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Sputum Cytological Changes in Rural Women of West Bengal Chronically Exposed to Low Level Groundwater Arsenic Contamination

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Abstract

Background: Groundwater arsenic (As) contamination is an alarming public health concern in West Bengal, India.

Methods: The effect of chronic low dose (11-50 µg/L) As ingestion on airway cells has been investigated in 605 never-smoking women (mean age 40 yr) in Nadia and North 24-Parganas district of West Bengal. Another 512 age-matched never-smoking women from control areas (As<10 µg/L) of these districts were enrolled as control. Cytology of spontaneously expectorated sputum was done following Pap staining, elastase enzyme in sputum cells was localized cyto chemically, and iron-laden macrophages (siderophages) were detected after Perl's Prussian blue staining. Plasma levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8) and C-reactive protein (CRP) were measured by ELISA.

Results: Groundwater (As) was 28.3 ± 13.51 µg/L in exposed and 2.72 ± 1.18 µg/L in control areas. Sputa of As-exposed women were 42% more cellular and contained significantly increased ($p<0.001$) numbers of neutrophils, eosinophils, lymphocytes and alveolar macrophages (AM) than control. In addition, they showed goblet cell hyperplasia, mucus hypersecretion, and sputum cell atypia such as multinucleated cells, and metaplasia of airway epithelial cells. As-exposure was associated with abundance of siderophages, excess production of elastase in sputum neutrophils and AM, and elevated plasma levels of TNF-α, IL-6, IL-8 and CRP ($p<0.001$).The observed changes were positively associated with As levels in ground water after controlling age, body mass index, education, occupation and family income as potential confounders.

Conclusion: Chronic exposure to even low level As (11-50 µg/L) may cause airway inflammation and sputum cytological alterations which in the long run may provoke respiratory diseases including lung cancer.

Keywords: Arsenic; Groundwater; Sputum; Cytology

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Introduction

Contamination of arsenic (As) in groundwater is a global public health problem. The Indian state of West Bengal envisages one of the serious As calamities of the world. About 26 million people in 9 out of the 19 districts of the state are drinking water from 1.3 million hand-pumped tube wells that contains a considerable amount of inorganic As [1]. Arsenic enters the human body either directly via drinking of As-contaminated water and ingestion of food cooked with water that contains As, or indirectly through intake of crops grown in soil irrigated with As-rich groundwater [2].

Prolonged As ingestion 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 [3] resulting in multi-organ damage [4]. Chronic exposure to As causes a wide range of adverse health effects, including increased risk of carcinogenesis in the skin, liver, lung and the urinary bladder [5]. It is important to mention in this context that inorganic As increases the risk of lung cancer irrespective of whether it is inhaled or ingested; the cancer risks relate to absorbed dose and are not dependent on the particular pathway of exposure [6]. In addition to malignant diseases, chronic As exposure is associated with

cardiovascular diseases [7] diabetes mellitus [8], neuropathies [9], liver disease [10, 11], and skin lesions such as rain drop pigmentation, hypopigmentation, hyperpigmentation, keratoses and skin cancers [12]. The respiratory toxicity of As include lung function reduction and chronic obstructive pulmonary disease (COPD), a progressive and potentially life-threatening lung condition [13-17].

Considering the wide range of adverse health conditions associated with cumulative As exposure, the World Health Organization (WHO) and the United States Environmental Protection Agency (US EPA) and have established a value of 10 µg As/L as the maximum contaminant level (MCL) for total As in drinking water [18, 19]. However, Bureau of Indian standards has set the legally enforceable limit of As in groundwater at 50 µg/L wherever alternative sources are not available [20]. In India, most of the health studies on As toxicity have been conducted in areas where the groundwater As level was above 50 µg/L. In contrast, very little is known about the health impact of chronic low dose As exposure (11-50 µg/L) despite the fact that several million people of West Bengal are still consuming water that contains As in this dose range [1].

Sputum cytology is recognized as a simple yet sensitive approach to understand, diagnose and monitor the initiation and progression of an underlying diseases in the airways and the inner lung [21, 22].Microscopic examination of sputum cells provides useful information regarding the pathophysiological changes in the lung tissue, because in the course of the development of pulmonary disease, the cells of the airways undergo a number of changes that are reflected in the sputum. In view of this, the present study employed sputum cytology to investigate whether chronic exposure to relatively low dose of As (11-50 µg/L) in groundwater elicits cellular changes in the airways that may lead to respiratory diseases.

Materials and Method

Participants and study areas

The study was conducted in Kalyani, Saguna and Naryanpur villages of Haringhata block in Nadia district, and Dhaltitha of Basirhat block in North 24-Parganas district of West Bengal, a state in eastern India. Among these villages, Narayanpur and Dhaltitha were in As endemic zone (As level in groundwater, 11-50 µg/L) while the As concentrations in groundwater of remaining three villages were within the WHO guideline of 10 µg/L. The villagers were invited to participate in this study through village panchayats (local administration), and non-government organizations.

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 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 palmar and plantar keratosis, hyperkeratosis, raindrop pigmentation (ii) under medication, (iii.) having a past history of malignancy, (iv.) pregnant and lactating women.

Demographic and socio-economic data and water samples were collected at the time of field visits. Arsenic induced skin lesion status was evaluated by a dermatologist. The study protocol was approved by the Institutional Ethics Committee of Chittaranjan National Cancer Institute, Kolkata. Informed consent was obtained from all the participants before enrollment for this study. The work was carried out in accordance with "World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964 and amended by the 59th WMA General Assembly, Seoul, South Korea, October 2008

Measurement of PM_{10} and $PM_{2.5}$ in indoor air

Particulate matter with aerodynamic diameter less than 10 µm (PM_{10}) and 2.5µm ($PM_{2.5}$) were measured in the cooking areas with a laser photometer (DustTrak™ Aerosol Monitor, model 8520, TSI Inc., MN, USA) following the procedure described earlier [23]. The monitoring was carried out for 3 consecutive days, 8 hours per day (07:00-15:00 hours), covering both cooking and non-cooking hours.

Collection of samples

Water samples from tube wells were collected in clean centrifuge tubes with 0.1% conc. HCl. Nail samples were collected from participants in zip locked plastic pouches and were coded.

Blood samples (3 ml) were collected from antecubital vein in vacutainer tubes with and without K_2EDTA as anticoagulant for plasma and serum samples of the participants.

The early morning sputum was collected from each subject for three consecutive days as the samples at this time of the day contain maximum number of exfoliated epithelial cells and alveolar macrophages. The participants were requested to rinse their mouth with sterile normal saline (0.9% NaCl) and to cough vigorously. The sputum was collected with 5 ml PBS in a sterile container.

Determination of arsenic concentration in nails by atomic absorption spectroscopy (AAS) using vapour generation assembly method (VGA)

Arsenic content of water samples was measured with AAS along with VGA as mentioned in our previous studies [17, 24].

Determination of arsenic concentration in nails by atomic absorption spectroscopy (AAS) using vapour generation assembly method (VGA) following microwave digestion

The nails were subjected to microwave digestion following the method of Samanta et al [25]. Nails weighing in between 0.1g to 0.5 g were taken in teflon vessels and pre-digested for 20 minutes with nitric acid and hydrogen peroxide in ratio 3:1. Subsequently the samples were digested in microwave digestion system (MARS 5, CEM Corporation, USA) with 100% efficiency at 160°C with 20 minutes holding time and 15 minutes ramping time. The cooled and digested nail samples were diluted to a volume of 10 ml and stored at 4°C until further analysis of arsenic content using AAS- VGA.

For the purpose of analysis, a stock solution of 1 mg/ml (1000 ppm) was prepared from As standard for AAS in milliQ water with 0.1% HCl. Subsequently standards were prepared with dilution of the stock solution. During standard preparation nails were pre-digested along with As in the range of 1, 5, 10 and 20 ppb for calibration before use. The AAS-VGA (Agilent VGA 77) was used for estimation of arsenic. Instrument was calibrated using 5M HCl in the acid channel and 0.6% $NaBH_4$ and 0.5% NaOH in the reduction channel in the working range of 01-20 ppb As standard. For pre reduction experiment sample was prepared in 5 M HCl and 20% KI was added and allowed to react up to 45 min at room temperature. For direct analysis unknown samples were diluted varying concentrations and pre reduced in the same way as standards. A standard curve was obtained with the prepared range of standards and unknown samples were analyzed with respect to the standard curve with the help of the software Spectra AA. Samples in triplicate were analyzed on AAS and the mean of each sample was calculated on the basis of three readings.

Assessment of sputum cytology

The non-transparent highly viscous parts of sputa were smeared on clean glass slides with the help of a sterile spatula. Efforts were made to make the smear thin and of uniform thickness throughout the slide. The smears were fixed immediately in dehydrated ethanol for 30 min for at the site of collection. Papanicolaou (Pap) staining was done following the method of Hughes and Dodds [26]. In brief, the slides were brought to water through graded ethanol and stained with aqueous solution of Harris' hematoxylin (Sigma Chem, USA) for 2 min, washed in running tap water and dehydrated in 95% ethanol. Thereafter, the slides were stained in Orange G mixture containing 1% Orange G6 (BDH, England) and 0.015 % phosphotungstic acid (BDH, England) in 95% ethanol, for 2 min. After washing in 95% ethanol the slides were stained in EA-50 mixture in ethanol that contains 0.225% light green SF (BDH, England), 0.05% of Bismarck brown (BDH, England), 0.225% Eosin yellow (BDH, England), 0.2 g phosphotungstic acid (BDH, England) and 0.05% aqueous solution of lithium carbonate (SRL, Mumbai, India) for 2 min and dehydration in absolute ethanol. The slides were cleared in xylene and mounted in distreneplasticiser xylene, 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 [27]. 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. Alveolar marcophages (AMs), bronchial epithelial cells, sputum neutrophils, eosinophils and lymphocytes, metaplasia of airway epithelial cells and goblet cell hyperplasia were identified by established criteria [28]. Koilocytosis which are generally associated with human papilloma virus (HPV) infections [29] were identified on the basis of morphological criteria [30]: perinuclear cytoplasmic vacuolization and pyknotic and usually eccentric nucleus. Differential distribution of sputum cells was expressed as percentage of total non-squamous cells. The samples were discarded if the percentage of squamous cells was greater than 80% or if the total number of non-squamous cells was less than 1000 [31].

Non-specific esterase staining for identification of alveolar macrophages

Non-specific esterase (NSE), a marker enzyme for macrophages, was evaluated cytochemically in sputum samples following the Fast Blue B method using alpha naphthyl acetate (Sigma Chem, USA) as the substrate. NSE activity was indicated by golden brown staining of macrophages, all other cells were negative. The number of NSE-positive cells was counted per hpf of light microscope.

Cytochemical localization of elastase

The elastase activity in alveolar macrophages and neutrophils in the airways was evaluated by simultaneous azo-dye coupling method using naphthol AS-D chloroacetate as substrate [32]. In brief, the sputum slides were fixed in buffered formalin for 10 min. Then the slides were incubated for 60 min at room temperature in reaction mixture containing 3 mg of naphthol AS-D chloroacetate (Sigma Chem, USA) in 0.5 ml of N,N-dimethylformamide (Sisco, India), 10 ml of phosphate buffer, pH 6.8 and 10 mg of fast Blue B (BDH, England). Following dehydration in graded ethanol, the slides were observed under microscope. Elastase activity was visualized as blue color and the enzyme activity was graded subjectively as low, moderate and high on the basis of intensity of color reaction.

Detection of iron deposition in lung: Perl's Prussian blue staining for siderophages

Perl's Prussian blue reaction was done to identify deposition of ferric iron (hemosiderin) in airway and inflammatory cells, especially the AM, by the method of Pearse [33]. The slides were fixed in 10% formalin for 10 min at room temperature. Then the slides were brought to water and were exposed to a fresh mixture of equal parts of 2% potassium ferrocyanide and 2% HCl for 45 minutes. The slides were then washed in distilled water, dehydrated through graded ethanol, cleared in xylene and mounted in distreneplasticiser xylene. Iron-containing AMs are known as siderophages. Presence of ferric iron in AM gives deep blue color reaction. Using a light microscope, a minimum of 10 high power fields at 400x magnifications was counted and the average frequency of siderophages was scored. The magnitude of iron deposition in macrophages was graded subjectively into 5 categories: 0, no stainable iron deposits, 1+, iron deposits visible in 1–25% of AM, 2+, iron deposits visible in 26–76% of AM; 3+, iron visible in 77–100% of AM and 4+, ferric iron deposits are visible by naked eye inspection. The Golde score was used to assess alveolar hemorrhage [34]. In slides stained for Perl's Prussian blue reaction, an average of 100 AM were graded for hemosiderin on a scale of 0-4, 0 being the minimum and 4 the maximum score for 100 macrophages. The mean score of 100 AM was calculated. Hemosiderin resorption was considered normal if the Golde score ranged from 0-20, medium from 20-70, and high when the score was more than 70 [34]. The scoring was done in a blinded fashion in all cases.

Measurement of the level of pro- inflammatory mediators

The levels of pro-inflammatory mediators- tumor necrosis factor alpha (TNF-α), interleukin -6 (IL-6), interleukin-8 (IL-8) and C-reactive protein (CRP), were measured in blood plasma by ELISA using commercially available kits following the manufacturers' protocols: kits manufactured by BD Biosciences, San Diego, USA for TNF-α and IL-6; Roche Diagnostics, Mannheim, Germany for IL-8; and IBL, Hamburg, Germany for CRP.

Statistical analysis of data

Results are expressed as mean ± SD (Standard deviation). The statistical significance between arsenic exposed and control groups were determined using the Student's t-test, Mann-Whitney U-test, Fisher's χ^2 test and multivariate logistic regression analysis. All results were computed and analyzed using the SPSS10.0 statistical software package (SPSS, Chicago, IL), and $p<0.05$ was considered significant.

Results

Demographic characteristics

The participants were stratified on the basis of As content in tube well water as control (below 10 µg/L) and exposed (above 10 µg/L). Of the 654 participants, 342 women aged 22-47 yr (median 41 yr) were in the exposed group. The remaining 312 women (median age 39 yr) were in the control group. The demographic characteristics of the participants have been previously described in our earlier reports [35]. The As-exposed and control women were comparable with respect to age, body mass index (BMI), education and type of fuel used for cooking. More than 90% women in both groups cooked daily for an average of 4 hr with biomass such as wood, cow dung cake, and agricultural wastes (jute sticks, hay, dried leaves, bamboo and husk). The indoor concentrations of PM_{10} and $PM_{2.5}$ were high in the households of both control and As-exposed women, but the inter-group differences were not statistically significant ($p>0.05$). The standard deviations of the mean PM values were high because the PM levels tended to increase remarkably during cooking time. The majority of the participants attended to agricultural works in addition to their household duties.

Arsenic levels in drinking water and nails

As reported earlier the As level in tube well water of the villages where the control women resided was 2.72 ± 1.18 (SD) µg/L**;** median 2.89 µg/L, range 0.89-5.07 µg/L**.** In contrast, the mean As concentration in the tube well water of As-endemic villages was 28.3 ± 13.51µg/L; median 29.9µg/L, range 11.5-43.1µg/L [35]**.** 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.

Few nail samples were analyzed at random from exposed and control group to have an estimate of the individualized chronic As exposure. The As level in the nail samples of the control people (N, 15; 2.23 ± 3.64 µg/g) was significantly lower than the mean As concentration of the low arsenic exposed people (N, 14; 35.18 ± 31.53 µg/g; $P=0.0004$). The As level in groundwater showed a positive correlation with As content in nails where Pearsons r was 0.7561 and 95% Confidence interval (CI) was 0.5439-0.8774. The correlation coefficient was highly significant $P<0.0001$. The regression coefficient (r^2) was 0.5716.

Sputum cytology

Sputum samples of As-exposed women had 42% more cells than the sputa of control women. They had 87.4 ± 14.5 (SD) cells per high power field (hpf) in contrast to 61.6 ±8.2 cells/hpf in controls ($p<0.0001$; **Figure 1**).Compared with control women, although there was a decline in neutrophil percentage, the absolute number of sputum neutrophils (number/hpf) in As-exposed women was 1.3-times more than that of controls ($p<0.001$; **Table 1**; **Figure 1a**).The relative distributions (%) of eosinophils (**Figure 1b**), lymphocytes (**Figure 1c**), AM (**Figure 1d**) and epithelial cells (**Figure 1e**)in sputum were higher in As-exposed women. The increases in the absolute number of eosinophils, lymphocytes, AM and epithelial cells were 4.2-, 1.6-, 1.9-, and 4.6-fold over control, respectively ($p<0.001$; **Table 1**).

Morphological changes in sputum cells

Epithelial cells

Aggregates or clumps of ciliated and non-ciliated columnar epithelial cells were present in 59 (9.7%) sputum samples of As- exposed women against 21 (4.1%) of control ($p=0.0056$). Ciliocytophthoria, characterized by the presence of disintegrated distal tufts of cytoplasm with intact cilia from columnar epithelial cells was present in 3.1% of sputum samples from As-exposed compared with 1.2% of control ($p=0.0828$, **Figure 2a**). Cytologic atypia such as multinucleated (more than two nuclei) airway epithelial cells were present in 6.8% sputum samples collected from As-exposed women against 2.3% of control ($p<0.05$; **Figure 2b**).In addition, metaplasia of epithelial cells was present in 71 (11.7 %) As-exposed subjects, whereas only 22 (4.3%) of control women had metaplasia (**Figure 2c**). The differences in the prevalence of metaplasia ($p=0.0003$) between control and As-exposed women were highly significant in Chi-square test ($p<0.001$). Koilocytes were present in sputum samples of 3.4% of As-exposed group compared with 0.8% of controls ($p=0.0036$; **Figure 2d**).The sputum samples of 11.6% As-exposed women showed heavy deposits of mucus (**Figure 2e**) with goblet cell hyperplasia compared with 3.9% samples of control women ($p<0.001$; **Table 2**; **Figure 2f**).

Inflammatory cells

In addition to significant increase in number, the AMs of As-exposed subjects exhibited several morphological changes. Expression of cytokeratin, detected by the characteristic eosinophilic cytoplasm in Pap-staining, was recorded in 8.9% of AM in As-exposed women against 3.2% of control ($p<0.001$). Besides, the AMs were smaller in size in the exposed subjects: cell diameter ranging from 11 to 28µm (median 16 µm) against 14-38 µm (median 22 µm) in control. Staining for NSE (a marker enzyme for macrophages) showed 'small sputum macrophages'

Table 1 Differential distribution of cells in sputum. The sub-headings Neutrophil, Lymphocyte, Alveolar macrophage and Non-squamous epithelial cells should come under the main heading parameters in the extreme left column

Parameters	Control (n=312)	Arsenic exposed (n=342)	Fold change in mean value over control
Neutrophil			
%	88.0 ± 5.9	82.5 ± 6.2	-1.06
Cells/hpf	54.2 ± 3.7	72.1 ± 5.0	1.33
Eosinophil			
%	0.6 ± 0.2	1.9 ± 0.7	3.16
Cells/hpf	0.4 ± 0.2	1.7 ± 0.7	4.25
Lymphocyte			
%	5.7 ± 0.6	6.5 ± 1.5	1.14
Cells/hpf	3.5 ± 0.4	5.7 ± 1.1	1.62
Alveolar macrophage			
%	4.8 ± 1.1	6.4 ± 1.5	1.33
Cells/hpf	2.9 ±0.7	5.6 ± 1.2	1.93
Non-squamous epithelial cells			
%	0.8 ± 0.3	2.6 ± 0.6	3.25
Cells/hpf	0.5 ± 0.2	2.3 ±0.5	4.60

Results are mean ± SD; p<0.001 compared with control in Student's t-test, hpf, high power field of microscope (400x)

Table 2 Sputum cytological alterations in arsenic-exposed women.

Cell Type	Morphological changes	Control (N=312)	As-exposed (N=342)	P value
Epithelial cells	Clumps of ciliated and non-ciliated columnar epithelial cells	4.1	9.7	0.0056
	Bi- and tri-nucleated epithelial cells	2.3	6.8	0.003
	Ciliocytophthoria	1.2	3.1	0.082
	Hyperkeratinized parabasal cells	3.2	8.9	0.0014
	Koilocytes	0.8	3.4	0.0036
Mucus and mucus-producing cells	Goblet cell hyperplasia	3.9	11.6	<0.0001
	Hypersecretion of mucus	15.5	24.4	0.0237
AMs	Hyperkeratinized AMs	1.2	3.8	0.0176
	Small sized AMs	7.2	16.4	0.0006

Results are expressed as percentage of women in each category

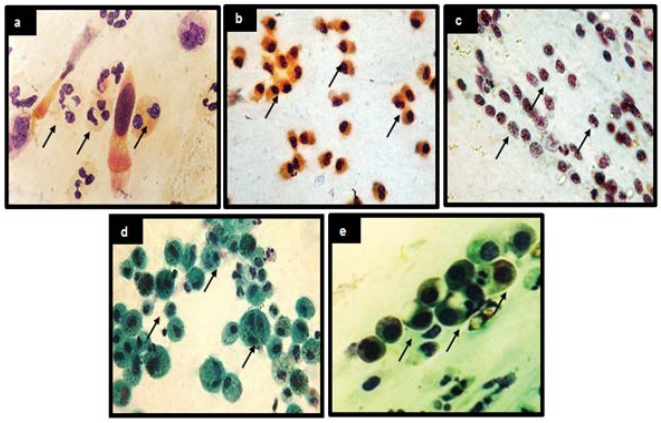

Figure 1 Photomicrographs of Pap-stained sputum samples of As-exposed women showing neutrophilia (a), eosinophilia (b), lymphocytes (c), alveolar macrophages (d) and epithelial cells (e). [Magnification 400 x (a, b); 1000 x (c-e)]

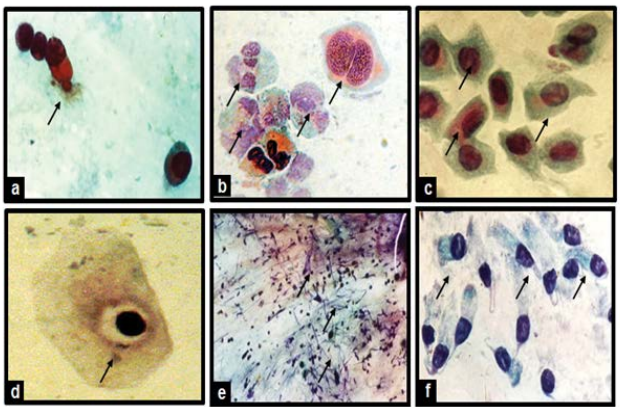

Figure 2 Photomicrographs of sputum (Pap stained) of As-exposed women showing ciliocytophthoria (a); multinucleated epithelial cell (b); metaplasia of airway epithelial cells (c); koilocyte (d); massive deposits of mucus (e) and goblet cell hyperplasia (f). [Original magnification, 1000x].

with a diameter of 10-15 µm [36] accounted for 16.4% of all macrophages in the As-exposed group compared with 7.2% of sputum macrophages in control women (p=0.0006).

Neutrophil and AM elastase

Elastase was detected in all sputum neutrophils and a section of AM (10.8% in control vs. 25.3% in As-exposed, p<0.001; **Table 3**).The intensity of staining reaction for neutrophil elastase was much more in As-exposed subjects. Subjective grading of enzyme activity in a scale of 1+ (mild), 2+ (moderate) to 3+ (high) revealed 81.7% of neutrophils of As-exposed women belonged to 3+ category against 58.9% of control (p=0.0029). Microscopical examination indicated increased production (intracellular localization) and greater release (extracellular staining) of elastase in airway neutrophils and AM of As-exposed subjects when compared with that of control (**Figure 3a and 3b** respectively).

Occult pulmonary hemorrhage

In order to investigate whether chronic exposure to As was associated with covert pulmonary hemorrhage, Perl's Prussian blue reaction was carried out for detection of hemosiderin iron in AM. Results showed that 29% of AM of the As-exposed women had iron deposits compared with 8% of iron-laden macrophages (siderophages) in sputum of control group (p<0.0001). The increase in the total number of siderophages in sputum was even greater (1.6 siderophages per hpf compared with 0.2 siderophage/hpf in control group; p<0.0001; **Figure 3c**). The As-exposed group also exhibited a much higher Golde score compared with the control (53 ± 36 (SD) compared with 12 ± 8 in controls; p<0.0001). Moreover, 3.6% of the As-exposed women had Golde score more than 100 compared with 0.4% of control women (p<0.0001),implying covert pulmonary hemorrhage in a sizable number of As-exposed women of this study.

Pro-inflammatory mediators

Since an excess of inflammatory cells was observed in the sputa of chronically As-exposed women, we measured the pro-inflammatory mediators in 90 randomly selected plasma samples, 45 each from control and As-exposed women. A marked increase (p<0.001) in the plasma levels of TNF-α, IL-6, IL-8 and CRP was observed in As-exposed women (**Table 4**).

Association between As exposure and sputum cytological changes and pro-inflammatory cytokine levels

In multivariate logistic regression analysis, after controlling age, BMI, education, own and husband's occupation, and family income as potential confounders, As concentration in tube well water was positively associated with sputum cell number per high power field (odds ratio [OR] =1.32, 95% confidence interval [95% CI] 1.08-1.76), goblet cell hyperplasia (OR=1.20, 95%CI 1.04-1.45), metaplasia (OR= 1.39, 95% CI 1.10-1.89), multinucleation of epithelial cells (OR=1.24, 95% CI 1.07-1.48), number of siderophages per hpf (OR=2.88, 95% CI 1.24-4.76), plasma TNF-α (OR=1.19, 95% CI 1.08-1.46), IL-6 (OR=1.30, 95% CI 1.11-1.52), IL-8 (OR=1.29, 95% CI 1.09-1.64),and CRP (OR=2.03, 95% CI 1.44-3.27).

Discussion

More than 90% of the population in West Bengal depends on groundwater to access safe drinking water through shallow tube well (<150 m deep) extraction systems. These tube wells are favored for the easy availability of aquifers, low-technology installation procedure, and affordable cost. Besides drinking, a substantial part of the total extracted groundwater is used for irrigation purposes to grow rice and vegetables in the dry season. Unfortunately, much of the groundwater extracted from these shallow alluvial aquifers is often enriched with As predominantly in the 3^+ (As (III), arsenite) and 5^+ (As (V), arsenate) oxidation states. Our study has shown that drinking of tube well water containing even low level of As (11-50 µg/L) for long can induce inflammation and several cytological changes in the airway cells. Cellular abnormalities range from cytological vaculation, multinucleated epithelial cells, absence of cilia and ciliocytophthoria and basal- and goblet-cell hyperplasia to metaplasia of airway epithelial cells. The basal cells represent a multipotent stem

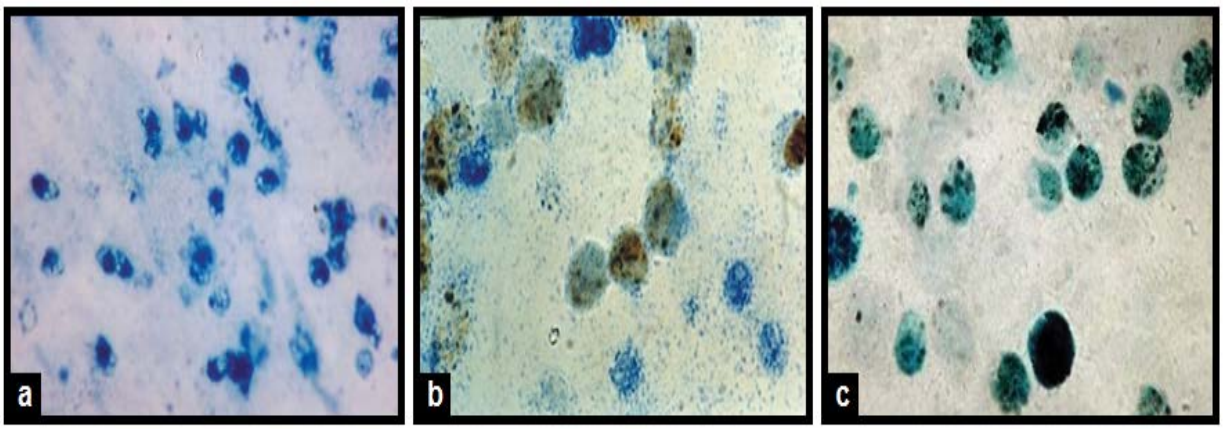

Figure 3 Photomicrographs of sputum of arsenic-exposed women showing elastase activity in neutrophils (a) and alveolar macrophages (b); and Prussian blue staining of siderophages (c). [Magnification 400 x (a); 1000 x (b, c)]

Table 3 Elastase activity in sputum neutrophils and macrophages.

Percentage of cells with different grades of activity	Control (N=312)	As-exposed (N=342)	P value
Sputum neutrophil			
0	0 ± 0	0 ± 0	
1+	1.3 ± 1.6	0 ± 0	<0.0001
2+	39.7 ± 3.2	18.1 ± 1.5*	<0.0001
3+	58.9 ± 14.7	81.7 ± 5.8*	<0.0001
Alveolar macrophage			
0	89.2 ± 11.6	74.7 ± 14.8*	<0.0001
1+	7.1 ± 3.2	13.3 ± 5.9*	<0.0001
2+	2.3 ± 0.7	7.9 ± 3.3*	<0.0001
3+	1.3 ± 0.5	4.0 ± 1.1*	<0.0001

Results are expressed as mean± SD; *, p<0.05 compared with control; 0, absence of elastase staining; 1+, mild; 2+, moderate, and 3+, high intensity of elastase staining under light microscope

Table 4 Plasma levels of pro-inflammatory mediators.

Pro- Inflammatory mediators	Control (N=312)	As-exposed (N=342)	P value
TNF (pg/ml)	11.5 ± 2.9	19.8 ± 4.6*	<0.0001
IL-6 (pg/ml)	11.3 ± 4.3	18.5 ± 4.9*	<0.0001
IL-8 (pg/ml)	14.6 ± 5.4	24.2 ± 7.3*	<0.0001
CRP (µg/ml)	1.8 ± 0.5	5.8 ± 2.8*	<0.0001

Results are mean ± SD; *, p<0.001 compared with control in Student's t-test.

cell population. They generate differentiated cells under normal physiological conditions as well as during epithelial repair [37]. Therefore, basal cell hyperplasia in As-exposed subjects may suggest injury to the airway epithelium. Increase in the number of multinucleated epithelial cells, on the other hand, indicates abnormality in cell cycle regulation presumably at the spindle check point. Presence of ciliocytophthoria in As-exposed women implies that As exposure increases the risk of respiratory viral infections [38]. Greater vulnerability towards viral infection among As-exposed women is apparent from a marked increase in the number of koilocytes. These cells appear as a consequence of infections by both low- and high-risk HPV [29]. HPV influences activity at the G2 cell cycle checkpoint [39], and increases the risk of lung cancer [40] and ductal carcinoma of the breast [29]. In both cases, increase in koilocytes has been recorded. In view of these, increased presence of koilocytes in sputum of As-exposed women may suggest greater risk of carcinogenesis in the airways. However, HPV-associated koilocytes have also been found in normal breast epithelium [29].

Compared with control women, goblet cells were found in increased numbers and mucus content was more in sputum of As-exposed women. These findings seemed interrelated, as goblet cells are the principal source of mucus. Excess production of mucus can plug the airways leading to recurrent infections [41, 42] and chronic inflammatory airway diseases [43, 44]. Although the precise mechanism of mucus hypersecretion among As-exposed women is currently unknown, various stimuli including endogenous oxidants induced by neutrophil elastase can induce it [45, 46]. Therefore, the observed increase in neutrophil elastase among As-exposed women may be implicated in part to excess production of mucus in the airways. Hyperplasia of mucus-secreting cells and loss of ciliated cells from the respiratory epithelium are common features of respiratory syncytial virus infection [47] and smoke inhalation [48]. On the basis of the present findings, chronic ingestion of low level of As in drinking water can be included in the list of potential toxic agents that can elicit such changes.

Small sputum macrophages represent only a minor fraction of macrophages (about 6%) in the airways of normal subjects [36]. But a remarkable (6-to 10-fold) increase in the percentage of these cells has been demonstrated in patients with chronic obstructive pulmonary disease [36]. In line with this, greater prevalence of these small macrophages in sputum of As-exposed women may suggest obstruction in lung function. Goblet cell hyperplasia and hypersecretion of mucus, as observed in the As-exposed group, support this assumption. Siderophages are iron-laden alveolar macrophages characteristically found in the airways of patients with diffuse alveolar hemorrhage [49, 50]. Increase in the number of these cells is recognized as an indicator of covert pulmonary hemorrhage and iron overload [51, 52]. Thus, marked increase in the number of siderophages in sputum of As-exposed women of this study may suggest occult pulmonary hemorrhage.

The underlying mechanism of the observed cellular changes in women who were chronically exposed to As in drinking water is yet to be elucidated. Inflammation, as observed in exposed subjects for an excess of inflammatory cells and proinflammatory cytokines, can play a role. Pro-inflammatory cytokines and chemokines, and matrix metalloproteinase which are involved in inflammation, have been shown to be increased in persons with As exposure [24, 53, 54]. Even low As exposure increases the matrix metalloproteinase in sputum [54, 55].

Cytological atypia in sputum that we have observed in excess among As-exposed women is in conformity with the findings among Chinese tin miners occupationally exposed to radon and As [56]. Atypia of sputum cells is an independent risk factor for lung cancer, and the risk increases with the degree of atypia for squamous cell carcinomas, small cell lung cancer and central lung cancer, with adjusted hazard ratios of 5.70 [56]. In the light of these reports, greater prevalence of metaplasia of airway epithelial

cells among As-exposed women of this study may suggest that long-term exposure to even low level of As via drinking water can increase the risk of carcinogenesis in the airways of never-smoking women. In agreement with this, higher incidence of lung cancer has been shown among women in West Bengal [57].

The majority of our results were based on sputum cytology. A great advantage of the technique is that it enables sampling of the airways in a non-invasive manner, in contrast with other methods such as bronchial biopsy, bronchial brushing and broncho-alveolar lavage, all of which require bronchoscopy, discomfort and risk that it entails [58]. Besides, sputum cytology is cost-effective, hence ideal for large population-based studies in the developing countries.

To our knowledge, this is the first study on the impact of chronic ingestion of low-level of As in drinking water on the cytological alterations of airway cells in never-smoking women at their reproductive age in India or elsewhere. Admittedly, this investigation has a number of limitations. First, being a cross-sectional study, it limits our inference on the causal relationship between As exposure and the observed cellular changes. Second, the As exposure assessment from food chain was not done. Third, it seems likely that factors other than groundwater As have influenced the changes observed among exposed women. For example, high level of indoor air pollution due to cooking with unprocessed biomass fuels adversely affects the lung function [59], and elicits inflammation [60] and cellular changes in the airways [22]. Likewise, agricultural activities and resultant exposure to agricultural pesticides could also be harmful to the lung [61]. Since agricultural activities and use of unprocessed solid biomass were slightly more prevalent among As-exposed women, sputum cytological changes could be attributed in part to these factors. Despite these shortcomings, however, the sample size of this study was large enough to conclude that the connection between chronic consumption of 11-50 µg/L of As-contaminated water and sputum cytological changes in rural non-smoking women were real rather than apparent. This conclusion is supported by the fact that biomass usage and participation in agriculture were not significantly different between control and exposed groups. Further, As concentration in the range of 11-50 µg/L showed positive association with total number of cells in sputum after controlling cooking with biomass and agricultural occupation as potential confounders. Moreover, we did not find any difference in the percentage of individuals having a mean upper arm circumference (MUAC) value of less than 23 cm (which suggests nutritional deficiency) between the control and exposed groups (data not shown), eliminating the possibility of nutritional deficiency as a contributor to cellular changes in the airways.

In essence, the study has shown that long term exposure to even low dose of groundwater As (11-50 µg/L) may initiate chronic inflammation in the airways. More over presence of inflammation and sputum atypia in low As exposed women might be indicative of risk of developing As associated respiratory diseases, covert pulmonary hemorrhage as well as malignancy of the lung cancer in the long run.

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