyperpigmentation, keratoses and hyperkeratosis [12]. As may cause both malignant and non-malignant respiratory diseases like lung function reduction and chronic obstructive pulmonary disease (COPD), a progressive and potentially life-threatening lung condition [13]. Considering these, the European Union (EU), the United States Environmental Protection Agency (US EPA) and WHO have established a value of 10 µg As/L as the maximum contaminant level (MCL) for total As in potable water [14]. However, the Indian national standards for groundwater As has remained 50 µg/L, five times higher than the WHO permissible limit [15]. Although the pulmonary and systemic toxicities of As above 50 µg/L are now well recognized, the health impact of chronic low dose As (11-50 µg/L) exposure is relatively unknown despite the fact that several million people of West Bengal are still drinking water that contains As above the MCL [1].

Inflammation is body's response to a variety of stress such as cellular damage, tissue injury and infection. It involves a cascade of events mediated by a large array of cells (e.g., mast cells, macrophages, neutrophils and lymphocytes) and molecules (inflammatory cytokines, free radicals, and Damage-Associated Molecular Pattern molecules) that locate invading pathogens or damaged tissue, alert and recruit other cells and molecules, eliminate the offending agents, and finally

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Received January 21, 2014; **Accepted** February 19, 2014; **Published** February 24, 2014

Citation: Sinha D, Mukherjee B, Bindhani B, Dutta K, Saha H, et al. (2014) Chronic Low Level Arsenic Exposure Inflicts Pulmonary and Systemic Inflammation. J Cancer Sci Ther 6: 062-069. doi:10.4172/1948-5956.1000250

restore the body to equilibrium [16]. Chronic inflammation, on the other hand, contributes to the pathobiology of many disease conditions including malignant neoplasms. The possible mechanisms by which inflammation can contribute to carcinogenesis include genetic and epigenetic changes causing inappropriate gene expression and consequent genomic instability that may lead to enhanced proliferation of initiated cells, resistance to apoptosis, angiogenesis and metastasis [17-19]. Chronic As exposures have been shown to induce skin, urinary, liver and lung cancers [20]. Investigators have suggested that As absorption results in oxidative stress and associated chromosomal abnormalities, DNA damage, reduced DNA repair, growth factor alteration, and altered cell signaling [21,22].

Inflammatory cells are particularly effective in producing most of the reactive oxygen species (ROS) that generate a highly oxidative environment within an organ of aerobic organisms. There have been reports that As evokes inflammation, increases serum levels of pro-inflammatory mediators interleukin-6 (IL-6) and interleukin-8 (IL-8) [23], and generates oxidative stress by increasing the production of ROS leading to oxidative tissue damage [24,25]. Besides, inorganic As and its metabolites can increase the production of nitric oxide (NO) via activation of inducible nitric oxide synthase (iNOS) [25]. Most of these investigations on As-associated inflammation have been carried out *in vitro* or in animal models and the human population studies deciphering the inflammatory response to As exposure have been limited to high level of exposures [26,27]. Therefore, it seems worthwhile to examine whether chronic low level As exposure elicits inflammation and oxidative stress that, in turn, can increase the risk of adverse health conditions including the development of cancer. With this view, the present study was designed to investigate whether chronic exposure to relatively low dose of As (11-50 µg/L) in drinking water elicits pulmonary and systemic inflammation in rural women of West Bengal.

Materials and Methods

Participants and study areas

Two hundred and sixty seven adult never-smoking women in the age group of 22-45 yr (median 39 yr) residing in 4 villages of Nadia district of West Bengal, India, were enrolled. The participants were subdivided into 2 groups on the basis of As in drinking water from the tube wells: (i) control (n=122, median age 39 yr) - from villages with relatively low level of As (<10 µg/L) (ii) exposed (n=145, median age 38 yr) from the same districts where the groundwater As level was in the range of 11-50 µg/L. They were invited to participate in this study through village *panchayats* (local administration), and non-government organizations after obtaining informed consent.

Inclusion and exclusion criteria

Inclusion criteria were (i) drinking water from the village tube wells for the past 10 years or more; (ii) non-smoker without prior history of COPD or other respiratory diseases, and non-chewer of tobacco, betel nut/ betel quid, and non-consumer of alcoholic beverages. Exclusion criteria were (i) having As-related skin lesions like palmer and planter keratosis, hyperkeratosis, raindrop pigmentation (ii) under medication, (iii) having a past history of malignant diseases, (iv) pregnant and lactating women. The study protocol was approved by the Ethics Committee of Chittaranjan National Cancer Institute.

Measurement of arsenic in water samples

The hand pumps of the tube wells were pressed so as to flow the water for 5 min and then the water samples were collected in sterile polyethylene bottles with 0.1% HCl. The As analysis in collected water samples was done by atomic absorption spectroscopy (AAS) using vapour generation assembly (VGA) according to the procedure of Behari and Prakash, 2006 [28]. In brief, a stock solution of 100 ppm (0.1 mg/L) As was prepared from As standard for AAS (Sigma-Aldrich) in milli Q water with 0.1% HCl. Subsequently a range of standards (1-50 µg/L) were prepared with dilution of the stock solution for calibration before use. The Atomic Absorption Spectrophotometer Duo (Agilent Technologies) equipped with vapour generation assembly (Agilent VGA 77) was used for the estimation of arsenic. The instrument was calibrated using 5 M HCl in the acid channel and 0.6% $NaBH_4$ and 0.5% NaOH in the reduction channel in the working range of 10-50 ppb As standard. For pre reduction experiment, the sample was prepared in 5 M HCl and 20% potassium iodide (KI) was added and allowed to react up to 45 min at room temperature. For direct analysis, the unknown samples were diluted in varying concentrations and pre reduced in the same way as the standards. A standard curve was drawn with the prepared range of standards and the concentrations of arsenic in the unknown samples were ascertained from the standard curve with the help of the software Spectra AA. The samples were analyzed in duplicate and three readings were taken for each sample. For quality control, inter-laboratory tests of the same samples were done along with School of Environmental Studies (SOES), Jadavpur University, Kolkata. The inter-laboratory differences were within 5-10%.

Assessment of sputum cytology

The early morning sputum was collected from each subject for three consecutive days. The participants were requested to rinse their mouth with sterile normal saline (0.9% NaCl) and to cough vigorously. The sputum was collected in a sterile container. The non-transparent highly viscous parts of sputum were smeared on clean glass slides with the help of a sterile spatula. The smears were fixed immediately at the site of collection in dehydrated ethanol for 30 min for Papanicolaou (Pap) staining following the procedure of Hughes and Dodds, 1968 [29]. Pap stained slides were coded and scored blindly. At least 500 cells in each slide (excluding squamous epithelial cells) were screened under light microscope (Leitz, Germany) by two independent observers. The samples were considered adequate and representative of the lower airways if squamous epithelial cell contamination was less than 20% and either cylindrical epithelial cells or alveolar macrophages (AM) or both were present [30]. Average number of cells in each high power field (40x objective x 10x eyepiece, i.e. 400x magnification) was calculated after counting at least 10 randomly selected fields. AM, basal and parabasal cells, goblet cells, ciliated and non-ciliated airway epithelial cells, sputum neutrophils, eosinophils and lymphocytes, metaplasia and dysplasia of airway epithelial cells, goblet cell hyperplasia, ciliocytophthoria and Curschmann's spirals were identified by established criteria [31]. The differential distribution of sputum cells was expressed as percentage of total non-squamous cells.

Hematology and measurement of cytokines by ELISA

Blood was collected between 10.00 and 11.00 am from the antecubital vein using 5-ml disposable plastic syringes into sterile EDTA-anticoagulated vacutainers and sterile no-additive vacutainers (Becton Dickinson, Franklin Lakes, NJ). Blood in the no-additive vacutainer was allowed to clot and serum was collected. Total and differential count of blood cells was done by standard procedures [32].

Enzyme-linked immunosorbent assay (ELISA) was used for

measurement of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), interleukin-10 (IL-10) in blood plasma, and C-reactive protein (CRP), cortisol, and nitric oxide (NO) levels in serum using commercially available kits. TNF-α, a pro-inflammatory cytokine, was measured using human ELISA kit # 550610 of BD Biosciences, San Diego, CA, USA with lowest detection level of 2 pg/ml; IL-8, a pro-inflammatory chemokine, by kit # 1967932 of Roche Diagnostics GmbH, Mannheim, Germany with lowest detection level of 6.2 pg/ml; IL-6, another pro-inflammatory cytokine, by kit of BD Biosciences, USA; IL-12, a pro-inflammatory cytokine, by BD Opt EIA # 559258, BD Biosciences, USA; IL-10, an anti-inflammatory cytokine, by kit # 550613, BD Biosciences, USA; CRP by kit # EU59131 of IBL, Hamburg, Germany with lowest detection level of 1 μg/ml; cortisol by ELISA kit # RE52061 of IBL, Hamburg, Germany with detection limit of 2.5 ng/ml; and NO level in serum was measured as nitrate by ELISA kit #98, Oxford Biomedical Research, Oxford, MI, USA with lowest detection level 1 pmol/μl.

MPO in neutrophils

Myeloperoxidase (MPO) enzyme, located within the primary granules of neutrophils, is considered as an indicator of neutrophil activation and corresponding inflammatory response. MPO activity was cytochemically localized in peripheral blood neutrophils smeared on glass slides following the procedure of Kaplow and Ladd, 1965 [33] using benzidine dihydrochloride. Quantitative measurement of MPO in isolated neutrophils from peripheral blood was determined by an assay involving ortho (O)-dianisidine [34]. Peroxidase present in neutrophils oxidizes O-dianisidine to produce a colored complex, which is spectrophotometrically read at 460 nm. The assay mixture, contained 0.3 ml of 0.1 M PBS (pH 6.0), 0.3 ml of 0.01 M H_2O_2, 0.5 ml of 0.02 M freshly prepared O-dianisidine (Loba Chem, Mumbai, India) in deionized water and 10 μl of neutrophil homogenate in a final volume of 3 ml. The neutrophil homogenate was added last in a cuvette with a path length of 1 cm and the change in absorbance in 460 nm was followed for 10 minutes. One unit of MPO is defined as that giving an increase in absorbance of 0.001 per minute. The specific activity of the enzyme is expressed as IU/mg protein.

Quantitative measurement of elastase in isolated neutrophil

The level of intracellular elastase in isolated blood neutrophils was measured spectrophotometrically following the procedure of Oltmann et al. [35]. It uses the chromogenic peptide N-methoxy-succinyl-Ala-Ala-Pro-Val-p-nitroanilide (MSAAP, Sigma Chem, USA) as substrate. It is a sensitive and specific assay for neutrophil elastase in which the enzyme hydrolyzes non-fluorescent substrate MSAAP into highly fluorescent p-nitroanilide. In brief, neutrophils isolated from EDTA-anticoagulated whole blood were adjusted at a cell concentration of 2.0×10^6/ml. Then 1.0 ml of the cell suspension was incubated with 1.0 ml of ice-cold lysis buffer that contains 20 mM Tris-HCl (SRL, Mumbai, India), 150 mM NaCl, 1 mM Na_2EDTA, 1% Triton X-100 (Sigma Chem, USA), and 1 μg/ml leupeptin, a protease inhibitor (Sigma Chem, USA). The cells were incubated with the lysis buffer for 20 min at 4°C in order to disintegrate the neutrophils so that the enzyme can be released into the cell lysate. Following incubation, the mixture was centrifuged at 5000 rpm for 5 min at 4°C, and 1.0 ml of the supernatant containing the released enzyme was collected. To the supernatant, 10% dimethylsulfoxide (DMSO, Sigma Chem, USA) and MSAAP substrate (2 mM) were added making the total volume to 1 ml at pH 7.5. The color developed in the sample was read at 410 nm in a spectrophotometer (Varian, model Carry 100, Austria) and the optical density (OD) of liberated p-nitroanilide was measured. A standard curve was prepared using varying concentrations (0.1-8.0 μg/ml) of purified human neutrophil elastase (HNE) purchased from Sigma Chemical, USA. Elastase enzyme activity in sample was extrapolated from the standard curve using OD of sample and standard.

Measurement of ROS generation

Generation of reactive oxygen species (ROS) in leukocytes in peripheral blood and airway cells in sputum was measured by flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA) [36]. In brief, 3 ml of EDTA-anticoagulated whole blood was centrifuged at 200 g at 4°C for 10 min and the buffy coat containing leukocytes and supernatant plasma were collected separately. An aliquot of 200 μl buffy coat was mixed with 300 μl of freshly collected plasma. The mixture was diluted with 1.0 ml of Hank's balanced salt solution (HBSS) containing NaCl (0.15 M) and HEPES (5 mM), pH 7.35. Thereafter DCFH-DA (0.5 mM; Sigma Chemicals, Saint Louis, MO, USA) solution in dimethyl formamide was added to the cell suspension and incubated at 37°C for 30 min in darkness. Finally the samples were washed in ice-cold phosphate-buffered saline and 10,000 events were acquired in flow cytometer (FACS Calibur with sorter, BD, San Jose, CA, USA) using Cell Quest software (BD, San Jose, CA, USA) equipped with a 488 nm argon laser and a 525 ± 10 nm band pass emission filter. Fluorescence was captured on fluorescence channel-1 with logarithmic amplification. Sputum cells were prepared as described earlier, and the generation of ROS was measured by flow cytometry like the blood cells acquiring 10,000 events. Respiratory burst and generation of ROS by the phagocytes resulted in green fluorescence that was recorded in and was expressed as mean fluorescence intensity (MFI) in arbitrary unit.

Detection of iNOS by immunocytochemistry (ICC)

Expression of iNOS in airway cells was localized by ICC following the procedure of Rajendran and Varkey, 2007 [37]. In essence, sputum was treated with 1% dithiothreitol (DTT, Sigma Chem, USA), kept on DMEM media (GIBCO Laboratories, USA) for 30 min, centrifuged at 1200 rpm for 4 min at 4°C. The cell pellet was washed in PBS, fixed in ethanol, washed in distilled water, and quenched in 3% hydrogen peroxide in methanol for 10 min in humid chamber. After washing, the cells were incubated with anti-human iNOS rabbit polyclonal IgG (Santa Cruz Biotechnology, USA) diluted 1:100 in PBS overnight at 4°C. The cells were then exposed to secondary antibody (goat anti-rabbit IgG-HRP: Santa Cruz Biotechnology, USA), incubated thereafter in horse-radish peroxidase substrate mixture for 45 min, observed under microscope.

Statistical analysis

Results are presented as mean ± standard deviation (SD) or median (interquartile range) with range in parentheses. The differences between groups and that between measurable parameters were determined by using Chi-square test or Student's t test, as applicable. Statistical analyses were performed using SPSS statistical software (Statistical Package for Social Sciences for windows, release 10.0, SPSS Inc., Chicago, IL). Multivariate logistic regression analysis was done to find association between As-exposure and other parameters after controlling potential confounders. Correlation was done by Spearman's rank correlation test. Statistical significance was assigned to $p<0.05$.

Results

Demographic characteristics of the participants

Demographic characteristics of control and As-exposed subjects who participated in this study are compared in Table 1. It is evident that the two groups were well-matched with respect to age, BMI, occupation and use of cooking fuel. However, exposed subjects had lower family income than the controls ($p<0.05$).

Arsenic in tube well water

The As level in tube well water of the villages where the control women resided was 2.72 ± 1.18 (SD) µg/L. The median value was 2.89 µg/L with a range of 0.89-5.07 µg/L. In contrast, the mean As concentration in the tube well water of As-endemic villages was 28.32 ± 13.51 µg/L. The median value was 29.9 µg/L with a range of 11.5-43.1 µg/L. The differences in the mean and median As concentrations in drinking water of these two areas were highly significant in Student's t-test ($p<0.0001$) and Mann-Whitney *U*-test ($p<0.0001$), respectively.

Hematological changes

Compared with the control group, the peripheral blood of As-exposed women showed 14.7% lower mean hemoglobin level, 14.8% lower erythrocyte count and 24.1% reduction in total leukocyte count. All these changes were statistically significant ($p<0.05$). The mean platelet count, in contrast, was 41.7% higher in As-exposed subjects ($p<0.05$; Table 2). The differential distribution of circulating leukocytes was also different between these two groups. The As-exposed women had 25% lower absolute number of neutrophils, 36% reduction in lymphocytes ($p<0.05$ in both cases), a mild 14% increase in the number of monocytes ($p>0.05$) but remarkable 67% increase ($p<0.001$) in the number of eosinophils. Immature neutrophils, such as myelocytes, metamyelocytes and band cells, represented 1-4% of total circulating neutrophils in controls, whereas these cells were more abundant (3-9%) in As-exposed subjects.

Parameter	Control (n= 122)	Arsenic exposed (n=145)	P value*
Age in year, median (range)	39 (22-44)	38 (21-45)	NS
Body mass index (kg/m^2), median (range)	22.7 (20.2-23.8)	22.3 (20.8-23.9)	NS
Years of schooling, median (range)	8 (0-12)	7 (0-12)	NS
Occupation (%)			
Household work only	40.2	37.9	NS
Household+Agricultural work	59.8	62.1	NS
Cooking fuel use at home (%)			
LPG, kerosene	16.4	13.8	NS
Biomass	83.6	86.2	NS
Members in family, median (range)	4 (3-7)	4 (4-9)	NS
Family income per month in US$, median (range)	88 (72-145)	72 (55-115)	<0.05

**Statistically analyzed by Chi-square test; NS, not significant*

Table 1: Socio-demographic characteristics of study population.

	Control (n=122)	Arsenic exposed (n=145)
Hemoglobin (g/dl)	14.3 ± 0.8	12.2 ± 0.9*
RBC (x10^6/µl)	5.4 ± 0.5	4.6 ± 0.6*
WBC, total (per µl)	7389 ± 1252	5608 ± 898*
Neutrophil/µl	5066 ± 387	3819 ± 268*
Eosinophil/µl	196 ± 27	327 ± 22*
Lymphocyte/µl	1924 ± 178	1234 ± 205*
Monocyte/µl	198 ± 28	226 ± 35
Platelet (x10^5/µl)	2.4 ± 0.4	3.4 ± 0.6*

*Results are mean ± SD, **p<0.05 compared with control*

Table 2: Hematological changes in arsenic-exposed subjects.

Changes in sputum cytology

The Pap-stained smears of spontaneously expectorated sputa of control and As-exposed women were evaluated microscopically to examine the impact of chronic low-level As exposure on the airway cells. Compared with control, the sputa of As-exposed women were 39% more cellular with increased number of inflammatory and epithelial cells. Greatest increase was found in the number of eosinophils which was 5-times higher than the control ($p<0.001$). The numbers of AM, lymphocytes and neutrophils were also 89%, 84%, and 28% higher than the control, respectively ($p<0.05$; Figure 1 and Table 3). Although the diameter of the AM of As-exposed subjects was not significantly different from that of controls (17.8 ± 6.7 µm vs. 18.6 ± 7.9 µm in control; $p>0.05$), the sputa of exposed women showed heavy deposits of mucus with goblet cell hyperplasia and aggregates of ciliated columnar epithelial cells. In essence, sputum cytology of As-exposed women suggests airway inflammation.

Circulating levels of pro-inflammatory mediators

The mean concentration of the pro-inflammatory cytokine TNF-α in blood plasma of As-exposed subjects was 53% higher than that of control ($p<0.001$). Similarly, the levels of plasma IL-8, IL-6 and IL-12 were 58%, 55%, and 63% higher than the control, respectively ($p<0.001$). In addition, the mean serum level of CRP of exposed women was 2.9-times higher than the control while the serum cortisol level was nearly doubled in these women ($p<0.001$; Table 4). Moreover, the As-exposed subjects had 61% more NO as nitrate in serum than

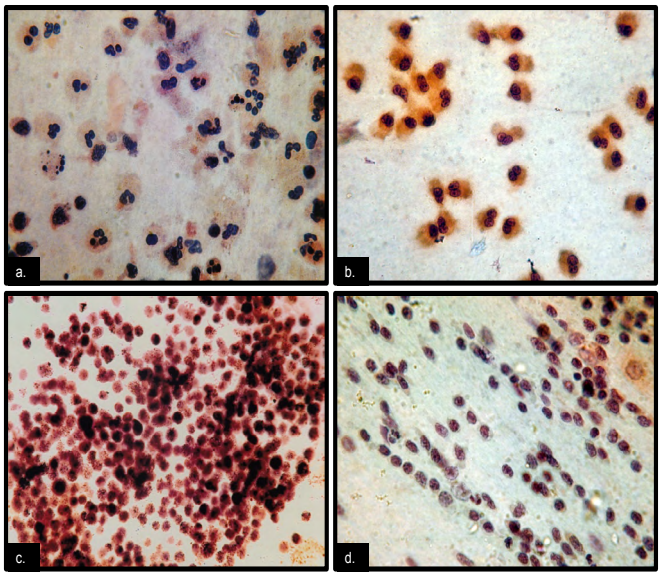

Figure 1: Photomicrographs of sputum samples of As-exposed women showing neutrophilia (a), eosinophilia (b), accumulation of alveolar macrophages (c) and lymphocytes (d), suggesting inflammation. Compared with control, the sputa of As-exposed women had 28% more neutrophils, 84% excess lymphocytes, 1.9-times more alveolar macrophages and 5-times more eosinophils. Pap-stained, original magnification 1000x.

Parameters	Control (n=122)	Arsenic exposed (n=145)
Total cells/hpf	56.5 ± 19.6	78.3 ± 28.4*
Neutrophil/hpf	49.8 ± 15.2	63.9 ± 22.6 *
Eosinophil/hpf	0.3 ± 0.1	1.5 ± 0.4*
Lymphocyte/hpf	3.2 ± 0.8	5.9 ± 1.8*
Alveolar macrophage/hpf	2.7 ± 0.7	5.1 ± 1.6*
Non-squamous epithelial cells/hpf	0.4 ± 0.2	1.8 ± 0.6*

*Results are mean ± SD; *p<0.05 compared with control in Student's t-test;, hpf, high power field of microscope (400x)*

Table 3: Differential distribution of cells in sputum.

Group	Control (n=122)	Arsenic-exposed (n=145)
Plasma TNF-α (pg/ml)	9.3 ± 3.2	14.2 ± 8.2*
Plasma IL-8 (pg/ml)	15.3. ± 3.8	24.1 ± 7.6*
Plasma IL-6 (pg/ml)	5.6 ± 1.9	8.7 ± 2.4*
Plasma IL-12 (pg/ml)	15.1 ± 2.8	24.6 ± 5.9*
Plasma IL-10 (pg/ml)	3.2 ± 0.3	2.5 ± 0.6*
Serum CRP (µg/ml)	3.2 ± 0.8	8.8 ± 2.4*
Serum cortisol (µg/dl)	21.3 ± 2.5	39.7 ± 4.8*
Serum nitric oxide (µM)	23.2 ± 7.2	37.3 ± 12.7*

Values are mean ± SD*; *p<0.001 compared with control in unpaired Student's t test*

Table 4: Concentrations of circulating pro- and anti-inflammatory mediators.

the controls (p<0.001; Table 4). In contrast, the plasma level of the anti-inflammatory cytokine IL-10 was 22% lower than the control (p<0.001). Collectively, the results suggest systemic inflammation in women who were chronically exposed to low level of As in drinking water. After controlling for age, occupation, education and family income as potential confounders, a strong positive association was found between As in drinking water with plasma TNF-α (OR=2.83, 95% CI: 1.74-4.16), IL-8 (OR=1.96, 95% CI: 1.48-3.18), IL-6 (OR=2.16, 95% CI: 1.48-3.78) and serum NO (OR=1.37, 95% CI 1.12-1.68).

MPO and elastase levels in neutrophils

The MPO level was 0.516 ± 0.10 (SD) Units/ml in As-exposed women against 0.264 ± 0.07 Units/ml in control (p<0.001), implying near doubling of this enzyme following chronic low level As exposure. Neutrophil elastase was also increased by 58% from 1.25 ± 0.31 µg/ml in controls to 1.97 ± 0.52 µg/ml in As-exposed subjects (p<0.05; Figure 2).

Differential ROS generation in blood and sputum

ROS generation was measured in blood and sputum cells by flow cytometry. Compared with control, the mean MFI of DCFH-DA was markedly lower (56.4 ± 24.6 in exposed vs. 662.4 ± 117.5 in control, p<0.001) in circulating leukocytes of As-exposed subjects (Figures 3a and 3b). In contrast, the mean MFI was 5-fold higher in sputum cells of the As-exposed women when compared with that of the control group (233.2 ± 44.7 in exposed vs. 43.7 ± 17.8 in control, p<0.001; Figures 3c and 3d). The results suggest remarkable decline in ROS generation in circulating white blood cells, but a substantial rise in ROS production by the airway cells following chronic ingestion of As-contaminated groundwater.

Changes in the expression of iNOS in airway epithelium

In ICC, immuno-staining for iNOS was detected in neutrophils of expectorated sputum of both control and As-exposed women. But the percentage of sputum neutrophils expressing iNOS activity was significantly higher in As-exposed women (21.7 ± 7.4 (SD) vs. 10.3 ± 3.8%, p<0.05; Figures 3e and 3f), suggesting up-regulation of nitric oxide production by airway neutrophils following chronic low-level As exposure.

Discussion

Several million inhabitants of eastern India drink tube well water contaminated with As below the Indian standard of 50 µg/L but above the WHO guideline of 10 µg/L. The objective of this study was to investigate whether drinking of tube well water contaminated with that low level of As (11-50 µg) for long causes inflammation and oxidative stress in the airways. For this we selected the endemic and control areas from the same district so that the participants come from same geographical area having similar ethnicity, socio-economy and demography. Our findings confirmed that chronic low-dose As ingestion elicits inflammatory response and oxidative stress in the airways.

Cytokines like TNF-α [38] and IL-6 [39] play pivotal role in mediating systemic inflammation in association with elevated levels of cortisol [39], NOS and NO [40]. We found rise in the circulating levels of these pro-inflammatory molecules in women who were chronically exposed to As. Cortisol, a stress hormone released from the adrenal glands following stimulation of the hypothalamus-pituitary-adrenal (HPA) axis, directly influences insulin levels and plays a role in mediating chronic inflammation [41]. Elevated level of CRP, an acute phase protein produced by the liver, could be due to direct action of As on the liver, or indirectly via the inflammatory cytokines that mediated CRP production in the liver [42]. As-induced injury to the hepatic cells may induce increased secretion of the inflammatory cytokines (IL-6, IL-8, TNF-α) which, in turn, contributes to cardiovascular risk [11].

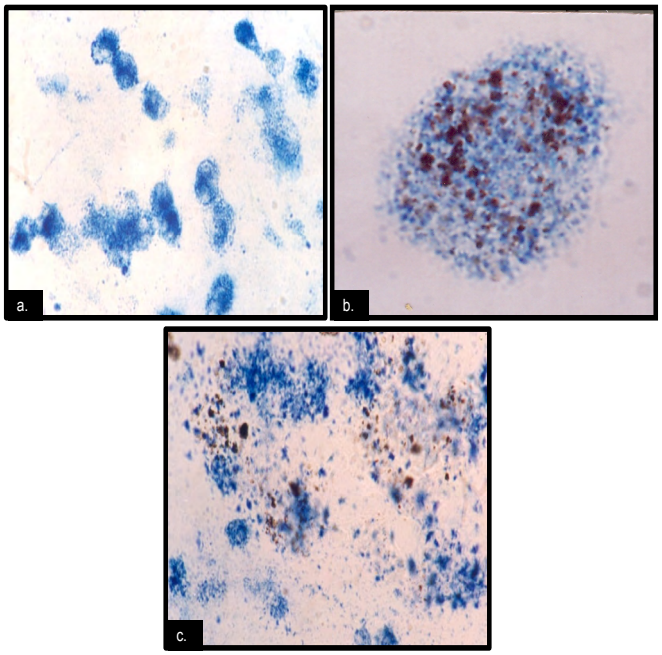

Figure 2: Photomicrographs showing cytochemical localization of high level of elastase enzyme in sputum neutrophils (a) and alveolar macrophages (b) of As-exposed women. In some exposed subjects, the enzyme was released from the cells into the surrounding medium (c) that may lead to tissue injury. The quantitative study showed 58% increase in the enzyme level in neutrophils of As-exposed women (1.97 ± 0.52 µg/ml vs. 1.25 ± 0.31 µg/ml in control, p<0.05). Elastase cytochemistry, original magnification 1000x.

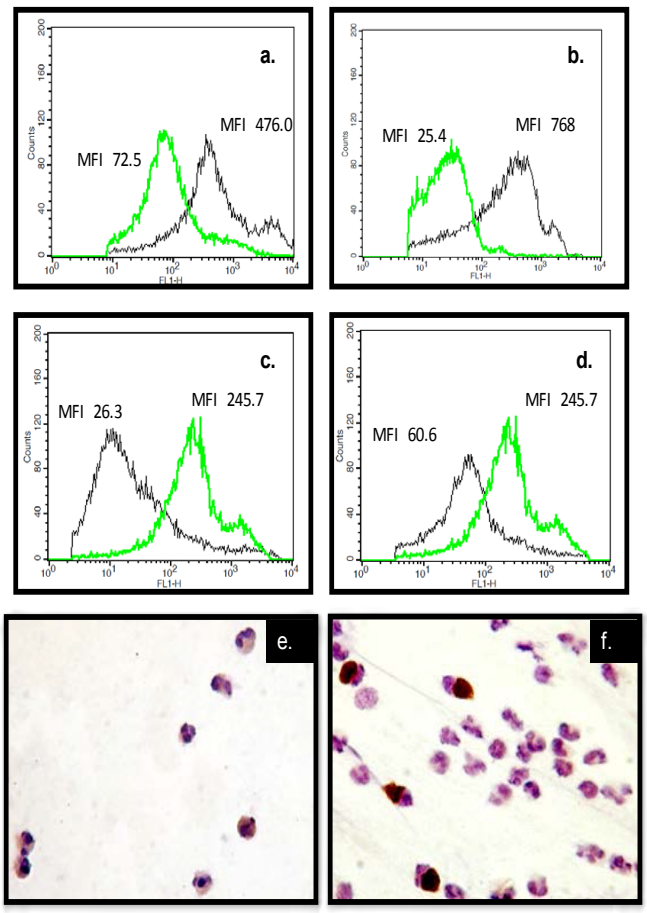

Figure 3: Oxidative stress in As-exposed subjects. Flow cytometric measurement of ROS generation in peripheral blood leukocytes (a and b) and airway cells in sputum (c and d) shows increased ROS generation in sputum but reduced in blood leukocytes of As-exposed women (green line). A total of 10,000 events were acquired for each sample, and the mean fluorescence intensities were 56.4 ± 24.6 in exposed vs. 662.4 ± 117.5 in control, $p<0.001$ for blood leukocytes and 233.2 ± 44.7 in exposed vs. 43.7 ± 17.8 in control, $p<0.001$ for sputum cells. The inducible nitric oxide synthase activity was also detected in a greater percentage of sputum neutrophils of As-exposed women (f) compared with control (e), Immunocytochemical staining counterstained with hematoxylin, original magnification 1000x.

Besides, higher CRP level can contribute to metabolic syndrome and cardiovascular disease [42]. Although the precise mechanism of the rise in pro-inflammatory molecules in circulation of exposed women is not known, As has been shown to induce over-expression of the concerned genes [43]. In general agreement with the present findings, earlier investigators have shown increased production of pro-inflammatory cytokines IL-8 [43,11] and IL-6 [11] in the respiratory epithelium following As exposure [5].

Neutrophils are among the first cells that migrate from blood to the tissues during inflammation. Neutrophil transmigration is driven largely by IL-8, a potent neutrophil chemoattractant. In case of lung injury, the AM produces TNF-α that in turn stimulate airway epithelial cells to produce IL-8. Although As can directly stimulate epithelial cells and neutrophils to produce IL-8 [44], concomitant rise in both IL-8 and TNF-α in As-exposed women of this study suggests indirect action of As via airway injury and TNF-α production by the AM. Marked rise in the number of AM in sputum, as observed in exposed women, supports this argument. As-exposed women had higher level of neutrophil elastase, a serine protease stored in the azurophilic granules of these cells. While the intracellular elastase degrades invading pathogens, extracellular elastase released by neutrophils assists in neutrophil migration to inflammatory sites by degrading extracellular matrix proteins [45]. Therefore, excess generation and release of neutrophil elastase in exposed women could be an adaptive measure to facilitate inflammation in order to combat airway injury.

Inflammation is pivotal in clearing the invading and offending agents and promoting tissue repair for restoration of homeostasis. Prolonged inflammation, however, can cause damage to the tissues, which in turn leads to the production of molecules that re-stimulate inflammation. This 'feed forward' loop sets in motion a vicious cycle of inflammation → damage → inflammation promoting organ dysfunction [46], metabolic [47] and cardiovascular diseases [11] and cancer [48]. Both IL-6 and TNF-α regulate the synthesis of other acute phase proteins which are established risk factors for atherosclerosis [49-54]. Circulating levels of IL-6, IL-8 and monocyte chemotactic protein-1 which are often elevated in As-exposed subjects [11], are considered early biomarkers of increased cardiovascular risk. The changes in the circulating levels of the inflammatory mediators among exposed women were positively associated with groundwater As levels after controlling for potential confounders. Therefore, it seems that the inflammatory response was evoked by the long-term ingestion of As-contaminated water. Like the present observation, As-mediated inflammation has been demonstrated in mice [54] and in human subjects [52]. Inflammation of the lung has been documented among the residents of Arizona, USA who were exposed to a relatively lower dose of As (about 20 μg/L) in drinking water [55].

We found enhanced ROS generation and increased iNOS activity in the airway cells of As-exposed women, implying oxidative stress in the airways. Our findings are consistent with the reports that As increases ROS generation in pulmonary as well as extrapulmonary sites [50]. The reason for the observed decline in ROS generation by circulating leukocytes is currently unknown, but the finding is important as it implies reduction in anti-bacterial defense following sustained low-dose As exposure. MPO, an important member of the oxido-reductase family, can increase the burden of ROS via conversion of hydrogen peroxide into hypochlorous acid and chloride anion. Therefore, increase in MPO in individuals chronically exposed to low (present findings) and high levels of groundwater As [51] assume clinical significance.

As-exposed women of this study had reduced hemoglobin, RBC and WBC levels. Policemen in Italy having chronic As exposure also showed reduced RBC, hematocrit and hemoglobin levels [52]. Anemia due to reduced deformability and premature destruction of RBCs is common in inflammation [53]. Among As-exposed subjects, we found several red cell abnormalities like aniso-poikilocytosis that suggest dysregulated erythropoiesis and the possibility of covert hemolysis. In addition, elevated circulating NO may cause impairment of RBC deformability and consequent anemia [53].

In essence, we found an association between low dose As exposure from drinking water and both airway (inflammatory changes in sputum cytology) and systemic inflammation (raised pro-inflammatory mediators and lower anti-inflammatory IL-10 in circulation). As chronic inflammation contributes to the development of many disease processes such as respiratory, cardiovascular and metabolic diseases, the findings are important from public health perspectives. Of particular important are the facts that all our participants were in child-

bearing age, and maternal As exposure elicits stress, inflammation and apoptosis and impacts gene expression in the newborn [26].

Admittedly, there are certain limitations of this study. First, self-reported demographic and medical information may suffer from bias. Second, the concentrations of As in groundwater can vary considerably within a small geographic area and depth of the well. Third, the As concentrations in drinking water, as documented in this study, represent only the current exposures. In order to minimize these limitations, we measured As in drinking water of all the tube wells in the study areas, and included women who were living at the same address for the past ten years or more. Besides, we followed a population-based study design and collected data by personal interview that might have lessened the bias, if any. However, we do not think that we have excluded the influence of all potential confounders through multivariate logistic regression analysis. Socio-economic status, nutrition, personal hygiene might have influenced the outcome. In addition, we did not have personal exposure data such as urinary arsenic or arsenic species (in urine or toenails), which could have shed additional light on individual arsenic methylation and subsequent health risk. Therefore, the findings should be considered in the light of these limitations. However, the sample size of this study is large enough to conclude that chronic exposure to even low level of As that is above the WHO limit but very much within the prevailing Indian Standard generates pulmonary as well as systemic inflammation. This could be part of a defense strategy to control As toxicity and to repair tissue injury. But an exaggerated and persistent inflammation could be detrimental for the lungs and the airways as many of the inflammatory products including proteolytic enzymes released by the recruited leukocytes damage the healthy tissue [56], making the body susceptible to infections [57], cardiovascular diseases [58] and malignant transformation [59,60]. Therefore, the findings highlight the immediate need for supply of As-free drinking water in the affected areas.

Acknowledgements

The authors would like to acknowledge the funding agency, Central Pollution Control Board, Govt. of India, for providing the grant support for the project. The authors would also like to thank Prof. (Dr.) Jaydip Biswas, Director, Chittaranjan National Cancer Institute for providing the infrastructural facilities.

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