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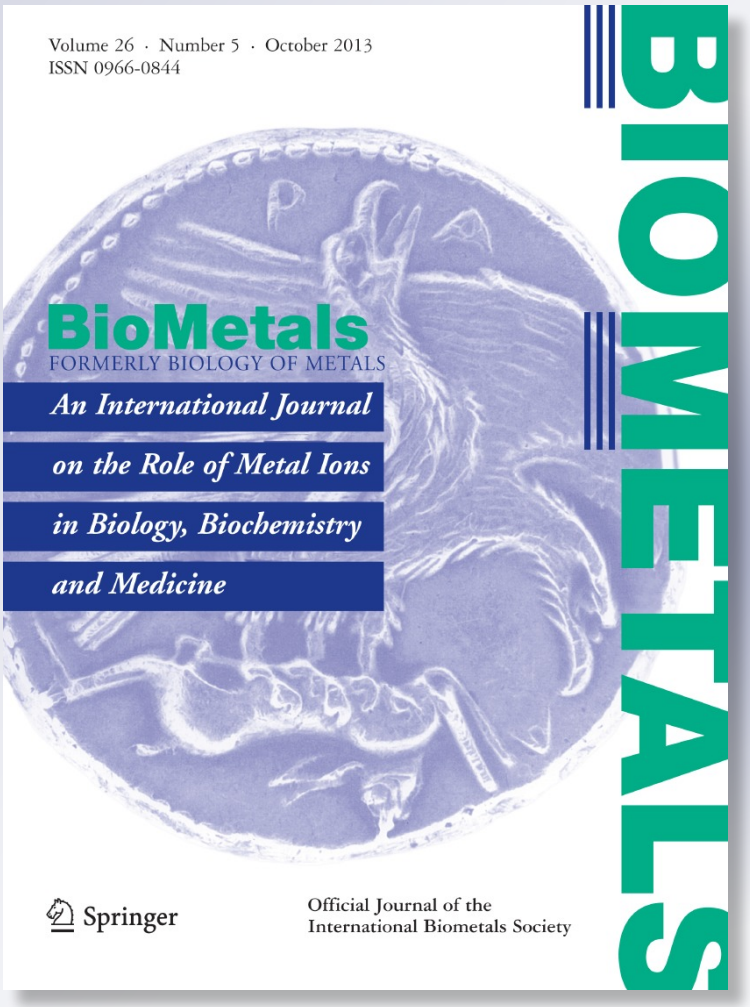

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Abstract Chronic exposure to arsenic through drinking water affects nearly 26 million individuals in West Bengal, India. Cytogenetic biomarkers like urothelial micronucleus (MN) are extensively used to monitor arsenic exposed population. In 2004–2005, 145 arsenic exposed individuals and 60 unexposed controls were surveyed of which 128 exposed individuals and 54 unexposed controls could be followed up in 2010–2011. In 2004–2005, the extent of arsenic content in the drinking water was 348.23 ± 102.67 μg/L, which was significantly lowered to 5.60 ± 10.83 μg/L in 2010–2011. Comparing the data obtained between 2004–2005 and 2010–2011, there was a significant decline in the MN frequency, when assayed in 2010–2011 compared to 2004–2005. Hence, we infer that urothelial MN can be utilized as a good biomarker in detecting remedial effects from toxicity of the low dose of arsenic through drinking water.

Keywords Arsenic · Groundwater · Genotoxicity · Micronuclei · Remediation

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Introduction

Arsenic, one of the most toxic inorganic carcinogen known to mankind and is of pandemic concern affecting more than 70 nations around the globe. The major source of arsenic contamination is through ground water which affects more than 150 million individuals (Brammer and Ravenscroft 2009; Polya and Charlet 2009). The situation however is severe in 9 out of 19 districts in West Bengal, India where 26 million individuals are exposed to arsenic contaminated drinking water which, is above the threshold limit of 10 μg/L (WHO 1996). According to the Agency of Toxic Substances and Disease Registry, arsenic has been given the top priority in the category of most hazardous substances adjudicating its incidence, degree of toxicity and accessibility to humans. Epidemiological investigations have demonstrated associations between arsenic ingestion and hyper-

pigmentation, keratosis of skin, anemia, burning sensation of the eyes, solid edema of the legs, liver fibrosis, chronic lung disease, gangrene of the toes (Blackfoot disease) and neuropathy as also, cancers of skin, lung, liver, bladder, kidney, and prostate (Tseng 1977; Chen et al. 1985, 1992; Guha Mazumder et al. 2001; Das et al. 2012; Paul et al. 2013). Skin lesions are recognized as the most sensitive end points of chronic arsenicosis and is considered as the hallmark of arsenic toxicity, which may either develop as early as 6 months or may take up to 10 years to develop (Ghosh et al. 2006). The first exposure to arsenic contaminated drinking water and development of the symptoms for arsenic toxicity depends on the concentration of arsenic in drinking water, volume of intake and the nutritional status of individuals, along with the genetic variability of an individual (Rahman et al. 2001; Ghosh et al. 2006). Since prolonged arsenic exposure results in multiple system disorders (Banerjee et al. 2011), hence bio-monitoring of this large arsenic exposed population is of extreme importance. A prolonged exposure to arsenic results in multi-system failures (Hall et al. 1991).

Although an imponderable number of individuals are bearing the brunt of chronic arsenic exposure and multi-pronged research approaches have been adopted to identify the mechanisms of arsenic toxicity and carcinogenicity; no appropriate clinical approach have yet been formulated to combat the menace. Biomarkers have always played an important role in establishing and identifying any biological effect of a carcinogen or mutagen. Cytogenetic biomarkers, namely chromosomal aberrations (CA), sister chromatid exchanges or micronuclei (MN) are traditionally used for bio-monitoring genotoxic effects in humans (Rossner et al. 2005; Ghosh et al. 2007). The ease of the technique and non-requirement of metaphasic cells made MN-assay from urothelial cells very popular (Ghosh et al. 2008). MN is a cytoplasmic inclusion within a cell, which is generated either by a chromosomal break or whole acentric chromosome that does not participate in formation of daughter nuclei during karyokinesis and remains alongside the main nuclear body in form of a smaller nucleus. Arsenic toxicity gives rise to certain reactive oxygen species (ROS), which contribute in shearing of the phosphate backbone of the nucleic acids, giving rise to chromosomal breaks (Yamanaka et al. 1990; Ghosh et al. 2008). Apart from that, several other reports indicate that MN frequency is a potent tool to understand genotoxic damage incurred by toxicants, in humans (Burgaz et al. 1995; Espinoza et al. 2008; Plöttner et al. 2012). Although MN studies in three different cell types e.g. lymphocytes, urothelial and oral epithelial cells are predictive of arsenic genotoxicity, relatively more studies were performed with urothelial cells due to its ease of availability (Basu et al. 2004; Ghosh et al. 2008). Urinary bladder serve as an appropriate index to monitor the genotoxicity induced by arsenic because the epithelial lining of the bladder is in direct contact with toxicant, both in it's organic and inorganic forms (Smith et al. 1993), as the human body release the toxicant maximally through urine (Buchet and Lauwerys 1985; Kurttio et al. 1998). Moreover extensive studies on arsenic and human health have implicated that MN frequency provides a correlative evidence of arsenic induced genotoxicity in humans, as observed in the exfoliated urothelial cells as well as in lymphocytes (Tian et al. 2001; Basu et al. 2004).

Ameliorative measures have been implemented by several governments all over the world, whereby they are reducing the arsenic content through drinking water and hence curb the arsenic intake. In our previous work, we conducted a two wave cross sectional study where a significant decrease in the dermatological health effects as well as cytogenetic damages were observed in a population exposed to arsenic through drinking water (Paul et al. 2013). Since the drinking water arsenic content was not lower than WHO permissible limit (10 μg/L), so the cytogenetic damages in exposed population decreased significantly, but were still higher than the arsenic unexposed individuals. In our present study the main objective was to find out the recovery in cytogenetic damage as measured by MN in urothelial cells in a population who were chronically exposed to arsenic, considering both arsenic-specific lesion and lesion free individuals, after they have consumed safe drinking water (<10 μg/L; WHO 1996) for at-least 5 years. Thus this shall identify briefly the genotoxic recovery achieved by drinking safe water by a human population having a chronic history of arsenic exposure. In this regard, an attempt had been made to compare the frequency of urothelial MN in a population at two different time points and understand the efficiency of this biological perspective in determining the degree of amelioration.

Materials and methods

Chemicals and reagents

Potassium chloride and tri-sodium citrate were purchased from Qualigens Fine Chemicals (India). Glacial acetic acid and methanol were purchased from E. Merck (India). Giemsa stain powder was bought from Sigma chemical company (St. Louis, MO, USA).

Study design

The study participants were recruited from the district of Nadia, which is one of the severely arsenic affected districts of West Bengal, in accordance to the government online register of West Bengal (WBPHED). Out of 17 affected blocks in Nadia, two blocks of Chakdah and Haringhata were selected by the method of probability proportional to size, where the size was calculated as a product of the total population in the village to the proportion of arsenic contaminated tube wells present in the village. From 103 and 71 villages in Chakdah and Haringhata respectively, six villages were chosen by the similar method. A total of 145 exposed individuals were selected from Nadia district during 2004–2005. Further, a follow up study was conducted in this population during 2010–2011, where a total of 128 exposed individuals participated. A total of 60 individuals unexposed to arsenic were recruited from district of Hoogly, West Bengal, using the similar method, while, 54 were available during the follow-up. Specific care was taken that the follow up individuals had consumed safe drinking water for at least 5 years.

The non-physician interviewer, alien to the clinical status of the participants, interviewed them on the basis of a structured questionnaire that elicited information about lifetime residential history, occupation, diet, and smoking habit. The detailed information on addiction, including smoking, chewing tobacco or addiction to alcohol was obtained. Then, an expert dermatologist examined each participant for the presence of arsenic-specific skin lesions. All the study participants provided informed consent. Water and urine samples were collected from these individuals on the same day in respective coded containers. This study was conducted in accord with the Helsinki II Declaration and approved by the Institutional ethics committee.

Cohort selection

After the dermatological examination, exposed population was subdivided in two groups; those with skin lesion and without skin lesion. In the parent population, 75 individuals had skin lesions referred as cohort 1 while 70 possessed no lesion referred as cohort 2. In the follow up study of the same samples, 61 individuals with skin lesion referred as cohort 1a and 67 individuals without skin lesion referred as cohort 2a. Similarly, the parent population of unexposed individuals was referred as cohort 3 ($N = 60$) and the follow up as cohort 3a ($N = 54$).

Dermatological criteria and gradation of arsenic toxicity

A careful examination of skin, by an expert dermatologist, identified pigmental changes and keratotic lesions with different degree of presentation. Scoring of severity of skin lesions were done as described previously (Guha Mazumder et al. 2010). Briefly, depending on the severity of the pigmentation and keratosis, both were categorized into mild, moderate and severe. If the observation of the pigmentation showed a physiological appearance of diffused, spotty pigmentation associated with leuco-melanosis, we considered the cases as mild, designation a score of 1–2 depending on the degree. For a moderate affect, the individuals showed a spotted pigmentation, visibly severe than the mild cases and the individuals were scored with either 3 or 4. Individuals with blotchy pigmentation were considered as severe cases and were scored 5–6. Many of these individuals showed pigmentation in the sub-lingual region. Similarly, keratosis were filtered into the categories of mild (1–2), moderate (3–4) and severe (5–6) where slightly thick papules having a thickness of <2 mm in palms and soles were considered mild. For moderate cases the papules were warty having a thickness of 2–5 mm. Cases possessing thick papules greater than 5 mm in thickness, large and discreet were considered severe cases. Any skin lesions that were diagnosed as probable or doubtful were, excluded from this study.

Estimation of arsenic content in the drinking water and urine

At both the time points (2004–2005 and 2010–2011), study participants were provided with acid-washed

(nitric acid–water [1 + 1]) plastic bottles for collection of drinking water (approx. 100 mL) samples into which, nitric acid (1.0 mL/L) was added later on as preservative, as conducted previously (Basu et al. 2004). Midstream of the first morning void were collected from the study participants in coded clean plastic bottles. After collection, the samples were stored in the icebox and brought to the laboratory and immediately kept at −20 °C until arsenic estimation was carried out. The details of the quality control and the procedure has been mentioned earlier (Ghosh et al. 2007). The arsenic content in water and other biological samples was analyzed at CSIR-Indian Institute of Chemical Biology, Kolkata and also at the School of Tropical Medicine, Kolkata. A Perkin-Elmer Model-3100 spectrometer at SOES, Kolkata and Model Analyst-700 spectrometer at our own institute equipped with a Hewlett-Packard Vectra computer with GEM software, Perkin-Elmer EDL System-2, arsenic lamp (lamp current 400 mA), illuminating the cuvette at 193.7 Å (specific for arsenic) were utilized for this purpose (Ghosh et al. 2006). For each sample, reading was taken in duplicate. Repeat sampling and rechecking in both the laboratories of same sample gave consistent results.

MN assay from urothelial cells

The urothelial cells were collected for MN assay following the method described by Titenko-Holland et al. (1994). Midstream of the first morning voids were collected (about 100 mL) from each study participants and the samples were brought to the laboratory on ice. Isolation of the exfoliated urothelial cells were done following a standard protocol, described earlier (Basu et al. 2004), with minor modifications. Briefly, the urothelial cells were recovered by centrifugation at 1,000 rpm, for 10 min from the first morning void of the study samples. The cell pellets were washed in 0.9 % NaCl, under the same condition of centrifugation. The cell density was checked with the phase contrast microscope, so as to achieve a desired density of 1.5–2 $\times$ 10^6 cells/mL. To achieve the target density, the cells were suspended in 0.9 % saline and centrifuged by either increasing or decreasing the dilution of the suspension. On reaching the desired concentration, 50 μL of the solution was spread on pre-heated (40 °C) glass slides, fixed and stained in Giemsa, which have been considered as a staining procedure to obtain MN frequency by several groups (Basu et al. 2004; Ghosh et al. 2006; Nersesyan et al. 2006; Ghosh et al. 2008; Speit et al. 2012; Araújo et al. 2013).

Scoring procedure

All the slides were first scanned under 20× magnification by Nikon Eclipse 80*i* microscope (Japan). Approximately 2,000 urothelial cells per individual were scored. Final scoring was done under oil, at 100×, based on the criteria as described earlier (Basu et al. 2004). Briefly, the unclear, smeared, clumped, over lapped or cells without non-intact nuclei were not recorded. Only those MN were noted which were spherical in shape and residing on the same focal plane, having lesser than a third of the diameter of the main nuclei. Care was taken that the nuclei scored were distinctly separated from the main nuclei and had similar colour and texture as with the main nuclei (Basu et al. 2004). Two slides for each sample were analysed. Data were checked blindly by two well trained individual scorers to minimize individual error and the average data were considered. MN score was represented as percent wise, as described by Titenko-Holland et al. (1994).

Statistical analyses

Since the number of individuals followed up slightly varied in 2010–2011 compared to 2004–2005, we have utilized one–way ANOVA with unpaired *t* test and Welch's Correction, to compare between the three study cohorts of the startup populations and follow ups. We have considered the MN data of only those individuals who were available at both the time points and hence have performed paired *t* test, as mentioned in Table 2. Unpaired *t* test with Tukey–Kramer multiple comparisons was conducted when comparing between cohorts in a single time point. All the statistical interpretation was done using InStat Graph Pad (San Diego, USA).

Results and discussion

Demographic characteristics of the study populations

Descriptive characteristics of the parent population for both 2004–2005 and 2010–2011 are summarized in

Table 1. No significant differences were found between the groups in age, gender distribution or tobacco usage. Smoking, mostly in form of *bidis* was the common addiction for the male participants in each cohort group, while the females were habituated towards chewing tobacco in form of betel leaves, areca nuts and slaked lime. Most of the male participants were cultivators and daily wage earners; while females were housewives, in general. Both the skin lesion (cohort 1 and cohort 1a) and no skin lesion group (cohort 2 and cohort 2a) were exposed to arsenic through drinking water at a similar extent as evident by arsenic content analysis. Moreover there was no arsenic mining in these study areas and the questionnaire records indicate that the sea food consumption was very rare in these participants. Hence, any chronic exposure due to arsenic in these study individuals mostly came from the drinking water. In 2010–2011, the arsenic content in drinking water was drastically reduced ($p < 0.0001$) to safe limit in the follow-up population (cohort 1a and cohort 2a) compared to their respective parent population, and this decrease reflected upon the arsenic content in the urine for the study cohorts at two different time points.

Table 1 Descriptive characteristics of the parent population studied during 2004–2005 and follow-up population in 2010–2011

	Study duration 2004–2005			Study duration 2010–11		
Parameters	Cohort 1	Cohort 2	Cohort 3	Cohort 1a	Cohort 2a	Cohort 3a
Total subjects	75	70	60	61	67	54
Male [*N* (%)]	48 (64.0)	48 (68.6)	36 (60.0)	40 (65.6)	46 (68.6)	33 (61.1)
Female [*N* (%)]	27 (36.0)	22 (31.4)	24 (40.0)	21 (34.4)	21 (31.3)	21 (38.9)
Age (years) [mean ± SD]	39.21 ± 12.33	37.97 ± 11.26	38.03 ± 13.17	37.93 ± 11.71	35.2 ± 10.56	37.23 ± 12.03
Overall tobacco usage	33 (44.0)	23 (32.8)	23 (38.3)	29 (47.5)	23 (34.3)	22 (40.7)
Smoking (bidi/cigarette)	18 (54.5)	21 (91.3)	17 (73.9)	18 (62.1)	21 (91.3)	16 (72.7)
Tobacco chewing	15 (45.5)	2 (8.7)	6 (26.1)	11 (37.9)	2 (8.7)	6 (9.1)
Non-tobacco users	42 (56.0)	47 (67.2)	37 (61.7)	32 (52.5)	44 (65.7)	32 (59.3)
Occupation						
Cultivation	24 (32.0)	15 (21.4)	6 (10.0)	21 (34.4)	15 (22.3)	5 (9.3)
Business	6 (8.0)	6 (8.6)	4 (6.7)	4 (6.6)	5 (7.5)	4 (7.4)
Daily wage earners	14 (18.7)	20 (28.6)	24 (40.0)	11 (18.0)	19 (28.4)	22 (40.7)
Service	3 (4.0)	4 (5.7)	2 (3.3)	2 (3.3)	8 (11.9)	2 (3.7)
Student	2 (2.7)	3 (4.3)	1 (1.7)	2 (3.3)	3 (4.5)	1 (1.8)
Housewife	25 (33.3)	18 (25.7)	22 (36.7)	21 (34.4)	17 (25.3)	20 (37.0)
Arsenic content [mean ± SD]						
Drinking water (μg/L)	348.23 ± 102.67	327.56 ± 115.23	BDL	5.60 ± 10.83**	8.53 ± 13.24**	BDL
Urine (μg/L)	642.31 ± 98.76$^{\#\#}$	598.64 ± 101.22$^{\#\#}$	11.62 ± 2.24	21.99 ± 1.27**$^{,\#\#}$	26.14 ± 3.92**$^{,\#\#}$	15.34 ± 1.93

BDL below detection level

** $p < 0.0001$, unpaired *t* test within same cohorts at two different time points, Welch corrected; $^{\#\#}$ $p < 0.001$, unpaired *t* test between (cohort 1 vs cohort 3 and cohort 2 vs cohort 3 in 2004–2005; cohort 1a vs 3a and cohort 2a vs 3a in 2010–2011), Tukey–Kramer multiple comparison

Reduction of cytogenetic damage by MN frequency distribution

The mean MN frequency in the urothelial cells of the three study groups at both the time point has been detailed in Table 2. The frequency of urothelial MN in exposed group was significantly higher compared to unexposed group. Interestingly, true to the expectation, the mean MN frequency in cohort 1a (1.84 ± 0.38) and cohort 2a (1.62 ± 0.77) was drastically reduced from their respective parent population of cohort 1 (5.15 ± 1.73) and cohort 2 (4.51 ± 1.27); while mean MN frequency in unexposed population remained almost similar in cohort 3 (1.21 ± 0.25) and cohort 3a (1.24 ± 0.33). Still, in 2010–2011, there was a significant difference in the MN frequency between the unexposed controls (cohort 3a), when compared with the exposed individuals (cohort 1a and 2a). Recently, in a two wave cross sectional study, we had observed a significant decrease in the MN-frequency, when arsenic in drinking water was significantly reduced but not below the maximum threshold limit (Paul et al. 2013). Hence, the present observation reflects the efficacy of recovery from genotoxic damage by arsenic, when individuals having chronic exposure to arsenic, consume "safe" drinking water according to WHO standards. This is one of the first study of it's kind where arsenic exposed individuals are being evaluated for the reduction of genotoxic damage when these individuals, having a chronic history of arsenic exposure, were consuming "safe" drinking water for at least 5 years.

Table 2 Micronuclei frequency profile (mean ± SD) at two time points (2004–2005 and 2010–2011) of the different study cohorts

Cohort (*N*)	2004–2005	2010–2011
Cohort 1 (61)	5.15 ± 1.73	1.84 ± 0.38**,##
Cohort 2 (67)	4.51 ± 1.27	1.62 ± 0.77**,##
Cohort 3 (54)	1.21 ± 0.25	1.24 ± 0.33

** $p < 0.001$ (paired t test) the samples were repeated in 2010–2011 (cohort 1a, 2a and 3a), from those collected in 2004–2005 (cohort 1, 2 and 3). Only those samples (*N*) were considered within the calculation, who were available at both the time points; ## $p < 0.001$ (Unpaired t test, Tukey–Kramer multiple comparison) between cohort 1a or 2a with 3a in 2010–2011. The MN frequency for approximately 2,000 urothelial cells were calculated and expressed as MN.1000 cells^{-1}. For the individual sample's MN data, refer Supplementary Data Table 1

Possible causes of cytogenotoxicity

Chronic exposure to arsenic induces generation of ROS within the body (Shi et al. 2004), following its detoxification to organic forms of arsenic (Concha et al. 2002). The ROS generation has direct detrimental effect on the chromatin integrity, leading to oxidation of the DNA back bone and inducing break (Jomova et al. 2011). This leads to the observation of various chromatin instability outcomes like CA and MN, which are utilized in monitoring various genotoxic damage incurred upon the cell by various xenobiotic agents apart from arsenic (Ghosh et al. 2007; Rossner et al. 2005). In humans, several studies have pointed out that exfoliated urothelial cells show induction of MN upon arsenic exposure (Biggs et al. 1997; Moore et al. 1997; Basu et al. 2004; Ghosh et al. 2006).

The present study elaborates that on decreasing the arsenic load through drinking water, below the recommended level (<10 μg/L), it was possible to reduce a bulk of the cytogenetic damage in a population having a chronic history of arsenic exposure. However this MN frequency was significantly higher ($p < 0.001$) in the study participants who has a history of chronic exposure (cohort 1a and 2a) compared to the unexposed cohort (3a). Although the total arsenic content in the urine samples of these three was quite low but, the amount of arsenic in the urine samples of cohort 1a and 2a were significantly high when compared to cohort 3a, even though both the cohorts (1a and 2a) were consuming safer drinking water. Thus, it indicates that apart from drinking water, there might be other sources of arsenic intake through their diet. Earlier report indicates that arsenic in rice is a major source of arsenic exposure in humans when there is no arsenic in the drinking water (Mondal et al. 2010). Very recently we have also found that arsenic in rice alone can induce genotoxic effects in human urothelial cells (Banerjee et al. 2013; communicated). This rural Bengal population consumes rice as staple food. So this slight increase in MN in cohort 1a and 2a may be due to the intake of arsenic through other dietary sources.

Conclusion

As mentioned earlier that although urothelial MN stands second best biomarker compared to other two

cell types (Ghosh et al. 2008), but due to the easy availability of the samples, the scope of studying MN in urothelial cells stands equally important to monitor the population exposed to low dose of arsenic. Our observations indicate that decline in arsenic consumption through drinking water can bring a major reduction in genetic toxicity within the urothelial cells. It is advisable that water for irrigation purposes should be under constant monitor as high arsenic in rice in this area has already been reported earlier (Mondal et al. 2010).

In future, we believe our study would call for better collaboration between biological researchers along with civil-engineering researchers to build up reservoirs or develop techniques to supply such arsenic free water to the people living in such arsenic-prone regions of the world.

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Conflict of interest None declared.

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