Protective effect of dietary phytochemicals against arsenite induced genotoxicity in mammalian V79 cells

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Chronic arsenic exposure causes skin diseases, gastrointestinal and neurological disorders, diabetes and cancer in various organs. Oxidative stress associated with arsenic exposure cause genetic instabilities and may initiate carcinogenesis. Phytochemicals present in vegetables, fruits, spices, tea, and medicinal plants, have shown to suppress experimental carcinogenesis in various organs. The aim of the present study was to elucidate the protective effect of some of the phytochemicals against the arsenite induced DNA damage in normal mammalian V79 cells. Comet assay was used for assessment of DNA damage and 2', 7'-dichlorofluorescein dihydroacetate for estimation of ROS generated by arsenite. The effect of the phytochemicals was observed during simultaneous treatment with arsenic, before arsenite exposure and during repair experiments. Of all the phytochemicals tested against arsenic, curcumin gave better protection during simultaneous treatment and resveratrol during pre treatment, which was evident both from comet assay and ROS generation experiments. During pre treatment a longer duration of treatment with lower dose of phytochemicals proved fruitful in reducing the genotoxicity. During repair experiments the phytochemicals enhanced recovery of DNA damage and ellagic acid gave promising results. The results indicated that natural phytochemicals may have the efficacy in reducing arsenic induced genotoxicity, in scavenging ROS and in enhancing the process of DNA repair in V79 cells.

Keywords: Arsenite, Dietary phytochemicals, Genotoxicity, V79 cells

Arsenic (As) contamination in drinking water has been a constant source of natural calamity. In the Gangetic delta region of West Bengal, India As contamination in drinking water is through tube wells where concentration of As ranges far above the WHO recommended limit of 10 µg/l. Individuals chronically exposed to arsenic are at an increased risk for various cancers, skin diseases, ischemic heart diseases, neurological problems and teratogenicity. Arsenite (As III) mainly found in deep anoxic wells have been most likely considered as environmental carcinogen. As III, has been reported to induce genetic alterations like chromosomal aberrations, micronuclei formation, gene amplification and known to block DNA repair. Lynn et al have reported that genotoxic effects may be the common etiology in As-induced carcinogenesis. Environmental levels of As III may produce free radicals mainly in the form of hydroxyl and superoxide radicals. Among the various modes of action for As related carcinogenesis, the oxidative stress mediated through reactive oxygen species (ROS) can directly or indirectly damage DNA, lipids and proteins. Genetic anomaly without effective DNA repair, leads to mutation and ultimately initiates carcinogenesis. Carcinogenicity of As III can be explained at least in part by it being a mutagen that depended on ROS for its activity. Some specific nutraceuticals contained in daily diet have been chosen for the present study. These include capsaicin (terpene found in green chillies and capsicum), curcumin (phenol found in rhizome of turmeric), ellagic acid (organic acid found in guava), fisetin (flavonoid found in strawberries), gallic acid (organic acid found in ginger), limolene (terpene found in citrus fruits like lemon and orange peel), lycopene (terpene found in tomato), quercetin (flavonoid found in citrus fruits, broccoli, onions, red grapes), resveratrol (polyphenol found in grapes) and rutin (flavonoid found in apricots, cherries and citrus fruits). All these compounds are reported to be excellent antioxidants and may have some chemopreventive role against arsenic toxicity. The genotoxic effect of arsenite and subsequent modulatory effect of the phytochemicals in
counteracting As toxicity has been reported in a normal mammalian cell line of Chinese hamster male lung fibroblasts V79. In present study potency of these natural compounds against the As III-induced DNA damage in Chinese hamster V79 cells has been reported.

Materials and Methods

**Chemicals** — Fetal bovine serum (FBS), gentamycin, minimal essential medium (MEM), penicillin, streptomycin, tris buffer and trypsin were purchased from GIBCO-BRL India Pvt Ltd (New Delhi, India). Curcumin [CAS No. 458-37-7], resveratrol [CAS No. 501-36-0], capsicain [CAS No. 404-86-4], fisetin [CAS No. 345909-34-4], ellagic acid [CAS No. 476-66-4], quercetin [CAS No. 6151-404-8], rutin [CAS No. 207671-50-9], limonene [CAS No. 502-65-8], gallic acid [CAS No. 149-91-7], Triton-X 100 [CAS No. 5989-27-5], lycopene [CAS No. 5002-93-1], ethidium bromide [CAS No. 1239-45-8], gentamycin, minimal essential medium (MEM), glucose, NaCl, KCl, CaCl2, MgCl2, NaOH, I M Na2EDTA; pH 7.4), stained with ethidium bromide (final concentration 40 µg/ml) and examined under a Nikon fluorescence microscope. 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.

**Culture of cells** — Routine culture of V79 cells was maintained in MEM supplemented with heat inactivated FBS (10%) and antibiotics (penicillin 100 units/ml, gentamycin 40 µg/ml) and streptomycin 10 µg/ml). For sub-culture, the medium was removed and attached cells were harvested with pre-warmed (37°C) trypsin solution (0.125%). Subsequently harvested cells were seeded in tissue culture plates with fresh media. Cells were grown at 37°C in a humidified atmosphere of 5% CO2/95% air.

**Single cell gel electrophoresis (SCGE) or comet assay** — As III-induced DNA single strand breaks were assessed by comet assay or single cell gel electrophoresis following the method of Singh et al14 with minor modifications. Briefly, cells (1x106) 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. Next 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 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 film.

Determination of intracellular ROS production — Measurement of intracellular ROS production gives an estimate of the oxidative stress created by any toxic agent inside the cell. It was carried out according to Balasubramanyam et al15. The dye, 2', 7'-dichlorofluorescein dihydroacetate (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 can be quantified by measuring its fluorescence. As III (500 µM)-treated cells were incubated with different phytochemicals (75 µM) for 1 h and resuspended in hepes buffered saline (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose, pH 7.4) loaded with the dye prior to each experiment. Cells were incubated with 10 µM DCFH-DA for 45 min. ROS levels were measured using spectrofluorimeter (Scanning Fluorescence Detector Waters, USA 474) with an excitation set at 485 nm and emission at 530 nm as a change in fluorescence because of the conversion of non-fluorescent DCFH-DA to the highly fluorescent compound DCF in the cells.

**Treatment of cells** — For comet assay CH V-79 cells were seeded in 35 mm tissue culture petri dishes at a cell density of 5 x 10⁵ cells per plate. Exponentially growing cells were subjected to different modes of treatment. For dosimetry analysis the V-79 cells were exposed with different concentrations of As III (50 µM, 100 µM, 250 µM and 500µM). The cells were treated at different modes:
(a) During simultaneous treatment cells were treated with the highest toxic dose of As III (500 µM) along with different doses of phytochemicals (10 µM, 25 µM, 50 µM and 75 µM) for 1 hr to assess the extent of reduction of DNA damage. (b) During the pre-treatment mode cells were treated with very low doses (5, 10, and 15 µM) of phytochemicals for 24 hr. Subsequently cells were washed with fresh medium and treated with different doses of arsenicals for 1 hr to examine the effect of pre-incubation of phytochemicals on As III-induced DNA damage. (c) During repair experiments the cells were initially exposed to As III (500 µM) treatment and subsequently subjected to undergo repair process either in presence or absence of phytochemicals (highest dose of 75 µM each). The recovery of DNA was monitored at 2 hr and 4 hr intervals.

The time for As exposure was carried out for 1 hr, 2 hr, 4 hr and 6 hr. Subsequently it was restricted to 1 hr irrespective of any type of treatment, as appreciable amount of DNA damage was observed during this period. Authors have also reported As III induced DNA damage with 1 hr treatment in V79 cells and human lymphocytes.

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 a control and treats all other groups against it.

Results and Discussion

The results indicated that As III was genotoxic to mammalian cells and induced DNA damage in CH V79 cells in a dose dependent manner (Fig. 1). The doses used (50 µM, 100 µM, 250 µM and 500 µM) showed significant induction of comet tail moment (P<0.001) with respect to control (not shown in Fig. 1) where there was no DNA damage. Chronic exposure to As can result in liver injury, peripheral neuropathy and an increased incidence of cancer of the lung, skin, bladder and liver. As III, a predominant form of As found in ground water, has been considered to be a potent environmental carcinogen. As III-mediated generation of DNA protein crosslinks and DNA strand lesions were observed in CH V79 cells. Formamidopyrimidine-DNA glycosylase enhanced As III-induced DNA strand breaks in phytohaemagglutinin (PHA)-stimulated and unstimulated lymphocytes.

Genotoxicity tests form an important part of risk assessment of potential carcinogens. Comet assay can be made applicable to virtually any cell line or tissue from which a healthy single cell suspension can be obtained. Using the alkaline version of the test, single and double strand breaks, alkali labile sites and incomplete excision repair sites can be detected.

In the present study comet assay was used to detect the genotoxicity of As III and the efficacy of phytochemicals in reducing the As III-induced DNA damage. Screening of the antigenotoxic effect of the natural plant products used (rutin, resveratrol, capsaicin, curcumin, ellagic acid, fisetin, quercetin, gallic acid, limonene and lycopene) was done with the highest possible doses used during simultaneous treatment (75 µM) and pre-treatment (15 µM) with As III (500 µM). The percentage of protection was calculated according to the formula 100 – [100 x/y] where x represented reduction in damage aided by the compounds and y, the As-induced damage (both x and y were measured in terms of comet tail moment). Of all the compounds used quercetin gave maximum protection of 77% during simultaneous treatment and 85.29% during pre-treatment (Table 1). During simultaneous treatment apart from gallic acid, lycopene and limonene all other compounds proved fruitful in reducing the comet tail moment induced by As III (500 µM). A comparative graphical
representation in decreasing the As III-induced DNA damage with different doses of various phytochemicals has been shown in Fig. 2. The decrease in DNA damage was significant ($P<0.001$) in a dose dependent manner with respect to cells where only As III (500 µM) was used and no phytochemical was administered. Fig. 3 represents the antigenotoxic effect of curcumin against As III (500 µM)-induced comet tail moment. The pre treatment results simulated similar effect of these natural compounds against the DNA damage caused by As III (500 µM) in V79 cells. But here it was interesting to observe that a longer period of incubation (24 hr) with very low doses (5, 10, 15 µM) of these compounds before As III (500 µM) exposure was effective in providing protection (Fig. 4). Distinct inter-individual variation of the phytochemicals in counteracting the As III-induced genetic imbalance has been represented with the IC$_{50}$ values (concentration of a compound needed to inhibit 50% of the DNA damage caused by As III) in Table 2. Though quercetin afforded maximum protection during screening of the phytochemicals, the IC$_{50}$ values indicated that curcumin was most effective during simultaneous treatment (35 µM) and resveratrol (7.5 µM) during pre-treatment (Fig. 5). Phytochemicals obtained from vegetables, fruits, spices, tea, herbs and medicinal plants, such as carotenoids, phenolic compounds and terpenoids, were shown to suppress experimental carcinogenesis in various organs$^{10}$. Previous findings of our group have also suggested that polyphenols present in tea and curcumin are very promising dietary factors which have shown antigenotoxic effect against As III in human lymphocytes$^{17,23}$.

The experimental findings elicited that As III (500 µM) caused an oxidative burst and increased the ROS generation in V 79 cells. Of all the compounds used curcumin proved most fruitful in reducing the generation of ROS during simultaneous treatment and resveratrol during pre-treatment (Table 3). Oxidative...
stress is caused due to an imbalance between the production of ROS and the biological system’s antioxidant mechanism that restricts the levels of reactive intermediates. ROS are free radicals that possess an unpaired electron in the outer valence shell of the molecule which makes them highly reactive in nature\textsuperscript{24,25}. They have low chemical specificity and can react with most molecules in their vicinity like proteins, lipids, carbohydrates and DNA. They try to gain stability by capturing the needed electron and don’t survive in their original state very long. When the attacked molecule looses its electron, it becomes a free radical itself, leading to a cascade of chain reaction, which finally leads to the disruption of a living cell. This leads to oxidative modifications of the genome, proteins, structural carbohydrates and lipids\textsuperscript{26}. The genetic instability and cytogenetic alterations are perhaps more likely to be associated with generation of ROS caused by As. It was also reported that As-insult in peripheral blood of humans increased the level of ROS and decreased the antioxidant capacity of plasma\textsuperscript{27}. 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\textsuperscript{28}. Phytochemicals are reported to exhibit a wide variety of biological activities including antioxidant and free radical scavenging activities\textsuperscript{29}. Flavonoids like quercetin and rutin are capable of scavenging ROS, chelating ferrous ions that play a vital role in the initiation of free radical reactions and suppressing the generation of hydroxyl radicals in the Fenton reaction\textsuperscript{30}. Tea and curcumin have also exhibited ROS quenching property in cultured human lymphocytes\textsuperscript{17,23}.

It was observed that subsequent to the treatment with As III (500 µM), the recovery of damage was enhanced in presence of phytochemicals (75 µM) than in their absence. The comparative recovery of DNA damage at different time intervals of the repair process has been represented in Fig. 4. Ellagic acid afforded the best DNA repair with a recovery of 66% during 2 hr and 80% during 4 hr intervals after DNA damage. As III not only causes DNA damage but also retards repair processes involved in break excision

Table 2 — IC\textsubscript{50} values (µM) of phytochemicals during simultaneous treatment and pre-treatment with As III (500 µM)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Simultaneous treatment</th>
<th>Pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>45.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>51.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Curcumin</td>
<td>36.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>57.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Quercetin</td>
<td>50.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Ellagaic Acid</td>
<td>50.0</td>
<td>9.5</td>
</tr>
<tr>
<td>Fisetin</td>
<td>48.0</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Fig. 3 — Single cell gel electrophoresis of V-79 cells showing As-induced comet tail (400×) and its reduction by curcumin. [(a) – Control; (b) – Treated with As III (500 µM); (c) – Treated with As III (500 µM) + curcumin (25 µM); and (d) – Treated with As III (500 µM) + curcumin (75 µM)]
repair and nucleotide excision repair. Previous findings from our laboratory showed that some of the natural compounds like resveratrol, curcumin, ellagic acid and indole-3-carbinol had great potency in recovery of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced DNA damage in cultured Chinese hamster lung fibroblast cells (CH V-79) [1].

Fig. 4 — Reduction in As III (500 µM) induced comet tail moment by pre-treatment with different doses of 7 phytochemicals [Rutin (RU), Resveratrol (RE), Capsaisin (CA), Curcumin (CU), Quercetin (QU), Fisetin (FI), Ellagic Acid (EA)]. Reduction of genotoxicity is significant (*P<0.001) with respect to As III-treated cells i.e. cells where phytochemical concentration is 0 µM.

Fig. 5 — Enhancement of repair activity as evident from SCGE in As III (500 µM) damaged V79 cells when post-incubated with 75 µM of different phytochemicals [Rutin (RU), Resveratrol (RE), Capsaisin (CA), Curcumin (CU), Quercetin (QU), Fisetin (FI), Ellagic Acid (EA)]. Repair with phytochemicals is significantly different (*P<0.001) with respect to As III treated cells undergoing repair without phytochemicals.

Table 3 — Quenching of ROS generation by phytochemicals during simultaneous treatment and pre treatment with and As III (500 µM)

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Dose of Phytochemicals (µM)</th>
<th>Simultaneous treatment</th>
<th>Pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>25</td>
<td>0.10 ± 0.02</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.16 ± 0.04</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>25</td>
<td>0.11 ± 0.04a</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.18 ± 0.02</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Curcumin</td>
<td>25</td>
<td>0.13 ± 0.01</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.14 ± 0.02a</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>25</td>
<td>0.09 ± 0.03a</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.20 ± 0.04</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Quercetin</td>
<td>25</td>
<td>0.12 ± 0.05</td>
<td>0.08 ± 0.02a</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.17 ± 0.03</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Ellagaic Acid</td>
<td>25</td>
<td>0.10 ± 0.06a</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.18 ± 0.04</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Fisetin</td>
<td>25</td>
<td>0.12 ± 0.05</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.19 ± 0.02</td>
<td>0.15 ± 0.03</td>
</tr>
</tbody>
</table>

* Reduction of ROS is significant at *P<0.005 level.
(ellagic acid) may have protective effect against the pro-oxidant activity of As III. Based on the IC \textsubscript{50} values it might be said that of all the compounds used curcumin (35 \(\mu\)M), exhibited best efficacy against As III during simultaneous treatment though and resveratrol during pre treatment (7.5 \(\mu\)M). These compounds were effective in scavenging As III generated ROS in the present study. The antioxidant property may contribute to the antigenotoxic effect both during simultaneous and pre-treatment experiments. Pre-treatment results also highlighted that long duration of pre exposure with low doses of phytochemicals may protect cells from As III-induced DNA damage. It is suggested that the residual amount of phytochemicals remaining within the cells even after complete removal from the medium may have afforded protection against the genetic damage. The effect of the natural compounds in induction of DNA repair has an added advantage against the arsenite induced genotoxicity. During repair experiments ellagic acid gave better recovery of DNA damage (66% during 2 hr and 80% during 4 hr intervals after DNA damage) in comparison to other compounds. The differences in protection afforded by various phytochemicals against As III toxicity needs to be studied in detail. Effect of these compounds on other antioxidant enzymes and repair enzymes affected by As are being studied. It may be concluded from the present study that dietary phytochemicals may have significant protective effect against As III-induced DNA damage, ROS generation and in induction of DNA repair in mammalian V79 cells.

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References
1 WHO Fact Sheet No.210, May 2001


