

Full Length Research Paper

Modulation of arsenic induced genotoxicity by curcumin in human lymphocytes

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Arsenic contamination of ground water is a vital health concern in West Bengal, India, where nine districts were affected. Oxidative stress created by arsenic may lead to genetic instability which may in turn lead to initiation of carcinogenesis. Management of arsenic problem at the preclinical stage by utilizing natural compounds could be a preventive strategy. The present study aims to bio-monitor the level of arsenic exposure in asymptomatic individuals by studying the DNA damage in lymphocytes and to use curcumin, an active ingredient of turmeric, in providing protection against arsenic toxicity. DNA damage was assessed by comet assay. Arsenic induced oxidative stress was observed by generation of reactive oxygen species (ROS). *In vitro* studies with human lymphocytes revealed that curcumin was effective in regression of arsenic induced ROS generation and thereby the DNA damage. The bio-monitoring of Chakdah block in West Bengal revealed that population residing in those areas exhibited severe DNA damage. When the same individuals were given curcumin for three months and monitored monthly, there was a remarkable regression in DNA damage as well as a reduction in ROS generation. Thus, curcumin may have some role in prevention and repair of the DNA damage caused by arsenic contaminated water.

Key words: Arsenic (As), DNA damage, comet assay, ROS, curcumin.

INTRODUCTION

The eastern side of the Bengal delta plain in India has been the worst affected region of groundwater arsenic contamination affecting millions of people residing in West Bengal and adjoining Bangladesh. Tubewells at the depth of 20 - 50 m yield maximum arsenic concentration in groundwater; which may exceed 500 µg/l compared to the maximum permissible limit of 50 µg/l in potable water (Pal, 2008). Chronic exposure to groundwater arsenic (As) contaminated has led to several clinical disorders like hyperpigmentation, keratosis, weakness, anemia, burning sensation of eyes, solid swelling of legs, liver fibrosis, chronic lung disease, gangrene of limbs, neuropathy and cancer at various sites (Guhamazumdar, 2003). DNA damage is the stepping stone for majority of malignancies and arsenic (As) has been proved to induce a number of genetic abnormalities both in *in vitro* and *in vivo* systems (Gebel, 2001). This genetic instability might be frequently associated with excessive generation of

reactive oxygen species (ROS) and malfunctioning of body's antioxidant defence mechanism (Hei, 1998). ROS create oxidative stress by targeting genome, proteins, structural carbohydrates and lipids proteins, lipids and DNA, which are the primary electron rich sites within the cells (Romero, 1998).

Curcumin (diferuloylmethane), a biologically active ingredient of *Curcuma longa*, which has potent anticancer properties as demonstrated in a plethora of human cancer cell lines/animal carcinogenesis model and also acts as a biological response modifier in various disorders (Iqbal, 2009). Curcumin showed modulatory effects on the levels of benzo[a]pyrene induced DNA adducts in the livers of rats by the [³²P]-post labeling assay method (Mukundan, 1993). Pretreatment with curcumin gave protection to lymphocytes against gamma-radiation induced cellular damage (Sriivasan, 2006). Antioxidant curcumin provide protection against chromosome damage produced by radiation (Thresiamma, 1998).

Human clinical trails indicated that curcumin showed no toxicity when administered at doses of 10 g/day

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(Aggarwal, 2003). Curcumin peaks in the serum within 1 - 2 h of oral intake and gradually declines within 12 h (Sharma, 2005). Piperine is an alkaloid found in *Piper nigrum*, the source of black pepper and white pepper. When used with curcumin as an adjuvant, it interferes with glucuronidation and increases its bioavailability (Anand, 2007). Piperine may enhance the bioavailability of curcumin by 2000% in humans (Shoba, 1998).

Previous reports from our laboratory had shown that curcumin could prevent DNA damage, ROS generation and lipid peroxidation caused by As III during *in vitro* experiments with human lymphocytes. Over and above, it also induced antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase (Mukherjee, 2007). In another report, we had shown that among various dietary phytochemicals used, curcumin gave the best protection against genotoxicity and ROS generation during simultaneous treatment with As III in mammalian V 79 cells (Roy, 2008).

In the present study, the endeavor was to investigate whether the *in vitro* and *in vivo* results obtained with curcumin could be translated for public health service against As toxicity. The work firstly tried to establish a bio-monitoring method for determining the level of chronic exposure of arsenic in asymptomatic individuals by studying the DNA damage in blood lymphocytes and secondly to use curcumin, an active ingredient of Indian spice turmeric, in providing protection against the toxic effects of arsenic. Comet assay was used to bio-monitor the level of DNA damage both during *in vitro* experiments and field studies. The comet assay, also called single cell gel electrophoresis (SCGE) assay allows detection and quantification of a broad spectrum of DNA damage induced by various agents in individual cells. Using the alkaline version of this test, single and double strand breaks, alkali labile sites and incomplete excision repair sites can be detected (Mckelvy-Martin, 1997). In comparison to other methods available for scoring DNA damage and repair like chromosomal aberration assay, micronucleus test, sister chromatid exchange and Ames test, comet assay is a very rapid, sensitive and cost effective technique. SCGE is applicable to virtually any cell line or tissue from which a healthy single cell suspension can be obtained (Tice, 2000).

MATERIALS AND METHODS

Chemicals

Curcumin [CAS No. 458-37-7], ethidium bromide [CAS No. 1239-45-8], Triton-X 100 [CAS No.9002-93-1], 2,7 dichlorofluorescein diacetate (DCFH-DA) [CAS No. 2044-85-1], histopaque 1077 were obtained from Sigma-Aldrich St Louis, MO, USA. RPMI-1640, fetal bovine serum (FBS), phytohaemagglutinin (PHA), gentamycin, penicillin, streptomycin and agarose (normal as well as low melting point) were obtained from Invitrogen. Curcumin capsules (Trade name, Cur Plus) were procured from Indsaff, India. Sodium arsenite (As III) was procured from Spectrochem India Pvt. Ltd..

Analysis of As (Arsenic) in water samples

As induced genotoxicity and oxidative stress was initially examined

in human lymphocytes from normal individuals. This study was further extended to a field trial in Chakdah block of Nadia district in West Bengal. So far, a population of 153 people in three villages (Chowgaccha, Manpur and Mathpara) of this block have been surveyed which recorded very high concentrations of As in village wells and tubewells ranging between 95 -175 µg/l. Samples of ground water (which was the only source of drinking water) were collected from the endemic villages taken under the field survey and were analyzed for their As level by Atomic Absorption Spectroscopy at the School of Environmental Studies, Jadavpur University, Kolkata.

Selection of volunteers

Before commencement of project, human ethical committee clearance was obtained for carrying out the project work. The work adhered to the principles expressed in the Declaration of Helsinki. A written informed consent form in local language was obtained from each of the volunteers prior to their recruitment in the field study. The volunteers selected for the field trial were non smoker males or females aged 25 - 55 years, with no chronic disease, no external manifestation of arsenic toxicity or abnormal blood report. For determining the basal level of DNA damage and ROS generation we had recruited 50 volunteers from our institute residing in Kolkata (control population) who were drinking arsenic free water. The water of these areas were tested free of arsenic (data not shown). Control blood samples were tested at the end of each month continuously for three months before curcumin intervention as well as in the subsequent three months after curcumin intake. Curcumin capsules were given for oral intake and nothing was added to the blood samples *in vitro*. The average values obtained from comet assay and ROS analysis of the institute volunteers had been taken as the control values for comparison with values of the affected individuals. In all the villages, 50% of the populations were given placebo (powder of pulses).

Sample collection and separation of lymphocytes

Freshly collected blood from volunteers was heparinised and carefully layered on top of histopaque and centrifuged at 1000 rpm for 20 min. The buffy coat interface, which represented the lymphocytes, was aspirated and again centrifuged at 1500 rpm for 15 min. The supernatant was discarded; pellets were disrupted and washed with normal saline. Finally, the lymphocytes were seeded in RPMI-1640 supplemented with 10% FBS and 20 µg/ml PHA. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂/95% air.

Treatment of human lymphocytes *in vitro*

For dosimetry analysis during the *in vitro* experiments, exponentially growing human lymphocytes were treated with different concentrations of As III (100, 250, 500 and 1000 µM). During simultaneous treatment, cells were treated with various doses of As III along with different doses of curcumin (10, 25 and 50 µM) for 1 h to assess the extent of DNA damage.

Exposure of arsenic for field volunteers

The volunteers recruited from the arsenic affected villages were neither treated with arsenic nor were their blood samples treated with As. They were recruited on the basis that they were continuously drinking As-contaminated water from the ground water sources throughout the experimentation period.

Table 1. Curcumin level in blood plasma as detected by HPLC analysis.

Village code (for use in this study)	Village	Number of individuals (n)	Curcumin level in blood plasma ($\mu\text{g/ml}$) at months after curcumin administration		
			1	2	3
a	Chowgaccha	26	2.45 ± 0.16	2.67 ± 0.32	2.67 ± 0.34
b	Manpur	26	2.65 ± 0.04	2.56 ± 0.22	2.61 ± 0.43
c	Mathpara	25	2.49 ± 0.23	2.59 ± 0.12	2.76 ± 0.52

Dosage of curcumin in the field trial

Dietary constituents derived from several plants including turmeric appear to have antigenotoxic and antioxidant properties which make them excellent chemo-preventive agents. A recent investigation suggested that combination of piperine (2.5 mg/Kg), a bio-availability enhancer with curcumin (20 mg/Kg) was effective against depressive disorders (Bhutani, 2009). In the present field study, a significantly high concentration of piperin was used with curcumin to increase its bio-availability. Curcumin with piperine (20:1) at a dose of 2 x 500 mg/day was administered to the As-exposed subjects for three consecutive months and were investigated at the end of each month to find out whether curcumin had any antigenotoxic and antioxidant effect against As. This was the first field study which had the goal to ascertain the possibility of using curcumin as a chemo-preventive intervention against the adverse effects of chronic arsenic exposure through contaminated drinking water.

Analysis of curcumin in blood samples

The sample preparation and analysis of curcumin in blood plasma were done with slight modifications of the method developed by Heath et al. (2003). Improper peak shapes were more often due to the secondary interaction (silanol activity). To optimize the chromatographic condition, during PDA detection, 0.1% trifluoroacetic acid (TFA) was added to the mobile phase to improve the peak shapes in the chromatogram.

Analytical procedure and sample preparation

Standard unknown sample (200 μl) was mixed with 80 μl of deionized water and spiked with curcumin (200 $\mu\text{g/ml}$). Extraction reagent that is 95% ethyl acetate / 5% methanol (500 μl) were added to each tube. All the tubes were then centrifuged at 13,500 rpm for 5 min. After centrifugation, the upper organic layers (500 μl) were carefully removed in clean microcentrifuge tubes. This organic layer was dried in a speed vacuum dryer (Speed Vac, SC 110, Savant). The extracted dried product was resuspended in 200 μl of prepared mobile phase reagent. The tubes were left at room temperature in the dark for at least 10 min. This was followed by

repeated vortex mixing and the contents were transferred to an injection sample vial (180 μl) for HPLC assay. Proper mixing was done at all stages using vortex mixer.

HPLC analytical run

Curcumin in plasma was quantified by isocratic HPLC method using ultraviolet detection at 425 nm. An aliquot (20 μl) was injected onto a reversed-phase column and eluted with a mobile phase containing a mixture of acetonitrile-methanol-water-acetic acid (41:23:36:1, v/v/v/v) along with TFA (0.1%). Flow rate was 1.0 ml /min. The quantitation of curcumin was based on a standard curve in plasma generated by using an external standard to spike plasma.

During analysis of curcumin by HPLC, a basal level was obtained which was the average of the amount of curcumin obtained in the blood plasma (0.049 $\mu\text{g/ml}$) before administration of curcumin without spiking. But the peak obtained for the basal level almost merged with the baseline noise. Therefore, for proper identification of the peak, the samples were spiked with a known concentration of curcumin. The analysis of the blood samples exhibited that the amount of curcumin obtained in the blood plasma increased after the 1st month and maintained a consistent level during the subsequent months of curcumin administration (Table 1).

Single cell gel electrophoresis (SCGE) or comet assay

As-induced DNA single strand breaks were assessed by comet assay or single cell gel electrophoresis following the method of N. P. Singh (Singh, 1988) with minor modifications. Briefly, cells (1×10^4) were suspended in 0.6% (w/v) low melting agarose and layered over a frosted microscopic slide previously coated with a layer of 0.75% normal melting agarose to ensure firm gripping. The slides were then kept at 4°C for solidification. Subsequently, slides were immersed in a lysis buffer [NaCl (2.5 M), Na₂EDTA (0.1 M), Tris (10 mM), NaOH (0.3 M), Triton X-100 (1%) and DMSO (10%) in a solution of pH 10] and left overnight for lysis of cell membrane and nuclear membrane. The slides were kept overnight for lysis at 4°C. The following day, slides were transferred into a horizontal electrophoresis chamber containing electrophoresis buffer (alkaline solution of 300 mM NaOH, 1 mM Na₂EDTA; pH 13.0) and presoaked for 20 min in order to unwind DNA. Electrophoresis was

Table 2. Dose dependent increase in comet tail moment with increase in dose of As III.

Dose of As III (μM)	Comet Tail moment (μm)
100	2.50 ± 0.29 *
250	3.72 ± 0.39 *
500	6.78 ± 0.26 *
1000	8.26 ± 0.23 *

Note: Significant induction of DNA damage by As III *($p < 0.001$) with respect to controls.

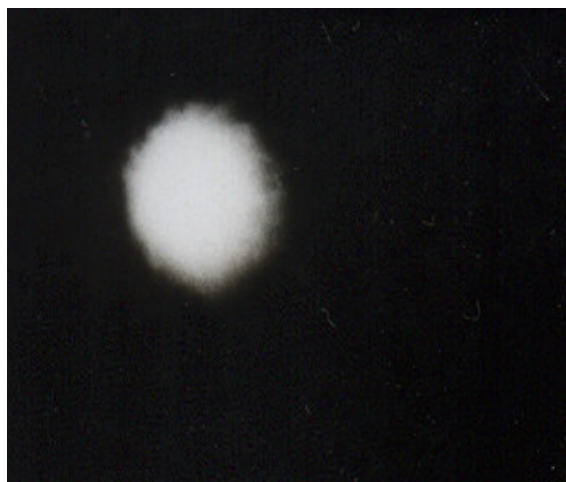


Figure 1a. Photograph representing regression of As-induced DNA damage by curcumin in normal human lymphocytes; Control.



Figure 1b. Photograph representing regression of As-induced DNA damage by curcumin in normal human lymphocytes; 1000 μM As.

then carried out for 20 min (300 mA, 20 V). Slides were then washed thrice with neutralizing buffer (Tris 0.4 M, pH 7.5), stained

with ethidium bromide (final concentration 40 $\mu\text{g/ml}$) and examined under a fluorescence microscope (Nikon). The cells were subjected to image analysis using Comet Assay Software Program (CASP). DNA damage was quantified by tail moment measurement, calculated by multiplying the total intensity of the comet tail and the tail length, measured from the center of the comet head. Photographs of single cells were taken at 400X magnification using 400 ASA 35 mm Kodak Gold film.

Determination of intracellular ROS production

The oxidative stress created by arsenic was examined by measurement of ROS generation. It was carried out according to Balasubramanyam et al. (2003). The non-fluorescent dye, DCFH-DA passively diffuses into the cells where the acetates are cleaved by intracellular esterases. The resulting diol is retained by the cell membrane. With the generation of ROS, this diol is oxidized to the fluorescent form 2', 7'-dichlorofluorescein (DCF) which is qualitatively detected by FACS analysis and quantitated by fluorimeter. As III (1000 μM)-treated cells were incubated with different concentration of curcumin (10, 25 and 50 μM) for 1 h and suspended in HBS buffer loaded with the dye, DCFH-DA 10 μM prior to each experiment. The lymphocytes from blood samples obtained from the field samples were separated for lymphocytes and directly suspended in HBS buffer with 10 μM DCFH-DA for assessment of ROS generation. Cells were incubated with dye in the dark for 45 min. ROS levels were measured using spectrofluorimeter (Waters, USA 474 Scanning Fluorescence Detector, with an excitation set at 485 nm and emission at 530 nm).

DCFH-DA (10 μM) was also used for FACS analysis in order to detect ROS generation. Cells were stained with the dye, suspended in PBS, kept in the dark for 45 min and analyzed in a flow cytometer (Becton Dickinson FACS calibre) equipped with a 488 nm argon laser and a 525 ± 10 nm band pass emission filter. Fluorescence was captured on a FL1H channel with logarithmic amplification. For each determination 10,000 cells were counted.

Statistical analysis

Statistical analysis was performed with SPSS 10.0 (one way ANOVA followed by Dunnett t-test, where significance level was set at 0.001). Dunnett t-test treats one group as control and all other groups against it.

RESULTS

In the present study, it was found that As III induced DNA damage in a dose dependent manner in normal human



Figure 1c. Photograph representing regression of As-induced DNA damage by curcumin in normal human lymphocytes after simultaneous treatment with 10 μ M curcumin.

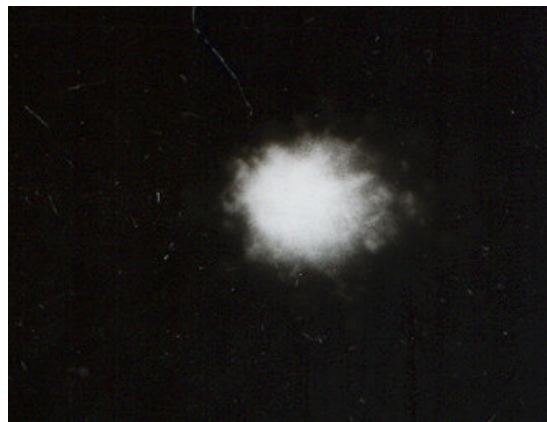


Figure 1e. Photograph representing regression of As-induced DNA damage by curcumin in normal human lymphocytes after simultaneous treatment with 50 μ M curcumin.



Figure 1d. Photograph representing regression of As-induced DNA damage by curcumin in normal human lymphocytes after simultaneous treatment with 25 μ M curcumin.

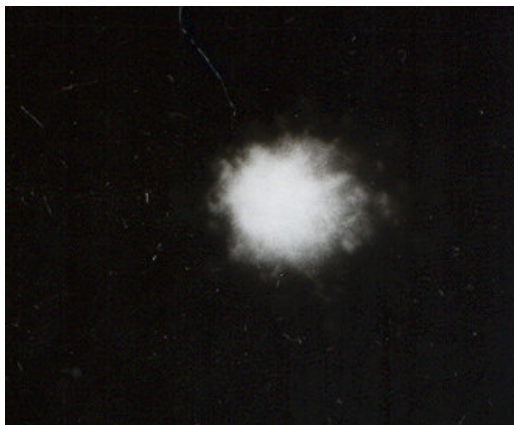


Figure 1e. Photograph representing regression of As-induced DNA damage by curcumin in normal human

lymphocytes after simultaneous treatment with 50 μ M curcumin.

lymphocytes as it is evident from the increase in comet tail moment (Table 2).

When the same lymphocytes were incubated simultaneously with different doses of curcumin (10, 25 and 50 μ M), there was a significant reduction in comet tail moment as exhibited in the Figure 1a,b,c,d,e. The oxidative stress created by As was evident from the increase in ROS generation as shown in Figure 2a and 2b. Curcumin proved effective in quenching the oxidative stress which was exhibited in fluorometric analyses (Figure 2a and 2b). The FACS analysis showed that the shift from the original light black peak to the dark black peak in case of *in vitro* experiments with human lymphocytes was due to oxidative burst caused by As III. This shift was brought back near the original peak after treatment with different doses of curcumin (Figure 2b).

The *in vitro* results gave us incentive to extrapolate our work to the highly endemic regions where we could test whether curcumin interventions could be used for prevention of such environmental calamities. Chakdah Block of Nadia District, about 160 Km from Kolkata was identified with thirty (30) villages, all having high levels of Arsenic in ground water ranging between 100 - 250 μ g / liter. Nine adjacent villages were selected as per demographic survey and water analysis. So far the work has been initiated in 3 villages namely, Chowgaccha, Manpur, and Mathpara whose demographic details have been given in Table 3.

The data clearly showed that the control values of comet did not show any significant difference either in presence or absence of curcumin (Figure 3). An average value of comet tail moment, obtained during first 3 months (in absence of curcumin) was 1.35 μ m and this value did not undergo much alteration during subsequent 3 months (in presence of curcumin intake).

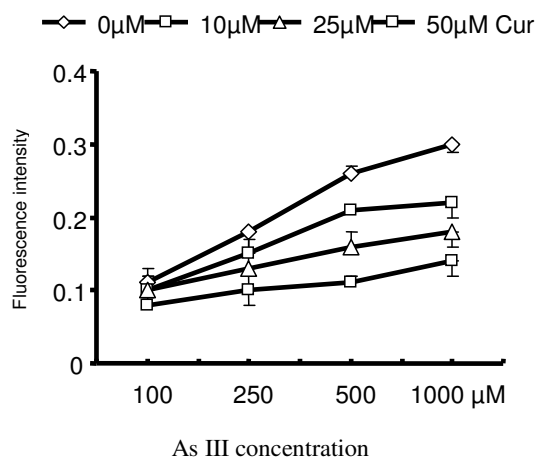


Figure 2a. As-induced ROS generation in a dose dependent manner in human lymphocytes which was effectively quenched with different doses of curcumin (Cur). Significant quenching was obtained with all doses of curcumin at As of 1000 μM ($p < 0.001 - 0.005$).

The chronic arsenic exposed villages surveyed, exhibited extensive DNA damage during the first three months in absence of curcumin. This was evident from the high comet tail moment values as shown in (Figure 4a,b,c). The mean difference of DNA damage during the first three months without curcumin was not significant ($p > 0.05$). When the same individuals were given curcumin with piperine (20:1) at a dose of 2 x 500 mg/day for three months and regularly monitored at the end of each month it was found that there was a remarkable decrease in DNA damage which has been represented in Figure 4. It was clear from the data that there was a significant reduction ($p < 0.001$) of As-induced comet tail moment in presence of curcumin than in absence of curcumin. The comparative profile of DNA damage with and without curcumin is shown in (Figure. 4a,b,c). The chronic exposure of As created an oxidative stress in the individuals residing in the endemic areas which was evident from the increase in ROS generation in comparison to the control population. Curcumin practically had no effect on the control population ($p > 0.05$). Placebo had no effect on treatment population ($p > 0.05$). In contrast, the treatment population receiving the curcumin administration for three months revealed a sharp quenching of ROS generation ($p < 0.001$) (Figure 5a,b,c).

DISCUSSION

The metalloid arsenic (As) had been a geo-environmental disaster for West Bengal in India. Various researchers have reported that frequencies of chromosomal aberrations, micronuclei and sister chromatid exchanges

increase in human population chronically exposed to arsenic (Basu, 2001). Reports suggest that As-induced in peripheral blood of humans increased the level of ROS and decreased the antioxidant capacity of plasma (Wu, 2001). The excess production of ROS beyond the cellular endogenous antioxidant balance might have been one of the prime factors of As-induced genotoxicity and thereby the related carcinogenicity. Lynn et al. (2000) reported that genotoxic effects may be the common etiology in As-induced carcinogenesis. Arsenic is a well known human carcinogen and DNA damage is prerequisite in As-induced carcinogenesis (Kessel, 2002).

In the present study, both the *in vitro* results and the field trials exhibited that As III induced DNA damage and generated ROS which might have been one of the plausible causes of the DNA damage. After a detailed demographic survey, a community based work was initiated in three villages namely Chowgaccha, Manpur and Mathpara. The non smokers were chosen to establish the fact that the DNA damage monitored was only due to As III and not due to any other DNA damaging agent like nicotine. Clinical manifestations of As intoxication were avoided because we wanted to investigate any abnormal indication in blood even before the manifestation of the actual symptoms which could be prevented.

Curcumin, a hydrophobic polyphenol has a wide spectrum of biological and pharmacological activities. It is a bis- α , β -unsaturated, β -diketone (diferuloylmethane), which exhibits keto-enol tautomerism having a predominant keto form in acidic and neutral solutions and stable enol form in alkaline medium (Anand, 2007). Due to polyphenolic structure and β -diketone functional group, curcumin is able to scavenge or neutralize free radicals by interacting with oxidative cascade, quenches oxygen and by chelating some metal ions inhibits peroxidation of membrane lipids thereby maintaining membrane integrity and their function (Pulla, 1994). Curcumin by virtue of its antioxidant properties blocked the oxidative stress created by methylglyoxal in human mononuclear cells (Chan, 2006). Curcumin protects islets against streptozotocin-induced oxidative stress by scavenging free radicals (Meghana, 2007). The HPLC analysis of the blood samples exhibited that the bio-availability of curcumin increased after 1st month of curcumin administration (4th month in experimentation period) and afterwards maintained a consistent level in the blood during the subsequent months (Table 3). Thus the attenuation of oxidative stress related DNA damage could be partially explained with rise in curcumin level in blood samples.

The bio-monitoring of the normal lymphocytes treated with As and the blood samples obtained from the affected areas in Chakdah block of West Bengal revealed severe DNA damage in the human lymphocytes. Curcumin intervention was found very effective as a preventive approach in reducing the genotoxicity as well as the oxidative stress caused by arsenic.

Conclusion

In the present investigation the reduction of ROS induced DNA damage by curcumin obtained during *in vitro* results was translated to the field survey where a large number of people were at the risk of drinking contaminated groundwater. Apart from quenching of ROS generation by curcumin, evaluation of oxidative stress with more biomarkers like lipid peroxidation, protein carbonyl, oxidative DNA adducts and analyses of antioxidants are ongoing as a part of the project work. Thus curcumin may have a practical implication in reducing the misery of affected people by preventing and repairing DNA damage caused by intake of arsenic contaminated water.

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