Shielding effect of anethole against arsenic induced genotoxicity in cultured human peripheral blood lymphocytes and effect of GSTO1 polymorphism

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Abstract

Chronic exposure of inorganic arsenic compounds is responsible for the manifestation of various tumours as well as other diseases. The principal mechanism behind arsenic toxicity is the induction of a strong oxidative stress with production of free radicals in cells. The present study was aimed to explore the shielding effect of anethole against oxidative damage induced by arsenic in cultured human peripheral blood lymphocytes and the effect of GSTO1 polymorphism. Sister chromatid exchange (SCE) frequency, comet tail moment and lipid peroxidation levels were used as biomarkers to assess the oxidative damage. Heparinised venous blood was collected from healthy individuals and treated with sodium arsenite (50 μ M) in the presence of anethole (25 and 50 μ M) for the analysis of shielding effect of anethole. For the genotyping of GSTO1, PCR RFLP method was adopted. A significant dose-dependent increase in the frequency of SCEs, tail moment and lipid peroxidation levels, was observed when lymphocytes were treated with sodium arsenite. Anethole in combination with sodium arsenite has shown a dose-dependent significant decrease in the frequency of SCEs, tail moment and lipid peroxidation levels. Genetic polymorphism of GSTO1 was found to effect individual susceptibility towards arsenic-mediated genotoxicity and was found insignificant when antigenotoxic effect of anethole was considered. GSTO1 mutant genotypes were found to have significant higher genotoxicity of sodium arsenite as compared to wild-type genotype. The results of the present study suggest ameliorative effects of anethole against arsenic-mediated genotoxic damage in cultured human peripheral blood lymphocytes. A significant effect of GSTO1 polymorphism was observed on genotoxicity of sodium arsenite.

Keywords Anethole · Oxidative stress · Sodium arsenite · Genotoxicity

Introduction

Arsenic (As) exposure is one of the major health problem worldwide. It is associated with various cancers and numerous other pathological effects (IARC 2012). It is estimated that over 100 million individuals are exposed to As on a regular basis and the rate of illness and death is quite disturbing (Pierce et al. 2012; Ferreccio et al. 2013). This

metalloid element exists in organic and inorganic forms. Latter is found in the environment as arsenite (AsIII) or arsenate (AsV) and considered as class I human carcinogen (IARC 2004, 2012). Inorganic As (iAs) is associated with adverse effects dependent on dose, duration and exposure regularity.

There is uncertainty in the studies concerning the mechanism of arsenic compound genotoxicity. However, in several studies, generation of oxidative stress via production of reactive oxygen species (ROS) is considered as one of the root cause of iAs toxicity (Flora 2011; Jomova et al. 2011). The oxidative DNA damage induced by iAs results in mutagenesis, carcinogenicity and several other pathologies.

Studies have shown that increased level of inorganic arsenic was associated with increased frequency of micronuclei, chromosome aberrations, and sister chromatid exchanges both in humans and animals (Faita et al. 2013) and also allied with risk of cancer of urinary bladder, lungs and many

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more deformities even in foetus (Chen et al. 2013; Lesseur et al. 2012).

Phytochemicals with inhibitory effects on mutagenesis and carcinogenesis have been identified in commonly used foods, vegetables, cereals, spices and beverages (Aggarwal et al. 2009; Kaur and Arora 2015). Anethole is volatile constituent of more than 20 spices (Newberne et al. 1999). It is a major component of sweet and bitter fennel and anise. It is used as a flavouring substance in chewing gums, baked goods, candy and ice-creams. Antigenotoxicity of anethole was studied in mice (Abraham 2001) and it was found that there is an increase in antigenotoxicity with increase in anethole dosage.

There is a whole range of genotoxicity endpoints to be followed directly in humans for the identification of genotoxic damage. The most frequently used endpoints includes chromosome aberrations, micronuclei, sister chromatid exchanges, single cell gel electrophoresis and 32P-postlabelling. Sister chromatid exchanges (SCE) involve interchanges of DNA replication products between sister's chromatids at apparently homologous loci, pointing to represent homologous recombination repair of DNA double strand breaks (Sonoda et al. 1999). They are analysed from second division metaphases using a staining method based on sister chromatid differentiation by bromodeoxyuridine labelling.

Single cell gel electrophoresis (SCGE), also known as comet assay (Singh et al. 1988), is one of the important biomarkers which estimate the DNA damage in terms of single strand breaks (SSB) in any accessible cells. In the comet assay, a damaged cell takes on resemblance of a comet, with head and tail regions. The head relates to the amount of DNA that still remains in the region of the nuclear matrix, whereas tail represents the damaged DNA migrating from the nucleus (Collins 2004).

Lipid peroxidation products of poly unsaturated fatty acids (PUFAs) are considered as biomarker of cellular injury significant for the analysis of oxidative DNA damage and cytotoxicity (Eder et al. 2008). Arsenic induces generation of reactive oxygen species which form complexes with lipids in cell membrane such as poly unsaturated fatty acids (PUFAs), these complexes react with thiobarbituric acid (TBA) to produce thiobarbituric acid reactive substances (TBARS) complexes. These TBARS complexes can be measured through spectrophotometer, where an increase in absorbance is considered as an indicator of increased lipid peroxidation (Buege and Aust 1978; Ohkawa et al. 1979).

Studies conducted in the last decades had proved the role of genetic polymorphism in determining individual's susceptibility to mutagens and carcinogens. The GST family genes (i.e., GSTP1, GSTO1, GSTO2, and GSTT1) had been studied and found to play a significant role in As metabolism. Arsenic biotransformation involves GSTO1 (Zakharyan et al. 2001) and GSTO2 enzymes (Whitbread et al. 2003)

that use glutathione (GSH) as a reducing agent (Buchet and Lauwerys 1987).

The present study was designed to study the protective effect of anethole against arsenic mediated genotoxic damage in human lymphocyte culture, using sister chromatid exchanges, comet and lipid peroxidation assays. GSTO1 polymorphism was also studied to analyse its effect on individual susceptibility to arsenic genotoxicity and anethole antigenotoxicity.

Materials and methods

Preparation of chemicals

Sodium arsenite and *trans* anethole (Sigma-Aldrich) were prepared in double-distilled water and DMSO (Himedia), respectively. The stock solution (1 mg/ml) was filtered sterile and stored at -20°C until use.

Subjects and sampling

Five milliliter of venous blood was collected from healthy individuals in vacutainer tubes containing sodium heparin as an anticoagulant for lymphocyte culture set-up. A consent form and questionnaire was also got filled by the donors for their consent and health status. Before commencement of the study, approval of the Human Ethics Committee of Kurukshetra University was obtained (no. IEC/14/367).

Human lymphocyte culture

Short-term peripheral blood lymphocyte (PBL) cultures were set up using earlier studied technique of Moorhead et al. (1960) with minor modifications. Cultures were set up in duplicate by adding (0.4 ml) whole heparinized blood into 5 ml of RPMI 1640 culture medium (Himedia) containing L-glutamine (1%), foetal calf serum (20%) (Himedia), penicillin (100 UI/ml) and streptomycin (100 µg/ml) solution (Himedia), and phytohaemagglutinin (2%) (Bangalore Genei). The cultures were incubated in 5% CO₂ at 37 °C for different durations.

Sister chromatid exchange assay

For sister chromatid exchange (SCE) analysis, short-term PBL cultures were set up. Sodium arsenite was added in different concentrations (10, 25, 50 and 100 µM) followed by anethole (25 and 50 µM) at the beginning of culture, 5-bromo-2-deoxyuridine (Sigma-Aldrich) in final concentration of 10 µg/ml culture was added after 24 h of incubation. The culture was incubated for another 48 h at 37 °C and $\pm 5\%$ CO₂. Two drops of colchicine in a final concentration

of 0.2 µg/ml were added 45 min prior to the harvesting. The cells were harvested by centrifugation and then treated with hypotonic solution (0.075 M KCl) and fixed in methanol:acetic acid (3:1). From a suspension of fixed cells, slides were prepared by the air drying method and stained with Hoechst 33258 and 4% Giemsa stain (Perry and Wolff 1974). Cells with > 10 SCE were considered as HFC (high frequency cell). For calculating the frequency of SCE per cell, fifty metaphases were analysed.

Single cell gel electrophoresis (SCGE) or comet assay

For comet assay, PBL cultures were set up following the methodology of Singh et al. (1988). Sodium arsenite was added to culture tubes in different concentrations (10–100 µM) to check its genotoxic effect. Simultaneously, antigenotoxic effect of anethole was also analysed at different doses (25 and 50 µM). The cultures were incubated for 24 h at 37 °C and $\pm 5\%$ CO₂. The cells were harvested by centrifugation and fixed. The harvested cells were suspended in 0.5% (w/v) low-melting agarose and layered over a frosted microscopic slide having first coat of 1% normal melting agarose for firm gripping. Then slides were kept at 4 °C for solidification for 10 min. A third layer was made with 0.5% (w/v) low-melting agarose, slides were kept for another 10 min. Afterwards, slides were immersed in 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] for 2 h at 4 °C. Then slides were transferred to electrophoresis chamber containing electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13.0) and pre-immersed for 20 min for unwinding the DNA which was followed by electrophoresis for 20 min (300 mA, 25V) at 4 °C using the method of Singh et al. (1988) with some minor modifications.

Slides were then kept in neutralizing buffer (Tris 0.4M, pH 7.5) for 10 min, stained with ethidium bromide (20 µg/ml) and later screened under a fluorescence microscope. The cells were subjected to image analysis using Lucia Comet (Version 7.12) software. The extent of DNA damage was analysed by tail moment measurement. The tail moment is calculated as the percentage of DNA in the tail multiplied by the length between the centre of the head and tail.

Lipid peroxidation assay

Lipid peroxidation is measured by the formation of thiobarbituric acid reactive substances (TBARS). Levels of TBARS in cell culture lysate were determined spectrophotometrically at 535 nm by the method defined by Ohkawa et al. (1979) with minor modifications. Short-term PBL culture was set up. Arsenic (50 µM)-treated cultures were supplemented with anethole (25 and 50 µM) for

the analysis of antioxidative effect. Control was also run simultaneously along with DMSO as a negative control and EMS as a positive control. The cultures were incubated in 5% CO₂ at 37 °C for 24 h. Treated cell cultures were harvested and washed with PBS (1%). The pellet was lysed with lysis buffer (Tris buffer, MgCl₂, EDTA, mercaptoethanol and glycerol). The lysate was centrifuged at 6000 rpm for 20 min at 4 °C and the supernatant was used for protein estimation by Lowry's method.

The reaction mixture tubes containing cell lysate, 0.1 ml of 10% SDS, 0.6 ml of 20% of glacial acetic acid, 0.6 ml of 0.8% TBA, and water (final volume 3.0 ml) were placed in boiling water bath for 1 h and immediately transferred to crushed ice for 10 min. Centrifugation was done at 5000 rpm for 10 min and supernatant was analysed spectrophotometrically at 535 nm against a blank devoid of cell lysate.

GSTO1 genotyping (rs4925, 419C to A)

Genomic DNA was isolated from 200 µl of whole blood by DNA extraction kit (Bangalore Genei). PCR RFLP was used to study the genetic polymorphism of *GSTO1* gene. The amplification reaction was carried out in a 25 µl volume containing 50–100 ng of genomic DNA as a template, 20 pmol/µl of each primer (Sigma-Aldrich), 200 µM of each dNTP (Bangalore Genei), 1× PCR buffer with 15 mM/l MgCl₂ (Bangalore Genei), and 0.7 units of Taq polymerase (Bangalore Genei). PCR was performed using reaction mixture with *GSTO1* primers (Fw-5'-AAA GTT GTT TCT TAA ACG TGCC-3' and Re-5'-AAG TGA CTT GGA AAG TGG GAA-3') (Marahatta et al. 2006). Initial denaturation was done at 94 °C for 10 min. A total of 35 temperature cycles were used: 94 °C at 1 min, 55 °C for 1 min, and 72 °C for 1 min. The last elongation step was extended to 10 min at 72 °C. The PCR products were analysed in 2% agarose gel. Amplification yielded 455 bp product which was then digested with *Cac8I* (New England Biolabs), and the products were analysed using 3% agarose gel. The genotypes were analysed as follows: homozygous wild-type C/C: 243, 145 and 67 bp; heterozygous C/A: 388, 243, 145 and 67 bp; homozygous variant A/A: 388 and 67 bp (rs4925, 419C to A).

Statistical analysis

All treatments were performed in duplicates and results were expressed as means $\pm$ S.E/S.D. The statistical analysis was performed using one-way analysis of variance (ANOVA) for multiple samples and Student's *t* test was used for comparing paired sample tests. All tests were performed using statistical package system SPSS 16.0.

Results

There are different biomarkers for assessing the genotoxicity. Biomarkers of effects are the reckonable changes that an individual undergoes, which indicates an exposure to a compound and may show resulting health effect. Sister chromatid exchange and comet assay are considered as the reliable, sensitive and simple methods for the analysis of genotoxic effects. In the present study, sister chromatid exchange and comet assay were used for the analysis of genotoxicity caused by sodium arsenite. Lipid peroxidation assay was used as a biomarker of oxidative stress.

There was dose-dependent increase in the frequency of SCE observed in cultures treated with sodium arsenite at concentration ranging from 10 to 100 μM (Fig. 1a). The number SCEs (8.72 ± 0.51) was significantly higher in arsenic treated culture when compared with frequency of SCEs (3.69 ± 0.29) in control. In comet assay, genotoxicity was measured in terms of tail moment value, similar trend as of SCE was observed in tail moment of arsenic-treated (10–100 μM) cultures. There was significantly higher tail moment (33.98 ± 2.69) in arsenic-treated culture as compared to control (2.31 ± 0.29) (Fig. 1b). Arsenic at concentration of 50 μM showed significantly higher genotoxicity in both assays.

Anethole was also analysed for having any genotoxic effects in a separate culture set treated with anethole (25

and 50 μM). It was found to be non genotoxic as there was no significant increase in SCEs as well as in tail moment as compared to control-untreated sample (Fig. 2a–d).

Anethole showed a protective effect against As-mediated genotoxicity in a dosimetry manner. There was a significant dose-dependent decrease ($P < 0.05$) in both frequency of SCE (4.91 ± 0.42) and tail moment (14.59 ± 0.31) in comparison to the PBL treated with As (Fig. 2a, b).

Anethole was also analysed for ameliorative effect against oxidative stress induced by arsenic. There was a significant ($P < 0.05$) decrease in lipid peroxidation observed when arsenic-treated cultures were supplemented with anethole (25 and 50 μM). Anethole showed a significant decrease in TBARS levels (10.8 ± 0.26) calculated from absorbance in comparison with arsenic-treated culture (Fig. 3).

Effect of GSTO1 polymorphism on arsenic-induced DNA damage

Individuals with different genotypes counter differently to xenobiotic exposure. Glutathione-S-transferase (GST) family involves group of genes coding enzymes of phase II detoxifications reactions and found to play a significant role in arsenic metabolism in the cellular response process against oxidative stress (Cimbaljevic et al. 2016).

Genetic polymorphism of GSTO1 gene had been associated with susceptibility to different types of cancer worldwide (Lesseur et al. 2012). In meta-analysis studies, population with mutant genotypes of GSTO1 was affected with higher levels to genotoxicity. The present study analysed the effect of GSTO1 polymorphism on arsenic-mediated DNA damage.

Significant increase in arsenic genotoxicity was observed in individuals with GSTO1 mutant genotypes as compared to wild type. However, no significant effect of GSTO1 polymorphism was observed on antigenotoxic potential of anethole against arsenic-induced DNA damage (Table 1; Fig. 4).

Fig. 1 **a** Dose-dependent increase in SCE with increase in As dose in cultured lymphocytes [$*P < 0.05$ (significant as compared to untreated)]. **b** Dose-dependent increase in comet tail moment with increase in As dose in cultured lymphocytes [$*P < 0.05$ (significant as compared to untreated)]

Discussion

Chronic exposure to iAs is associated to an increased risk of skin, bladder, lung, kidney cancers, as well as cardiovascular (James et al. 2015) and several other pathologies (Wu et al. 2014). In a recent study, arsenic is said to be an upcoming threat in North India after chromium (IMG Report 2015).

Phytochemicals with ameliorative effects against genotoxicity have been observed in dietary elements such as fruits, spices (Aggarwal et al. 2008; Kaur and Arora 2015). Shaha et al. (2011) considered fennel as an excellent source of natural antioxidants to combat oxidative stress. Fennel plant fruit extract (anethole 80%) had been studied for genotoxicity and antigenotoxicity in *Drosophila melanogaster* using

Fig. 2 **a** Dose-dependent decrease in SCEs with increase in dose of anethole in cultured lymphocytes against arsenic-mediated genotoxicity [$*P < 0.05$ (significant as compared to untreated), *As* arsenic, *An* anethole, *EMS* ethyl methane sulphonate]. **b** Dose-dependent decrease in tail moment with increase in dose of Anethole in cultured lymphocytes against arsenic-mediated genotoxicity [$*P < 0.05$ (significant as compared to untreated), *As* arsenic, *An* anethole, *EMS* ethyl methane sulphonate]. **c** Comet assays of lymphocytes in (A) control (B) arsenic treated (C) anethole treated (a, c—undamaged cells, b—damaged cells). **d** Sister chromatid exchanges in lymphocytes in (A) control (B) arsenic treated (C) anethole treated

somatic mutation and recombination test (SMART) and results showed a significant protective potential in eyes spot against methyl methane sulphonate (Amkiss et al. 2013).

In the present study, antigenotoxic and antioxidative properties of anethole have been explored against arsenic-induced genotoxicity in PBL. The results of the present study

Fig. 3 Dose-dependent decrease in TBARS level with increase in dose of anethole in cultured lymphocytes against arsenic-mediated genotoxicity [$*P < 0.05$ (significant as compared to untreated), *As* arsenic, *An* anethole, *EMS* ethyl methane sulphonate]

Table 1 Effect of *GSTO1* polymorphism on arsenic-induced genotoxicity and anethole (An) antigenotoxicity

Biomarker	Control	Arsenic (50 μm)	Arsenic (50 μm) + anethole (50 μm)
Wild genotype			
SCE ± S.E	3.63 ± 0.31	8.26 ± 0.39 ^a	4.87 ± 0.35 ^b
Tail moment ± S.D	14.59 ± 0.31	25.52 ± 19.6 ^a	18.28 ± 9.23 ^b
TBARS ± S.E	3.42 ± 0.19	18.04 ± 0.28 ^a	10.41 ± 0.23 ^b
Mutant genotype			
SCE ± S.E	4.83 ± 0.46	10.13 ± 0.38 ^{a*}	5.33 ± 0.31 ^{b*}
Tail moment ± S.D	17.62 ± 0.46	34.12 ± 20.61 ^{a*}	26.91 ± 11.9 ^{b*}
TBARS ± S.E	5.56 ± 0.17	19.93 ± 0.34 ^{a*}	10.31 ± 0.22 ^{b*}

S.E standard error, *S.D* standard deviation

^a $P < 0.05$ (significant as compared to wild genotype)

^b $P > 0.05$ (non-significant as compared to wild genotype)

^a $P < 0.05$ (significant as compared to untreated)

^b $P \leq 0.05$ (significant as compared to sodium arsenite (As) treatment)

Fig. 4 *GSTO1* genotyping [homozygous wild (lane 2, 3, 4, 9), heterozygous (lane 5,6)]

showed a shielding effect of anethole in a dose-dependent manner against arsenic-mediated genotoxicity and oxidative stress. There was a significant decrease in SCE frequency and comet tail moment at arsenic toxic dosage (50 μ M) in cultures when supplemented with anethole (25 and 50 μ M). Similarly, anethole proved protective against arsenic-induced lipid peroxidation. It significantly decreased the TBARS levels in arsenic-treated cultures.

Our results agree with the study of Abraham (2001), regarding dose-dependent antigenotoxic effect of anethole (40–400 mg/kg body weight) in mice against cyclophosphamide, procarbazine, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and urethane assessed by mice bone marrow micronucleus test. Anethole also found to inhibit ethyl methane sulfonate-mediated genotoxicity. Avani and Rao (2009) studied the protective role of vitamin A against As-induced genotoxicity in humans. Similar findings observed with other vitamins which reduce the risk of As pathologies if taken in higher doses in routine (Zablotska et al. 2008). In a recent study, nanocurcumin found to have a protective effect against As in rats (Sankar et al. 2013).

Concerning the effect of GST omega gene (GSTO1 and GSTO2) polymorphism on arsenic-exposed population, study in Taiwan showed that the GSTO2 *Asn142Asp* variant is associated with an increased risk of urothelial carcinoma (Hsu et al. 2011) and associated with major risk of As-related bladder cancer (Lesseur et al. 2012). In another study, GSTO1 *Al40D* polymorphism was found to be associated with a lower mono methyl arsenious acid (MMA%) (Chen et al. 2012) and also linked to marked elevated risk of carotid atherosclerosis in subjects having high As exposure with mutant GSTO risk haplotypes (Heish et al. 2011).

Results of our present study have showed significant higher frequency of sister chromatid exchanges, comet tail moment and TBARS levels in GSTO1 mutants than wild type both in control- and arsenic-treated samples. It has been observed that individuals with mutant genotypes were susceptible to arsenic-induced DNA damage and oxidative stress studied by different biomarkers. However, no effect of genetic polymorphism of GSTO1 was observed on antigenotoxic potential of anethole.

To the best of our knowledge, no study has been conducted on healthy population regarding the effect of GSTO1 polymorphism on arsenic-mediated genotoxicity and oxidative stress. A few in vitro studies regarding effect of GSTM1 and GSTT1 polymorphism effect on damage induced by different genotoxins in cultured PBL showed both significant and non-significant results (Park et al. 2002; Kumar et al. 2009).

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of the present work.

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