

Review Article

Arsenic Induced Health Effects, Genetic Damage and Genetic Variants in the Population Exposed to Arsenic through Drinking Water in West Bengal

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(Received on 09 October 2013; Revised on 17 February 2014; Accepted on 19 February 2014)

Arsenic is a potent human carcinogen, which affects millions of people around the world causing deleterious health impacts including cancer and death. In India, West Bengal is the worst affected state where more than 26 million people are exposed chronically to arsenic by drinking heavily contaminated ground water. Several hypotheses have been associated with arsenic-induced carcinogenesis including chromosomal abnormalities, oxidative stress, altered DNA repair, p53 gene suppression, gene amplification, transformation and altered growth factors leading to increased cell proliferation and carcinogenesis. In addition to that, it has been hypothesised that altered DNA methylation patterns might contribute to arsenic-induced carcinogenesis. Even then, the mechanisms leading to arsenic-induced carcinogenesis are poorly understood. We have been trying to identify the contenders that contribute to arsenic susceptibility, toxicity and carcinogenicity in the arsenic exposed rural population of West Bengal. In this article, attempts have been made to summarise the work that we have carried out so far in the said human population exposed to arsenic through drinking water.

Key Words: Arsenic; Health Effects; Genetic Damage; Genetic Susceptibility; Immunosuppression; Mitigation

Introduction

Arsenic is a major environmental contaminant and more than 137 million people in 70 countries of the world are affected by drinking heavily contaminated ground water (Modal *et al.*, 2010). In India, West Bengal is the worst affected state where more than 26 million people in 9 districts are affected by drinking arsenic-laden drinking water that contains arsenic much above the permissible limits of 10µg/l laid down by WHO (Chakraborty *et al.*, 2009). Chronic arsenic exposure through ingestion of arsenic-contaminated water has resulted in various hazardous health outcomes including noncancerous (raindrop pigmentation and hyper pigmentation), precancerous (palmer, planter and palmo-planter hyperkeratosis), and cancerous skin lesions (basal cell carcinoma [BCC], squamous cell carcinoma [SCC], and Bowen's diseases [BD]). These skin lesions may

develop within the latency period of 6 months to 10 years from first exposure to arsenic. The appearance of skin lesions depends on the concentration of arsenic in drinking water, volume of intake and the health and nutritional status of individuals exposed to arsenic (Rahman *et al.*, 2003). It also causes cancers of liver, kidney, bladder, and other internal organs. Other symptoms of chronic arsenic poisoning include anaemia, burning sensation of the eyes, solid edema of the legs, liver fibrosis, chronic lung disease, gangrene of the toes (Blackfoot disease) and neuropathy (Guha Mazumder *et al.*, 2001). During our epidemiological survey we have found peripheral neuropathy, respiratory problems and conjunctival irritations in the eyes are the most commonly occurring non-dermatological health effects in our study population. Although arsenic-induced skin lesions are considered as hallmarks of chronic arsenic toxicity, only 15-20% of the total population show

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arsenic-induced skin lesions and the rest do not (Banerjee *et al.*, 2011), indicating that genetic variations might play an important role in arsenic susceptibility, toxicity and carcinogenicity. We have been trying to find out cause of arsenic susceptibility, toxicity and carcinogenicity in the arsenic exposed rural population of West Bengal. In this study, we have summarised the work that we have done so far in this population who are exposed to arsenic through drinking water.

Evaluation of Arsenic Induced Health Effects and Genetic Damage

1. Identification of the Arsenic Exposed Populations in the Districts of North 24 Parganas, Nadia and Murshidabad, West Bengal

Water and other biological samples (urine, nail, hair and blood) were collected from the arsenic exposed individuals of three highly arsenic-affected districts of West Bengal i.e. North 24 Parganas, Nadia and Murshidabad. Five villages from the four administrative blocks (Gaighata, Habra, Deganga and Baduria) of North 24 Parganas, two villages i.e. Dasdia and Ghetugachi from Haringhata block of the district of Nadia and Bhagabangola-I and II, Hariharpara and Pomaipur blocks from the Murshidabad district were selected. The control subjects were chosen from East Midnapur district of the same state with little or no history of arsenic exposure. The study subjects were matched with respect to age, sex and socio-economic status. Individuals ranging from 18 to 60 years of age with at least 10 years of arsenic exposure were selected as arsenic exposed study participants. Occupationally, majority of the study participants were farmers and household workers. An interview was performed based on a structured questionnaire that elicited information about demographic factors, life-style, occupation, diet, smoking, medical and residential histories (Ghosh *et al.*, 2007a). An expert dermatologist, with fifteen years of experience identified the characteristic arsenic-induced skin lesions and helped in the recruitment of the exposed study participants. He also confirmed that the other group had no arsenic-induced skin lesions. Then

specialists in the fields of ophthalmology, neurology and respiratory diseases examined each participant to diagnose non-dermatological health effects in them. Urine, water, nail, hair and blood samples were collected only from those subjects who provided informed consent to participate in the study. This study was conducted in accord with the Helsinki II Declaration and approved by the Institutional Ethics Committee of CSIR - Indian Institute of Chemical Biology.

2. Collection of Water and Biological Samples and Exposure Assessment

Blood samples were collected from a total of 1600 (880 males and 720 females) arsenic exposed individuals comprising 853 individuals with skin lesions (459 males and 394 females) and 747 individuals without skin lesions (421 males and 326 females) from three arsenic affected districts of West Bengal. The control blood samples were collected from the 1200 participants (671 males and 529 females) living in the East Midnapur district of the same state. The collected samples included drinking water (100 ml approx.), urine (100 ml), nails (approx. 250-500 mg) and hair (approx. 300-500 mg). The samples were analysed by Atomic Absorption Spectrometer. A Perkin-Elmer Model-3100 (Boston, MA) spectrometer equipped with a Hewlett-Packard (Houston, TX) Vectra computer with GEM software, Perkin-Elmer EDL System-2, arsenic lamp (lamp current 380 mA) was utilised for the purpose.

3. Assessment of Different Major Diseases in Arsenic Exposed Population

Skin lesions are considered as hallmarks of chronic arsenic toxicity. Dermatologists identified noncancerous: raindrop pigmentation, hypo- and hyper-pigmentation, precancerous palmer and plantar hyperkeratosis, and cancerous lesions like BD, BCC, and SCC in our study population. However, these skin lesions often appear after a latency period of 6 months up to 10 years or more from the initial exposure and the degree of arsenic susceptibility in humans is often determined by individual genetic variability. So, we made an attempt to compare the prevalence of three

known major arsenic-induced health effects i.e. peripheral neuropathy, respiratory distress and eye problems in the individuals with and without skin lesions drinking same arsenic contaminated water. Attempts have also been made to compare the results with a group of unexposed individuals.

(a) Clinical Symptoms Recorded

Since three major diseases i.e. peripheral neuropathy, respiratory diseases and eye problems (mainly conjunctivital irritations of the eyes) were very common non-dermatological health effects in the arsenic exposed populations reported earlier, so we have mainly examined and recorded these three different diseases in our study population in addition to the arsenic induced skin lesions. Each subject was examined by a team of four expert physicians, which included dermatologist, neurologist, ophthalmologist and respiratory disease specialist.

(b) Dermatological Symptoms

Since skin lesions are hall marks of chronic arsenic exposure, each subject was examined by a skin specialist. Long-term exposure to arsenic causes changes in skin pigmentation and hyperkeratosis. This promotes ulceration of skin and accelerates the risk of skin cancer. Hallmark signs of chronic arsenicism are raindrop pigmentation, palmar and plantar hyperkeratoses, hypo and hyperpigmentation. The type, extent and duration of different types of skin lesions were noted. Patients were carefully examined for different skin lesions, Raynaud's phenomenon and ulcer, gangrene, BD or cancer. Significant skin lesions were captured in the digital camera for future record.

(c) Peripheral Neuropathy

Peripheral neuropathy is a common complication of arsenic poisoning, which affects both sensory and motor nerves. The criteria recorded were: (i) pain and paraesthesias in stocking and glove distribution, (ii) numbness, (iii) weakness and (iv) muscle cramp. The different signs of clinical neuropathy were anaesthesia or hypoesthesia (no or reduced sensation) to touch, pain, temperature, pressure, vibration, calf tenderness, power and deep tendon reflexes. All the

confirmed and suspected neuropathy individuals were ultimately sent to the National Neuroscience Centre, Kolkata for the Nerve Conduction Velocity (NCV) test and Electro-myograph (EMG) test for the confirmation of neurological disorder.

(d) Respiratory Diseases

Respiratory diseases are very common in the arsenic exposed populations. Ingestion of inorganic arsenic in drinking water results in pulmonary effects manifested by cough, chest sounds in lungs and shortness of breath. All the unexposed and exposed participants were thoroughly examined by an expert respiratory disease specialist in medical camps. We have focused on the prevalence of respiratory signs and symptoms assessed in the survey including cough, chest sound and shortness of breath. Cough due to seasonal variation and chronic bronchitis or bronchial asthma patients were not considered in these cases. Participants were questioned about their smoking habits since it is a confounding factor in lung cancer and chronic obstructive pulmonary diseases (COPD) related mortality. Cases suspected for interstitial lung disease (ILD) were carefully examined.

(e) Eye Problems

Individuals exposed to chronic arsenicism were suffering from various vision-related problems. Each individual was examined by an eye specialist. Conjunctivitis, conjunctival irritation with burning sensation and watering were recorded since these were the common eye symptoms of arsenic exposed individuals. Only watering or congestion was excluded from our study. Refractive errors and cataract were also not considered since these may be age related problems and their link to arsenic toxicity will be questionable.

The results are shown in Fig. 1. The frequency of arsenic-induced peripheral neuropathy, respiratory problems, and conjunctival irritation of the eyes were significantly higher in exposed individuals compared to the unexposed individuals. Within the exposed group, the incidences of all three diseases were significantly higher in individuals with skin lesions compared to the individuals without skin lesions. In

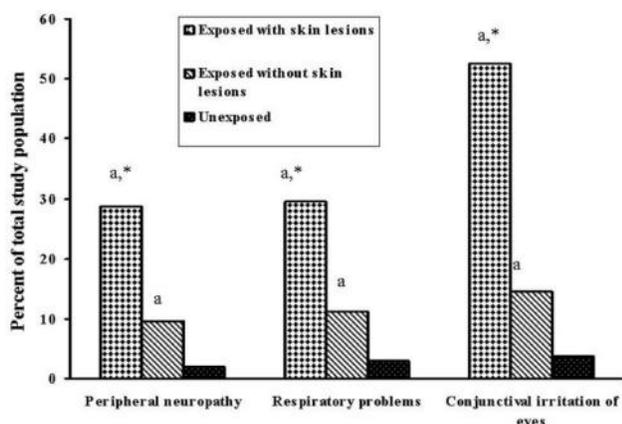


Fig. 1: Arsenic induced non-dermatological health effects. Students Unpaired T Test was performed. ^a $p < 0.001$ when compared with unexposed individuals, ^{*} $p < 0.001$ when compared to the exposed individuals without skin lesions. $N=853$ for individuals with skin lesions; $N=747$ for individuals without skin lesions; $N=1200$ for unexposed individuals

another similar work by Majumdar *et al.* (2009), a cross-sectional study was carried out in South 24 Parganas of West Bengal and a correlation of systemic manifestations in relation to arsenic exposure was found in subjects having no arsenic induced skin lesions. Results showed that in persons without arsenic induced skin lesions and drinking water with arsenic content more than 50 ppb, the frequency of occurrence of various clinical manifestations like weakness, anaemia, diarrhoea, hepatomegaly and lung disease was found to be significantly higher than to those taking water with arsenic content below that level. This, together with our results confirmed that although individuals with skin lesions are more susceptible to arsenic induced toxicity and carcinogenicity but those individuals who did not manifest any skin lesions yet, were also at considerable risk for developing such health related problems.

4. Assessment of Immunological Changes in Arsenic Exposed Population

(a) Analysis of T-Cell Proliferation and Cytokine Secretion

Higher incidences of opportunistic infections are found in the arsenic exposed individuals, indicating that their immune systems may be impaired somehow.

Thus, the effect of arsenic on T-cell proliferation and cytokine secretion was observed in 20 individuals with arsenic induced skin-lesions and the results were compared with 18 arsenic unexposed individuals. A marked dose dependent suppression of Concanavalin A (Con A) induced T-cell proliferation was observed in the arsenic exposed individuals when compared with the unexposed ($p < 0.001$) individuals. This correlated with a significant decrease in the levels of secreted cytokines by the T-cells (TNF- α , IFN- γ , IL2, IL10, IL5 and IL4) in the exposed individuals ($p < 0.001$) (Biswas R *et al.*, 2008).

(b) Arsenic Induces Apoptosis via Mitochondria Mediated Pathway

Although arsenic is a potent human carcinogen, interestingly, it is also known to induce apoptosis *in vitro* in various cancer cells at lower concentrations. The induction of apoptosis was studied in human peripheral blood mononuclear cells (PBMC) of 30 arsenic exposed individuals with skin lesions by annexin V-FITC staining and compared with 28 unexposed individuals. Attempts have also been made to find out the probable mechanism by which the cells were undergoing apoptosis. The percentage of apoptotic cells in both T lymphocytes and monocytes, in the individuals with skin lesions was significantly higher when compared to unexposed individuals ($p < 0.01$). In these exposed individuals with skin lesions, elevated levels of intracellular reactive oxygen species (ROS) resulted in mitochondrial membrane permeability, increased cytochrome *c* release, leading to increased downstream caspase activity. Arsenic-induced DNA damage was confirmed by DNA ladder formation and confocal microscopy. Arsenic reduced Bcl-2/Bax ratio and also resulted in cell cycle arrest of PBMC in G_0/G_1 phase. Thus, it can be concluded that arsenic exposure causes apoptosis via the mitochondria-mediated pathway (Banerjee *et al.*, 2008a). Our results correlated with previous *in vitro* studies in mice where arsenic induced mitochondria mediated apoptosis occurred in liver of the treated mice. Arsenic induced liver injury was found to be associated with increased oxidative stress in liver mitochondria and alteration of mitochondrial permeability transition (MPT). Altered MPT

facilitated cytochrome c release in the cytosol, activation of caspase 9 and caspase 3 activities resulting in apoptotic cell death (Santra *et al.*, 2007).

(c) Chronic Arsenic Exposure Impairs Macrophage Functions in the Exposed Individuals

Owing to the established roles of human macrophages in immune defence, the effect of chronic arsenic exposure upon these major hematopoietic cells was investigated in 70 arsenic-exposed individuals with skin lesions and 64 unexposed individuals. Human monocyte-derived macrophages were prepared from peripheral blood mononuclear cells, by culture of the adherent cells for 6 days in medium supplemented with granulocyte-monocyte colony stimulating factor. Parameters studied included cell adhesion capacity, expression of CD54 and F-actin, nitric oxide production, phagocytic capacity, and effect of arsenic on Rho A-ROCK pathway. In macrophages of exposed individuals when compared to unexposed group, there was cell rounding accompanied with a significant ($p < 0.001$) loss of cell adhesion capacity, decrease in nitric oxide production, impaired phagocytic capacity, and decreased CD 54 and F-actin expression. Additionally, chronic arsenic exposure affected Rho A-ROCK pathway, which in turn impaired macrophage functions. Since macrophages are important immune mediators, arsenic induced impairment of macrophage functions could contribute significantly to arsenic-induced immuno-suppression as is observed in the exposed individuals (Banerjee *et al.*, 2009).

5. Study of Mechanism of Erythrocyte Death in Human Population Exposed to Arsenic through Drinking Water

The mechanism of haemolysis in arsenic exposed population is controversial till date. Arsenic has been reported to exert its toxic effects through oxidative denaturation of hemoglobin. Later investigations have contradicted this theory and proposed a non-oxidant mechanism suggesting membrane transport as the target site for arsenic action. A comprehensive study on the red blood cell environment may provide indications on the mechanism of premature destruction of these cells in arsenic toxicity. The

objective of this study was to understand the sequence of toxic events in arsenic induced premature destruction of erythrocytes. Blood samples were collected from unexposed and exposed individuals (50 samples each). Morphologic characterisations of erythrocytes were done with scanning electron microscope (SEM).

Results revealed transformation of smooth discoid red cells into evaginated echinocytic form in the exposed individuals. Further distortion converted reversible echinocytes to irreversible spherocytocytes. Arsenic toxicity increased membrane microviscosity along with an elevation of cholesterol/phospholipid ratio, which hampered the flexibility of red cell membrane and made them less deformable. Significant increase in the binding of merocyanine 540 with erythrocyte membrane due to arsenic exposure indicated disruption of lipid packing in the outer leaflet of the cell membrane resulting from altered trans-bilayer phospholipid asymmetry. Arsenic induced eryptosis was characterised by cell shrinkage and exposure of phosphatidylserine at the cell surface. Furthermore, metabolic starvation with depletion of cellular ATP triggered apoptotic removal of erythrocytes from circulation. Significant decrease in reduced glutathione content indicating defective antioxidant capacity was coupled with enhancement of malondialdehyde and protein carbonyl levels, which pointed to oxidative damage to erythrocyte membrane. Arsenic toxicity intervened into red cell membrane integrity eventually leading to membrane destabilisation and haemoglobin release. The study depicted the involvement of both erythrophagocytosis and haemolysis in the destruction of human erythrocytes during chronic arsenic exposure (Biswas D *et al.*, 2008).

6. Assessment of Genetic Damage in Individuals Exposed to Arsenic through Drinking Water

A cross-sectional biomarker study was conducted to evaluate and compare the cytogenetic damage as measured by micronuclei (MN) in peripheral blood lymphocytes, oral mucosa cells and urothelial cells from the inhabitants of North 24 Parganas, one of

the arsenic affected districts. The 3 cell types were collected from 163 residents exposed to high levels of arsenic in drinking water ($214.7213 \pm 9.0273 \mu\text{g/l}$), and from 154 unexposed subjects residing in the unaffected Midnapur district with very little or no exposure to arsenic through drinking water ($9.2017 \pm 0.3157 \mu\text{g/l}$). Our analysis revealed that MN frequencies in the exposed group were significantly elevated to 5.33 fold over unexposed levels for lymphocytes, 4.63 fold for oral mucosa cells, and 4.71 fold for urothelial cells (increases in MN frequencies significant at $p < 0.01$). The results indicate that chronic ingestion of arsenic in drinking water by the exposed subjects is linked to the enhanced incidence of MN in all the 3 cell types; slightly higher level of MN being observed in lymphocytes compared to oral mucosa and urothelial cells (Basu *et al.*, 2004).

Assessment of genetic damage in the human populations exposed to arsenic through drinking water were also carried out as measured by chromosomal aberrations (CA) and sister chromatid exchanges (SCE) in the individuals with and without skin lesions. A significant ($p < 0.01$) increase in CA and SCE were observed in both the skin lesion and no skin lesion groups when compared to unexposed individuals. Elevated mean values ($p < 0.01$) of the percentage of aberrant cells (8.08%) and SCEs per cell (7.26) were also observed in the exposed individuals in comparison to controls (1.96% and 5.95, respectively). Within the two exposed groups, arsenic induced higher incidence of CA and SCE in the skin lesion than the no skin lesion group. These results suggested that no skin lesion individuals had relatively lower sensitivity and susceptibility towards induction of genetic damage by arsenic compared to the individuals with skin lesions (Mahata *et al.*, 2003).

Again, a matched case-control study was performed to examine whether biomarkers such as chromosomal aberrations can predict the development of arsenic induced Bowen's (*in situ* carcinoma) diseases. Chromosomal aberrations (both chromosome and chromatid types) and mitotic index were analysed from the lymphocytes of 25 cases of Bowen's patient which was compared to matched control from the individuals with arsenic induced skin

lesions such as raindrop pigmentation, keratosis of palm and sole, hypo and hyper pigmentation. Chromosomal aberrations/cell, chromosome type aberrations and total percentage of aberrant cells were significantly higher in cases compared to control ($p < 0.01$). These results suggest that chromosomal aberrations can be used for cancer risk assessment of the population exposed to arsenic through drinking water (Ghosh *et al.*, 2007b).

To detect DNA damage, alkaline comet assay was performed from lymphocytes of 30 individuals residing in North 24 Parganas district and exposed to high levels of arsenic ($247.12 \pm 18.93 \mu\text{g/l}$) in drinking water with evidence of high arsenic contents in nail ($4.20 \pm 0.67 \mu\text{g/g}$), hair ($2.06 \pm 0.20 \mu\text{g/g}$) and urine ($259.75 \pm 33.89 \mu\text{g/l}$) samples and manifesting arsenical skin lesions. Unexposed samples were collected from 30 residents of the unaffected East Midnapur district with very little or no exposure to arsenic ($7.69 \pm 0.49 \mu\text{g/l}$) in drinking water. The results were evaluated principally by manual comet analysis and partly by computerised image analysis. Both the analytical methods exhibited a high degree of agreement in results. The exposed participants expressed significantly higher DNA damage ($p < 0.01$) in their lymphocytes than the unexposed participants. Significant positive trend effects of comet lengths in relation to arsenic levels in water prove that DNA damage can be used as a sensitive biomarker of carcinogen exposure. This study demonstrated that arsenic induced significant DNA damage in the exposed participants, which could correspond to a higher susceptibility to arsenic induced toxicity and carcinogenicity (Basu *et al.*, 2005).

Our results can be correlated with several other studies around the world. Higher incidences of micronuclei induction was found in a Chilean population exposed to arsenic through drinking water as compared to the control group (Martinez *et al.*, 2004). Again, it was found that arsenic induced extensive DNA damage in a chronically exposed population of West Bengal (Biswas *et al.*, 2010). In this work, the authors found a protective role of curcumin in combating arsenic induced toxicity. In

another study, attempt has been made to find the genotoxic potential of arsenic at its reference dose in mice, at doses equivalent to its human reference dose as well as at higher doses. Significant increases in the frequencies of chromosome abnormalities in the bone marrow cells were found over the control level upon exposure to all the doses of arsenic including its reference dose (Kesari *et al.*, 2012). In a very recent study, an attempt was made to find out oxidative DNA damage and repair in children exposed to low levels of arsenic in utero and during early childhood. Results showed that levels of salivary 8-OHdG in exposed children were significantly higher (~4-fold, $P < 0.01$), whereas levels of urinary 8-OHdG excretion and salivary hOGG1 expression were significantly lower in exposed children (~3-fold, $P < 0.05$), suggesting a defect in hOGG1 that resulted in ineffective cleavage of 8-OHdG, which play critical role in oxidative DNA damage (Hinhumpatch *et al.*, 2013). Another *in vitro* study showed that arsenic is cytotoxic and genotoxic to human lung primary cells but lung fibroblasts are more sensitive to arsenic than epithelial cells (Xie *et al.*, 2014). Thus, there are sufficient evidences to conclude that arsenic is genotoxic in nature and causes extensive DNA damage.

7. DNA Repair Deficiency May be the Cause of Arsenic Susceptibility

In another study, alkaline comet assay, CA study and Challenge assay were carried out in the lymphocytes to find out the DNA damage and DNA repair capacity in individuals both with and without skin lesion. Sixty arsenic exposed (30 individuals with arsenic-induced skin lesions and 30 without skin lesions but drinking similar arsenic contaminated water) and 30 arsenic unexposed individuals were recruited as study participants. Individuals with and without skin lesions had comparable levels of arsenic exposure and urinary arsenic content compared to the unexposed individuals. Both the exposed subgroups (with and without skin lesions) had significantly higher DNA damage as can be seen from significantly higher values of each of the three parameters i.e. Olive Tail Moment (2.51 ± 1.40 and 2.76 ± 1.39 respectively), % of tail DNA (13.40 ± 3.51 and 14.05 ± 4.71

respectively) and tail length (13.54 ± 4.38 and 11.85 ± 5.51 respectively) [$p < 0.001$ in each case] compared the unexposed group (OTM = 0.55 ± 0.83 , % Of Tail DNA = 4.29 ± 1.49 and Tail Length = 2.20 ± 0.72 respectively). However, within the exposed population, there was no significant difference in any of these three parameters, in between the two subgroups ($p > 0.05$). Both the exposed subgroups had significantly higher incidence of CA (both CA/cell and % of aberrant cells) [$p < 0.001$] than the unexposed group. However, within the exposed group, individuals exhibiting arsenic-induced skin lesions have significantly higher incidence of CA (both CA/cell and % of aberrant cells) [$p < 0.01$] than the group of individuals without skin lesion. There was no significant difference in the basal DNA damage level, in terms of each of the three parameters used in between the two subgroups. However, significant difference in the level of damaged DNA existed in each of the three parameters used in arsenic exposed individuals with and without skin lesions respectively [$p < 0.0001$ in each case] was seen in between the two subgroups after the induction of damage and subsequent repair. Thus the deficiency in DNA repair capacities in the individuals with skin lesions emerged as a prime contender for arsenic susceptibility (Banerjee M *et al.*, 2008).

Genetic Variations and Susceptibility in Arsenic Exposed Population

1. Analysis of Purine Nucleoside Phosphorylase (PNP) and Arsenic Methyltransferase (As3MT) Gene Polymorphisms in the Arsenic Exposed Individuals

Individual variability in arsenic metabolism may be the underlying fact of individual susceptibility towards arsenic induced skin lesions as well as skin cancer. PNP enzyme has been found to act as the arsenate reductase that converts arsenate (AsV) to arsenite (AsIII) and thus plays an important role in arsenic metabolism i.e. biotransformation. A number of *in vivo* and *in vitro* studies established that As3MT enzyme is indispensable for conversion of the arsenic metabolites to their corresponding methylated products. Methylation of inorganic arsenic to form

methylarsonic acid and dimethylarsinic acid is an important reaction in arsenic biotransformation. Because of the importance of this metabolic reaction in humans, both *PNP* and *AS3MT*, were chosen as candidate genes whose functionally significant genetic polymorphisms might contribute to risk for arsenic-dependent carcinogenesis. A cross-sectional study recruiting total 428 unrelated individuals (229 cases i.e. individuals having arsenic induced skin lesions and 199 controls i.e. individuals without arsenic induced skin lesions but drinking arsenic contaminated water at similar concentration) from severely affected districts of West Bengal for the study of the genotypic distribution of *PNP* and *AS3MT* variants. A total exon-promoter screening of *PNP* gene by PCR-sequencing method revealed that three exonic polymorphisms (H20H, G51S and P57P) were showing high minor allele frequencies and differential distribution in case and control individuals. Results show (Table 1) significant genotypic difference between case and control groups. Again, screening of the entire coding region revealed only one exonic SNP (Met287Thr) in our population, with a heterozygosity of about 9%, similar to studies with African-American and white American populations. Thus the result (Table 1) depict that the individuals carrying these genotypes are showing an increased tendency of developing arsenic induced skin lesions and hence increased susceptibility towards arsenic toxicity (De Chaudhuri *et al.*, 2008).

2. Role of *p53* Gene Polymorphisms in Arsenic Susceptibility

Inactivation of *p53* gene function is frequently observed in various human cancers. The inheritance of a *p53* mutation has been associated with a high risk of cancer at an early age. The various mechanisms by which the *p53* protein is inactivated include allelic loss, point mutations, and by forming a complex with viral oncoproteins. Among these sequence variants the codon 72 polymorphism which changes an Arginine (Arg) to a Proline (Pro) due to a G to C transversion, along with concomitant loss of a *BstU1* polymorphic site, was the first to be described and has since been subject to many epidemiological studies that explore the possible association of this

polymorphism with the risk of different cancer types. So, the objective of this study was to find out the association of a particular *p53* haplotype (the loss of a 16 bp duplication allele at intron3-Arg at codon 72-absence of *Nci1* at intron 6) with the arsenic induced skin lesions mainly keratosis. Genotyping of *p53* was done on the basis of PCR and RFLP. Blood samples were collected from unrelated 189 individuals without skin lesions and 177 skin-lesion (keratosis) donors. Restriction enzyme *BstU1* (Arg72Pro) and *Nci I* (G>A in intron 6) were used and analysed by PAGE. 16 base pair deletion/duplication (S/D) were assessed by PCR and PAGE analysis. Results showed (Table 2) that R/R (OR 2.086, 95% CI 1.257-3.457) and S/S (OR 2.086, 95% CI 1.318-3.299) genotype has higher risk for development of arsenic induced keratosis (De Chaudhuri *et al.*, 2006).

3. Role of *ERCC2* Codon 751 Polymorphism in Arsenic Susceptibility

ERCC2 is a nucleotide repair pathway gene, whose protein product has a helicase activity and plays a key role in mending DNA damage, especially those induced by inorganic chemicals. *ERCC2* codon 751 polymorphism (A→C; Lys→Gln) is implicated in several types of cancer. The aim of the study was to find out any possible association of *ERCC2* codon 751 polymorphism with arsenic specific premalignant hyperkeratosis. 165 individuals with arsenic specific hyperkeratosis and 153 individuals with no arsenic specific skin lesions were genotyped by PCR-RFLP (*EarI*) method, and the distribution of the three possible genotypes in the two study groups was found out. AA genotype was significantly over represented in the arsenic induced hyperkeratosis exhibiting group, indicating that it is strongly associated with the development of arsenic specific precancerous hyperkeratosis. Individuals with *ERCC2* codon 751 AA genotype were at 4.77 fold higher risk (OR 4.77, 95% CI 2.75 – 8.23) of developing arsenic induced hyperkeratosis than those with CC or AC genotype (Table 2). In order to validate this observation, chromosomal aberration study was done. Individuals with hyperkeratosis were seen to exhibit a significantly higher frequency of both CA/cell and percentage of aberrant cells compared to the no skin

Table 1: Genetic variants associated with arsenic induced skin lesions in the exposed population

| Genotype | Exposed individuals without skin lesions | Exposed individuals with skin lesions | OR (95% CI) | p value | Authors |
|----------------------------|--|---------------------------------------|------------------|---------|------------------------------------|
| PNP | | | | | |
| C>T (codon 20) | | | | | |
| CC | 160 (80.4) | 162(70.7) | 1.00 (Referent) | 0.02 | De Chaudhuri <i>et al.</i> , 2008 |
| CT/TT | 39 (19.6) | 67 (29.3) | 1.69 (1.08-2.66) | | |
| G > A (codon 51) | | | | | |
| G/G | 154 (81.05) | 160 (72.07) | 1.00 (Referent) | 0.04 | |
| GA / AA | 36 (18.95) | 62 (27.93) | 1.66 (1.04–2.64) | | |
| C > T (codon 57) | | | | | |
| CC | 151 (80.75) | 158 (71.5) | 1.00 (Referent) | 0.04 | |
| CT/TT | 36 (19.25) | 63 (28.5) | 1.67 (1.05-2.66) | | |
| AS3MT | | | | | |
| T>C (codon 287) | | | | | |
| TT | 181(90.95) | 206 (90.75) | 1.00 (Referent) | 0.94 | |
| TC/CC | 18 (9.05) | 21 (9.25) | 1.02 (0.53-1.98) | | |
| XRCC3 | | | | | |
| C allele | 303 (72.49) | 344 (83.49) | 1.00 (Referent) | 0.0002 | Kundu <i>et al.</i> , 2011 |
| T allele | 115 (27.51) | 68 (16.50) | 0.52 (0.37-0.72) | | |
| CC | 107 (51.19) | 144 (69.9) | 1.00 (Referent) | | |
| CT | 89 | 56 | 0.47 (0.31-0.71) | 0.0004 | |
| TT | 13 | 06 | 0.34 (0.1-0.93) | 0.033 | |
| CT/TT | 102 (48.8) | 62 (30.1) | 0.45(0.30-0.67) | 0.0001 | |
| TNF- (-308G>A) | | | | | |
| GG | 168 (88.4) | 148 (71.6) | 1.0 (Referent) | < 0.001 | Banerjee <i>et al.</i> , 2011 |
| GA/AA | 22 (11.6) | 59 (28.5) | 3.04 (1.78–5.21) | | |
| IL10 (-3575 T>A) | | | | | |
| TT | 157 (83.2) | 145 (71.1) | 1.0 (Referent) | | |
| TA/AA | 33 (16.8) | 62 (28.9) | 2.03 (1.26–3.28) | 0.0046 | |
| NALP2 (1052A>E) | | | | | |
| CC | 103 (48.35) | 128 (58.44) | 1.0 (Referent) | | Bhattacharjee <i>et al.</i> , 2013 |
| CA/AA | 110 (51.64) | 91 (41.55) | 0.67 (0.46-0.97) | 0.042 | |

lesion group ($p < 0.01$). A statistically significant increase in both the parameters (CA/cell and percentage of aberrant cells) was observed in the individuals with AA genotype compared with those with either AC or CC genotype combined ($P < 0.01$) in either of the two study groups (with hyperkeratosis

and without arsenic-induced skin lesions) as also in the total study population (comprising the two study groups combined). The data suggested significant role of *ERCC2* in arsenic susceptibility (Banerjee *et al.*, 2007).

4. Association of XRCC3 T241M Polymorphism in Arsenic Exposed Population

T241M polymorphism in *XRCC3* (a homologous recombination repair pathway gene) is widely studied for its association with several cancers so a case-control study was carried out to find the association of *XRCC3* T241M polymorphism with arsenic-induced precancerous and non-cancerous disease outcomes. The study involved 206 cases with arsenic-induced skin lesions and 215 controls without arsenic-induced skin lesions having similar arsenic exposure. *XRCC3* T241M polymorphism was determined using conventional PCR-sequencing method. Association with chromosomal aberration, arsenic-induced neuropathy and ocular diseases were also evaluated. The data revealed that presence of at least one Met allele (Met/Met or Thr/Met) was protective towards development of arsenic-induced skin lesions (Table 1), peripheral neuropathy [OR=0.49; 95% CI: 0.30-0.82] and conjunctivitis [OR=0.60; 95% CI: 0.40-0.92]. A significant correlation was also observed between protective genotype and decreased frequency of chromosomal aberrations. Thus, the results reveal the protective role of Met allele against the arsenic-induced skin lesions, chromosomal instability, peripheral neuropathy and conjunctivitis (Kundu *et al.*, 2011).

5. Role of Polymorphisms in the TNF- α and IL10 Gene Promoters in Imparting Risk of Causing Arsenic-Induced Skin Lesions and other Non-dermatological Health Effects

Chronic arsenic exposure has been associated with impairment of immune systems in the exposed individuals. Because cytokines are important immune mediators, alteration in expression of these gene products may lead to arsenic-specific disease manifestations. The aim of the present work was to investigate the association between the *TNF- α* -308G>A (rs1800629) and *IL10*-3575T>A (rs1800890) polymorphisms and arsenic-induced dermatological and non-dermatological health outcomes. A case-control study was conducted involving 207 cases with arsenic-induced skin lesions

and 190 controls without skin lesions having similar arsenic exposure in West Bengal. The polymorphisms were determined using conventional PCR-sequencing method. ELISA was done to determine the serum levels of the two cytokines tumour necrosis factor α (TNF- α) and interleukin 10 (IL10). Associations between the polymorphisms studied and non-dermatological health effects in the study subjects were determined from our epidemiological survey data. Individuals with GA/AA (-308 TNF- α) and TA/AA (-3575 IL10) genotypes were at higher risk of developing arsenic-induced skin lesions (Table 1), ocular, and respiratory diseases. Also, the -308 TNF A allele corresponded to a higher production of TNF- α , and -3575 IL10 A allele corresponded to a lower production of IL10. Thus, the polymorphisms studied impart significant risk toward development of arsenic-induced dermatological and non-dermatological health effects in the chronically exposed study population of West Bengal (Banerjee *et al.*, 2011).

6. Association of NALP2 Polymorphism with Arsenic Induced Skin Lesions and other Health Effects

Previous studies have indicated that arsenic targets immune system and is associated with characteristic immuno-suppression, which may further adversely affect respiratory function. Hence, a total of 432 arsenic-exposed individuals, of which 219 individuals were with characteristic arsenic-induced skin lesions (cases) and 213 individuals were without arsenic-induced skin lesions (controls), from arsenic-exposed districts of West Bengal, were recruited to find any probable association between arsenicism and the exonic single nucleotide polymorphisms (SNPs) in *NALP2* gene, an important component of inflammasome complex. The entire coding region (exon) was screened in all the study participants. Among 9 SNPs found in *NALP2* gene, the A1052E polymorphism (at least with one minor allele), was significantly overrepresented in controls (Table 1) and hence implies decreased risk toward the development of skin lesions. Since, development of non-dermatological health effects are also important factors to properly look into, attempts have also been made to correlate the genetic variation of

NALP2 with the extent of cytogenetic damage as measured by chromosomal aberration assay and adverse health effects including peripheral neuropathy, eye problem and respiratory diseases in the study population. Results show that individuals with the protective genotype had less chromosomal aberration ($p < 0.05$), and were also less susceptible toward arsenic-related respiratory diseases. These findings suggest that *NALP2* A1052E SNP plays an important role towards development of arsenic-induced skin lesions, chromosomal damage and respiratory diseases (Bhattacharjee *et al.*, 2013).

In this context, it may be mentioned that in a genome-wide association study involving arsenic exposed individuals of neighbouring Bangladeshi population, chromosome 10q24.32 variants were found to be associated with arsenic metabolism and toxicity. The observed patterns of associations suggested that MMA% and DMA% had distinct genetic determinants and supported the hypothesis that DMA is the less toxic of these two methylated arsenic species (Pierce *et al.*, 2012). In another study, Engstrom *et al.* (2013) assessed the influence of genetic variation in AS3MT on DNA methylation and

gene expression within 10q24, in people exposed to arsenic in drinking water. Results showed that in the Argentinean women, the major AS3MT haplotype, associated with more efficient arsenic metabolism, showed increased methylation of AS3MT ($p = 10^{-6}$) and also differential methylation of several other genes. Similar, but weaker, associations between AS3MT haplotype and DNA methylation in 10q24 were observed in cord blood of the Bangladeshi population. Thus, it might be concluded that single nucleotide polymorphisms in different genes have significant contributions in causing arsenic susceptibility in different populations around the world.

7. Epigenetic Modifications of DAPK and p16 Genes Contribute to Arsenic-Induced Skin Lesions and Non-Dermatological Health Effects

In addition to several hypotheses associated with arsenic-induced carcinogenesis, it has been speculated that altered DNA methylation patterns might contribute to arsenic-induced carcinogenesis. Again, promoter hypermethylation of *DAPK* and *p16* genes with resultant gene inactivation and

Table 2: Genetic variants associated with premalignant skin lesions (hyperkeratosis) in the arsenic exposed population

| Genotype | Exposed individuals without skin lesions N(%) | Exposed individuals with hyperkeratosis N(%) | OR (95% CI) | Authors |
|-----------------|---|--|---------------------|-----------------------------------|
| ERCC2 | | | | |
| AC/CC | 131 (85.62) | 90 (54.55) | 1.00 (Referent) | Banerjee <i>et al.</i> , 2007 |
| AA | 22 (14.38) | 75 (45.45) | 4.77 (2.75–8.23) | |
| p53 | | | | |
| Intron 3 | | | | |
| 1/2+2/2 | 55 (30.39) | 31 (17.71) | 1.00 (Referent) | |
| 1/1 | 126 (69.61) | 144 (82.29) | 2.086 (1.257-3.457) | |
| Codon 72 | | | | |
| 1/1 +1/2 | 135 (74.59) | 105 (60.0) | 1.00 (Referent) | De Chaudhuri <i>et al.</i> , 2006 |
| 2/2 | 46(25.41) | 70 (40.0) | 2.086 (1.318-3.299) | |
| Intron 6 | | | | |
| 1/2 +2/2 | 143 (99.31) | 127 (96.21) | 1.00 (Referent) | |
| 1/1 | 1(0.69) | 5(3.79) | 5.312 (0.6-46.96) | |

Note : OR: odds ratio; CI: Confidence interval

development of different types of cancers have been found previously. To elucidate the role of promoter methylation in arsenic-induced dermatological and non-dermatological health effects, methylation status of *p16* and *DAPK* genes was determined. So, we made an attempt to correlate epigenetic modifications of the tumour suppressor genes with dermatological and nondermatological health outcomes in a population chronically exposed to arsenic. A case-control study was conducted involving 72 individuals with arsenic-induced skin lesions (cases) and 50 individuals without skin lesions (controls), having similar arsenic exposure through drinking water. Methylation status was determined by bisulfite conversion of genomic DNA and methylation-specific PCR. Expression of the genes was determined by real-time PCR and Western blot analysis. Associations between the promoter methylation status and non-dermatological health effects were determined from epidemiological survey data. Significant hypermethylation was found in the promoters of both *DAPK* and *p16* genes in the cases compared with the controls resulting in down regulation of both the genes in the cases. There was a 3.4-fold decrease in the expression of death-associated protein kinase and 2.2-fold decrease in gene expression of *p16* in the cases compared to the controls, the lowest expression being in the cancer tissues. Promoter hypermethylation of the genes was also associated with higher risk of developing arsenic-induced skin lesions, peripheral neuropathy, ocular and respiratory diseases (Banerjee N *et al.*, 2013). There have been other previous studies, which support our work. Chanda *et al.* (2006) associated methylation of p53 and p16 gene in with arsenic exposure in another exposed population of West Bengal. They found significant and dose dependent hypermethylation of promoter region of *p53* and *p16* genes in arsenic-exposed people compared to the control subjects. However, a small subgroup of cases showed hypomethylation with high arsenic exposure. In another study, it was also found that arsenic exposure was positively associated with genomic PBMC-DNA methylation in a dose-dependent manner in a neighbouring Bangladeshi population (Pilsner *et al.*, 2007). In a very recent dose-response study of arsenic exposure and global

methylation in PBMC-DNA in Bangladeshi adults (Niedzwiecki *et al.*, 2013), it was found that the concentration of arsenic in drinking water was positively associated with global methylation of PBMC DNA over a wide range of arsenic concentrations. Very recently, in another study from our group it has been found that promoter hypomethylation of ERCC2 has resulted in impaired CAK-Cdk7 activity by increased association of CAK complex with ERCC2. This in turn inhibited the normal functioning of the CAK-complex, thus impairing DNA-repair (Paul *et al.*, 2014) in the exposed individuals. Thus, epigenetic modifications, particularly DNA methylation, contributed significantly to arsenic susceptibility in the exposed population of this part of the subcontinent. Not only that, studies from other regions of the world also established the role of epigenetic modifications in arsenic induced toxicity and carcinogenicity (Liu *et al.*, 2013; Yang *et al.*, 2014; Gribble *et al.*, 2014).

Arsenic Mitigation

1. A Two Wave Cross-Sectional Study for Arsenic Mitigation

Arsenic exposure thus causes several deleterious impacts including cancers and death. At present, providing arsenic-free drinking water is considered to be the best remedial measure to combat arsenic toxicity, as medications for this purpose, are yet to be developed. Owing to governmental efforts, there has been a decrease in the arsenic concentration in drinking water, raising the possibility of remediation. A cross-sectional study was conducted, where 189 arsenic exposed individuals with skin lesions and 171 unexposed individuals were recruited at two time points, 2005-06 and 2010-11, with concomitant decrease in the level of arsenic exposure via drinking water in the exposed skin lesion group in 2010-11. Parameters studied included dermatological, non-dermatological health status and cytogenetic damage. Decrease of arsenic exposure (190.1 mg/l to 37.94 mg/l) resulted in significant decline in the number of individuals having dermatological disorders ($p < 0.01$) and in the severity of each dermatological outcome ($p < 0.0001$). Micronucleus formation in urothelial

cells and lymphocytes decreased significantly ($p < 0.001$). However, there was a significant ($p < 0.001$) rise in the incidence of each of the non-dermatological diseases like, peripheral neuropathy, conjunctivitis and respiratory distress over the period. Thirteen (6.87%) of the initially recruited individuals with skin lesions died of cancer, in this period. Results show that drinking arsenic-safe water could reduce dermatological symptoms and cytogenetic damage but it was unable to counter the non-dermatological symptoms in these exposed individuals. Thus, chronic arsenic exposure has a far-reaching consequence in the chronically exposed population of West Bengal (Paul *et al.*, 2013). Similar observations have been made by other workers also (Oshikawa *et al.*, 2001; Guha Mazumder *et al.*, 2003; Sun *et al.*, 2006).

In another study by our group 145 arsenic exposed individuals and 60 unexposed controls were surveyed in 2004-2005. Of these, 128 exposed individuals and 54 unexposed controls could be followed up in 2010-2011. The extent of DNA damage was measured by micronuclei assay at the two time points. Results showed that, there was a significant decline in the MN frequency, when assayed in 2010-2011 compared to 2004-2005. Hence, it was concluded that urothelial MN can be utilised as a good biomarker in detecting remedial effects from toxicity of the low dose of arsenic through drinking water (Paul *et al.*, 2013a).

2. Rice is a Potential Source of Arsenic Exposure to Humans

More than 3,000,000,000 people across the world consume rice as a staple food. Arsenic contents of such rice vary widely, ranging from 20-900 $\mu\text{g}/\text{kg}$. Recently rice has also been identified as a major exposure route, as evidenced by observations of a strong association between rice consumption and urinary arsenic. Indeed, it is often the most important human exposure route where drinking water arsenic concentrations are less than 50 $\mu\text{g}/\text{l}$ (Mondal *et al.*, 2008; 2010). To our knowledge, there are to date no studies that demonstrate such deleterious health impacts in humans consuming high arsenic rice in the absence of exposure through drinking water. We therefore designed a study to determine if cooked rice

arsenic content on its own is sufficient to give rise to genotoxic effects in humans. We have chosen 417 arsenic exposed individuals from three districts of West Bengal, namely, Murshidabad, Nadia and East Midnapur where arsenic content in drinking water was $< 10 \mu\text{g}/\text{l}$. The entire study population was divided into 6 exposure groups based on the arsenic content in rice and micronuclei formation in their urothelial cells was recorded. Results show that the rice arsenic content of $> 200 \mu\text{g}/\text{kg}$ is associated with significant increased genetic damage as is evident from the increased micronuclei formation in the urothelial cells of the arsenic exposed individuals. So, when arsenic content in rice exceeds this limit, it is sufficient to give rise to significant amounts of genetic damage, even when there is little exposure through drinking water. It is interesting to note that while about 40% (102 of 256) of the samples collected from highly groundwater-arsenic exposed areas showed cooked rice arsenic above 200 $\mu\text{g}/\text{kg}$, only about 2% (3 of 161) of the samples collected from relatively groundwater-arsenic unexposed areas showed the same elevated arsenic concentrations. These observations may be due to the fact that irrigation of rice paddy fields in these regions is done by using high arsenic groundwater. Interestingly, suitable cooking methods (Signes *et al.*, 2008; Raab *et al.*, 2009) and cooking with low arsenic waters can reduce arsenic exposure to some extent. Management strategies to reduce arsenic accumulation in rice have been summarised by Meharg and Zhao (2012) amongst others and include promoting plaque formation and the use of aerobic cultivation processes (Tripathi *et al.*, 2007; Tuli *et al.*, 2010). Thus, there are many effective and potentially effective management strategies for reducing arsenic exposure from rice in regions where exposure from drinking water is insignificant (Banerjee *et al.*, 2013).

In this context, it may be mentioned that there are several other mitigation strategies that have been adopted by other arsenic affected countries of the world to fight arsenic menace. Previously, Hug *et al.* (2008) reported that different groundwater compositions require different approaches to arsenic mitigation in Vietnam and Bangladesh. It has also been found that vermicompost was the most effective

organic amendment in reducing arsenic load of grain followed by municipal sludge (Pati and Mukhopadhyay, 2009). In addition to the use of very deep tube wells, Neumann *et al.* (2013) also reported the use of composite iron matrix filter for effective removal of arsenic from drinking water in Bangladesh, which has severe problems of arsenic toxicity. In another study, Majumder *et al.* (2013) has reported the efficacy of indigenous soil microbes in arsenic mitigation from contaminated alluvial soil of India. In this study, selected arsenic-volatilising indigenous soil bacteria were isolated and their ability to form volatile arsenicals from toxic inorganic arsenic was assessed. Approximately 37% of As III (under aerobic conditions) and 30% As V (under anaerobic conditions) were volatilised by new bacterial isolates in 3 days. In contrast to genetically modified organism, indigenous soil bacteria was capable of removing 16% of arsenic from contaminated soil during 60 days incubation period while applied with a low-cost organic nutrient supplement (farm yard manure). Thus, it might be said that the use of mitigation strategies either alone or in combination might help us to reduce arsenic burden from drinking water and food materials of the affected regions of the world.

Conclusion

In order to conclude we might say that, chronic arsenic exposure results in different dermatological and non-dermatological consequences like non-cancerous, pre-cancerous, cancerous skin lesions, peripheral neuropathy, respiratory problems and conjunctivitis of the eyes. Again, increased apoptosis of the immune cells, decrease in T cell proliferation and impaired macrophage functions in the arsenic exposed individuals are leading to immunosuppression in them. Moreover, arsenic causes genetic damage in the exposed population as is evidenced by chromosomal aberration and micronuclei formation in them and DNA repair deficiency is found to be the cause of arsenic susceptibility. Additionally, our results show that genetic variations in different

individuals are important contenders of arsenic susceptibility and carcinogenicity. So, genetic damage, genetic variants in different genes, deficiency in DNA repair mechanism, immunosuppression and epigenetic modifications contribute to arsenic susceptibility, toxicity and carcinogenicity in the rural population of West Bengal. Results from the cross-sectional study shows that drinking arsenic-safe water could reduce dermatological symptoms and cytogenetic damage but was unable to counter the non-dermatological symptoms in these exposed individuals. Recently, rice has been found to be an important source of arsenic exposure in addition to drinking water, which causes significant DNA damage. Of late, various action plans have been employed in West Bengal to combat arsenic toxicity from drinking water. Steps have been taken to supply arsenic free potable drinking water in all the arsenic affected and surrounding villages under the “Bharat Nirman” programme. The action plan envisages 349 Piped Water Supply Schemes to cover 3413 villages and 16.6 million populations beyond permissible limit. To avoid arsenic contamination from ground water and to augment the available water from major irrigation schemes, establishment of more number of surface water based schemes, such as Lift Irrigation Schemes for irrigation and drinking water on location specific basis has been encouraged in this region (West Bengal State action plan on climate change). This with other mitigation strategies including using less contaminated water for cultivation, use of rice and other crop varieties which accumulate less arsenic and bioremediation should be employed to overcome the arsenic calamity in West Bengal, the worst arsenic affected state of India.

Acknowledgements

The authors are thankful to Council for Scientific and Industrial Research (CSIR), India, for providing research associateship (RA) to N.B. and funding the Emeritus Scientist Project (21(0885)/12/EMR-II) of A.K.G.

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