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RESEARCH ARTICLE

Arsenic induced biochemical and genotoxic effects and its amelioration by diallyl trisulphide in rats

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Abstract

Purpose: Arsenic (As) is a well documented human carcinogen. Conversely, its mechanisms of toxic deed and carcinogenic possible in animals have not been indisputable. So, this study was conducted to investigate the biochemical and genotoxic possessions in male wistar rats treated with As, DATS with As and As and vitamin C (positive control).

Method: Five groups of six male rats, each weighing approximately 180-190 g, were orally administered, daily once for 28 days with a dose of 5 mg/kg BW of sodium arsenate. A control group was also made of 6 animals treated with vehicles alone. At the end of experimentation, the animals were sacrificed, and Chromosome and micronuclei preparation was obtained from bone marrow cells. The activities of plasma 8-OHdG, as well as the number of structural chromosomal aberrations (SCA), frequency of micronuclei (MN), mitotic index and DNA damage in the bone marrow cells were estimated.

Result: Arsenic exposure significantly increased ($p<0.05$) the activities of plasma 8-OHdG, as well as the number of structural chromosomal aberrations (SCA), frequency of micronuclei (MN) and DNA damages in the bone marrow cells. In contrast, the mitotic index in these cells was significantly reduced ($p<0.05$). In animals treated with DATS, the altered parameters were significantly recovered.

Conclusion: These findings indicate that 8-OHdG is novel biomarkers for arsenic-induced genotoxicity. Our results also demonstrate that As has a strong genotoxic potential, as measured by the bone marrow SCA and MN tests in wistar rats. DATS has shown the anti-genotoxic effect against As induced genotoxicity in rats.

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Introduction

Arsenic is an omnipresent element present in food, soil, water and air, and it is released into the environment from both anthropogenic and man-made sources [1,2]. The major inorganic forms of arsenic include the trivalent meta arsenite As^{+3} and the pentavalent arsenate As^{+5}. Trivalent arsenic form has a higher affinity for thiol groups [3] and is more cytotoxic and genotoxic than As^{+5} [4]. Organisms that accumulate the trivalent intermediates are thought to be greater risk of arsenic- induced ailments [4]. Some of the organic forms include the methylated metabolites monomethylarsonic acid (MMA), dimethylarsenic acid (DMA) and trimethylarsine oxide (TMAO) as well as arsenobetaine (AsB), arsenocholine and arsenosugars. More than 80% of commercially utilized arsenic compounds are used to manufacture products with agricultural applications such as insecticides, herbicides, fungicides, algaecides, sheep dips, wood preservatives, dye-stuffs, and medicines for the eradication of tapeworms in sheep and cattle. Arsenic compounds have been used for at least a century in the treatment of syphilis, yaws, amoebic dysentery, and trypanosomiasis [5].

Genotoxicity endpoints such as micronuclei, sister chromatid exchanges, chromosome aberrations and aneuploidy have been reported for inhabitants of nine arsenic-affected districts of West Bengal, India

[6]. Industrial resources of arsenic to human workers consist of vineyards, ceramics, glass making, smelting and refining of metallic ores, through production and utilize of arsenic containing agricultural products like pesticides and herbicides. Exposure to arsenic occurs through the oral route (ingestion), inhalation, dermal contact, and the parenteral route to some scope. Humans can be exposed to arsenic through the intake of air, food and water [7]. Epidemiological and clinical studies indicate that arsenic is a paradoxical human carcinogen that does not easily induce cancer in animal models [8]. The toxicity of arsenic depends on its chemical state. Inorganic arsenic in its trivalent form is more toxic than pentavalent arsenic. The toxicity of arsenic also depends on the exposure dose, frequency and duration, the biological species, age, and gender, as well as on individual susceptibilities, genetic and nutritional factors [9,10]. By binding to thiol or sulfhydryl groups on proteins, As (III) can inactivate over 200 enzymes. This is the likely mechanism responsible for arsenic's widespread effects on different organ system. As (V) can replace phosphate, which is involved in many biochemical pathways [11-13].

Analysis for genotoxicity have designated that arsenic compounds restrain DNA repair, and stimulate chromosomal aberrations, sister-chromatid exchanges, and micronuclei formation in both human and rodent cells in culture [14-18] and in cells of exposed humans [10]. An *invitro* cell transformation studies in the absence of animal models, become a useful means of obtaining information on the carcinogenic mechanisms of arsenic toxicity. Arsenic and arsenical compounds are toxic to and provoke morphological transformations of Syrian hamster embryo (SHE) cells as well as mouse C3H10T1/2 cells and BALB/3T3 cells [19–21]. It has been reported that arsenic trioxide induces DNA damage in human lymphophytes, [22], colon cancer cells [23] and also in mice leukocytes [24] as based on the comet assay. Arsenic compounds have also been revealed to provoke gene amplification, seize cells in mitosis, hinder DNA repair, and induce expression of the c-fos gene and the oxidative stress protein heme oxygenase in mammalian cells [25,26].They have been concerned as advertisers and co mutagens for a range of toxic agents [27].

Considering the toxic effects of arsenic is complicated because it exists in many different inorganic and organic compounds, and its toxicity varies according to its oxidation state, its solubility and many other factors including the exposure dose, frequency and duration, the biological species, age and gender, as well as individual susceptibilities, genetic and nutritional factors [28–30]. Most cases of human toxicity from arsenic have been associated with exposure to inorganic arsenic. Interest in the toxicity of arsenic has been heightened by recent reports of large populations in West Bengal, Bangladesh, Taiwan, China, Mexico, Argentina, Chile, Finland and Hungary that have been exposed to high concentrations of arsenic in their drinking water and are displaying various clinico-pathological conditions, the major effects being skin alterations and skin cancer. General health effects that are associated with arsenic exposure include cardiovascular and peripheral vascular disease, developmental anomalies, neurologic and neurobehavioral disorders, diabetes, hearing loss, portal fibrosis, hematologic disorders (anemia, leucopenia and eosinophilia) and multiple cancers: significantly higher standardized mortality rates and cumulative mortality rates for cancers of the skin, lung, liver, urinary bladder, kidney, and colon in many areas of arsenic pollution [9,31,32].

Although arsenic and arsenic containing compounds has been the subject of important toxicology research, there exists a lack of appropriate animal model for carcinogenicity assessment, as well as a scarcity of scientific data describing the tissue distribution of arsenic in relation to the biomarkers of arsenic-induced genotoxicity in *invivo* systems. Cytogenetic biomarkers (SCA, MN) play an important role in toxicological hazard evaluation as the first step towards quantification of cancers. Biomarkers serve as internal indicators of environmental or occupational exposures and have the potential for the prevention of effects of carcinogen exposure by early detection. The possible use of biomarkers representing intermediate steps in the exposure-to-disease scale to estimate health risk in human populations has gained growing interest. A number of naturally occurring dietary constituents are known that suppress genotoxic damage by these xenobiotics through various intracellular and extracellular mechanisms. Therefore, the present work was undertaken to investigate the genotoxic effects of As in wistar rats through structural chromosomal aberrations (SCA), micronuclei (MN) formation and mitotic index (MI) in bone marrow cells and to study the geno protective effects of DATS against As induced genotoxic effects in rats as some work has been done with DATS indicating them to inhibit arsenic induced free radicals and ameliorates oxidative stress induced by arsenic in rats [33,34].

Material and Methods

Chemicals

Diallyl trisulfide was purchased from Lukang Cisen Pharmaceutical Co., Ltd. (Shangdong,

China). Arsenic was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals used for the present study were procured from Sigma chemical Co. (St. Louis, MO, USA) and other chemicals and solvent were of certified analytical grade and purchased from S.D. Fine Chemicals, Mumbai or Himedia Laboratories Pvt. Ltd., Mumbai, India. Reagent kits were obtained from span Diagnostics, Mumbai, India. Methanol, glacial acetic acid, and superfrost microscope slides were purchased from Fischer-Scientific Houston, TX, USA. Potassium chloride solution (0.075 M) and Giemsa stain stock solution (0.4%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hanks Balanced Salt Solution was purchased from GIBCO (Grand Island, NY, USA). Fetal Bovine Serum (FBS) was obtained from Hyclone (Logan, UT).

Animals and experimental design

The experiment was carrying out on healthy male albino rats of Wistar strain reared and acquired from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College Annamalai University. The animal treatment and protocol employed were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 885/2012/CPCSEA). The experimental animal of body weight ranging from 170-190g were used in the study and housed in polypropylene cages lined with husk. The rats were maintained in normal day and night schedule (12 hour light: 12 hour dark). Each rat was given pellet foodstuff from Amrut laboratory animal feed, Pune, India for feeding and water *ad libitum*. In the present study, sodium arsenate was administered intragastrically at a dose of 5 mg/kg body weight/day for 4 weeks and DATS was administer 90 minutes before the administration of As. In the experiment, a total of 30 rats were used. The rats were divided into 5 groups of 8 rats each.

Group 1: Control rats treated with normal saline and corn oil for 28 days.

Group 2: Rats received As as sodium arsenate (5 mg/kg body weight) in normal saline for 28 days.

Group 3: Rats received As (5 mg/kg body weight) with oral pre-administration of DATS 80 mg/kg body weight) for 28 days

Group 4: Normal rats received DATS (80 mg/kg body weight) dissolved in corn oil and administered orally for 28 days.

Group 5: As (5 mg/kg.BW) + Vitamin 'C' (100 mg/kg.BW).

Chromosome Aberration Assay

The rats were sacrificed by cervical dislocation 24 h after administration of the last dose for chromosome aberration assay. Cytogenetic analysis was performed on bone marrow cells according to the recommendations of Preston et al. [35], with slight modifications. Experimental animals were injected (intraperitoneal) with colchicine (4 mg/kg) 1.5 h prior to sacrifice. Both femora were dissected out and cleaned of any adhering muscle. Bone-marrow cells were collected from both femora by flushing in KCL (0.075 M, at 37°C) and incubated at 37°C for 25 min. Collected cells were centrifuged at 2000 × g for 10 min, and fixed in aceto-methanol (acetic acid: methanol, 1:3, v/v). Centrifugation and fixation were repeated five times at an interval of 20 min. The cells were resuspended in a small volume of fixative, dropped onto chilled slides, flame-dried and stained the following day with freshly prepared 2% Giemsa stain for 3–5 min, and washed in distilled water to remove excess stain.

Mitotic index determination

The mitotic index was used to determine the rate of cell division. The slides prepared for the assessment of chromosomal aberrations were also used for calculating the mitotic index. Randomly selected views on the slides were monitored to determine the number of dividing cells (metaphase stage) and the total number of cells. At least 1000 cells were examined in each preparation.

Micronucleus test

Rats were sacrificed by cervical dislocation 30 h after the last treatment. The frequency of micronucleated cells in femoral bone marrow was evaluated according to the procedure of Schmid [36], with slight modifications as reported by Agarwal and Chauhan [37]. The bone marrow was flushed out from both femora using 2 mL of Fetal Calf Serum and Hanks Balanced Salt Solution (3:1) and centrifuged at 2000 × g for 10 min. The supernatant was discarded. Evenly spread bone marrow smears were stained using the May-Grunwald and Giemsa protocol.

Scoring of slides

Bone marrow preparations for the analysis of chromosome aberrations in metaphase cells were obtained using the technique by Preston et al., [35]. The slides were stained with Giemsa. Well-spread metaphases presenting 42±1 chromosomes were analyzed. One hundred metaphases per animals were screened to a total of 500metaphases for each treatment and control to obtain the total number of chromosomal aberrations. The mitotic indices were obtained by counting the number of mitotic cells in 1000 cells per animal to a total of 5000 cells per treatment and control. The mitotic index was calculated as the ratio of the number of dividing cells to the total number of cells, multiplied by 100. A total of 3000 cells/treatment were scored, on coded slides to evaluate the frequency of micronucleated cells in bone marrow under an Olympus microscope.

Assessment of DNA damage

DNA damage was assessed by using alkaline single cell electrophoresis (comet assay) according to the method of Singh and Mc Coy [38]. Single cell suspensions from tissues like liver and kidney were prepared following the procedure of Singh et al [39]. One hundred µl of 1% NMPA in phosphate buffered saline was dropped on to frosted slide immediately covered with cover slip and kept for 10 min in refrigerator for it to solidify. Then, cover slips were removed and 100 µl of LMPA coated cells (100 µl of cell suspension in HBSS and 100 µl of LMPA) were added to the slides. The cover slips were replaced and the slides were kept in the refrigerator for another 10 min to solidify the LMPA. After this, the cover slips were removed and a top layer of 100 µl of LMPA was added the slides were again cooled for 10 min.

After removal of cover slips, the slides were immersed in cold lysing solution. The slides were kept in dark at 4ºC for at least 1 hr. To prevent the occurrence of additional DNA damage, the following steps were performed under dim light. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank. The unit was filled with a freshly made electrophoresis buffer to a level of 0.25 cm above the slides. The cells were exposed to alkali for 20 min to allow for DNA unwinding.

An electric current of 25 V and 300mA was applied for 20 min to electrophoresis. After electrophoresis, the slides were placed horizontally, and neutralized with Tris- HCl. Finally, 50 µl of ethidium bromide was added to each slide and covered with a cover slip and analyzed using a fluorescence microscope (Nikon, Japan) with a calibrated scale in ocular. Images of 50 randomly selected cells were analyzed from each sample. For each cell, the length of the image was measured and was expressed as micrometers (µm) with the help of software Komet V– single cell gel electrophoresis, Version V, 2001, Marketed by Kinetic Imaging Limited, UK.

8-hydroxydeoxyguanosine (8-OHdG) estimation

Plasma samples from each group were diluted twice and filtered through 20 µm filters and subsequently filtered samples were used for 8-OHdG analysis. The 8-OHdG analysis was performed using the NWLS^{TM} 8-OHdG ELISA kit (Northwest Life Science Specialities, LLC, Vancouver, WA). The protein content in each sample was estimated using DC protein Assay kit. The results were expressed as 8-OHdG (ng/mg of protein).

Statistical analysis

Values are given as mean ± S.D. for six rats in each group. The data for various biochemical parameters were analyzed by analysis of variance (ANOVA) using SPSS version 13.0 (SPSS, Cary, NC, USA) and DMRT was use to obtained individual comparison. A value of $P<0.05$ was considered to indicate a significant difference between groups.

Results

Chromosome aberrations

Table 1 summarized the results of chromosomal aberrations in bone marrow cells of male albino rats treated with As (5 mg/kg). The chromosomal aberrations were represented in the form of chromatid deletions, dicentric, fragment, centromeric separation, ring and polyploidy. These structural and numerical types were identified and calculated relative to the control. Rats treated with DATS and As exhibited significant ($p<0.05$) reduction in chromosomal aberrations induced by As. The frequency of total chromosomal aberrations was highly significantly increased in As treatment for 28 days (51.42 ±25.31) when compared with the control (1.68 ±1.2). Rats treated with DATS and As significantly decreased (11.38 ±7.11) when compared with the As treated group. DATS alone treated group show significantly lower chromosomal aberrations when compared with the control.

Table 1 Chromosomal aberration in rat bone marrow cells after treatment with Arsenic and DATS.

Group	Chromosomal aberrations						No. of Aberrations	Average No. of Aberration ± S.D
	R	D.C	F	C.S	D	Polyploidy		
Control	2	0	1	0	3	2	8	1.68 ±1.2[a]
As	74	12	56	60	38	19	300	51.42 ±25.31[b]
As+ DATS	31	4	21	28	15	6	105	11.38 ±7.11[c]
DATS	1	0	1	2	2	6	6	1.06 ±1.02[d]
As+Vitamin C	40	8	37	37	20	7	147	12.98 ±13.59[e]

Abbreviations used- R: ring, D.C: dicentric, F: fragment, C.S: centric separation, D: deletion. Values were mean ± S.D. (n=6); As: arsenic, DATS: diallyl trisulfide. Values not sharing a common superscript letter (a–e) differ significantly at $p<0.05$ (DMRT).

Micronuclei induction

The micronuclei frequencies in bone marrow cells of rats exposed to DATS and As are summarized in Fig. 1. As was found to significantly ($p<0.05$) induced an increased in micronuclei frequency in bone marrow cells of rats (4.50±1.24) when compared to controls rats (2.20±1.01). Rats treated with DATS and As exhibited significant reduction in micronucleated cells. Rats treated with DATS and As significantly decreased micronuclei frequency (2.93±1.02) when compared with the As treated (4.50±1.24). DATS alone treated rats show significantly ($p<0.05$) lower micronuclei frequency (2±1.01) when compared with the control. The mean numbers of micronucleated cells were 2.20±1.01, 4.5±1.24, 2.93±1.02, 2±1.01 and 3.2±1.02 per 1000 cells for control, arsenic, arsenic plus DATS, DATS alone and arsenic with vitamin C respectively.

Fig. 1: Effect of As and DATS on micronuclei induction

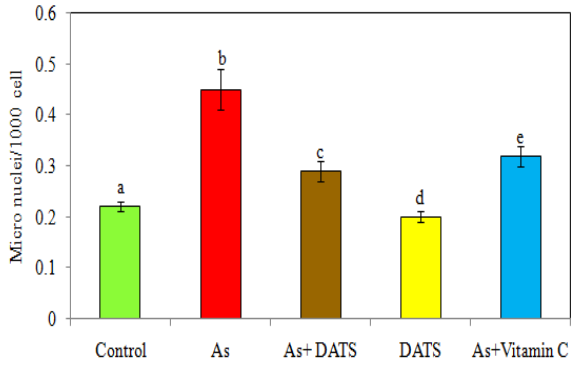

Fig. 1: Effect of As and DATS on micronuclei induction for bone marrow cells of control and experimental rats. Values are mean ± SD for 6 rats in each group. Values not sharing a common superscript letter (a–e) differ significantly at $p<0.05$ (DMRT).

Mitotic index

The mitotic index was used to determine the rate of cell division in bone marrow cells of rats exposed to As. It was found that the mitotic index significantly ($p<0.05$) decreased in the arsenic (6.81±0.17) treated rats when compared to control rats (12.06±0.24). A mitotic index of 11.21±0.32 was recorded in DATS and As treated rats showing improve cell division in bone marrow cells of rats when compared with the As alone treated rats (Fig. 2). A significant ($p<0.05$) different was found between the control and DATS alone treated rats.

Fig. 2: Effect of As and DATS on mitotic index

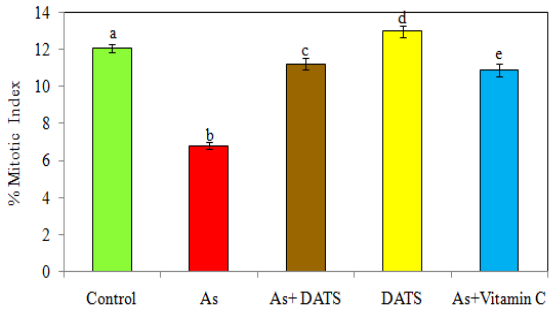

Fig. 2: Effect of As and DATS on mitotic index for bone marrow cells of control and experimental rats. Values are mean ± SD for 6 rats in each group. Values not sharing a common superscript letter (a–e) differ significantly at $p<0.05$ (DMRT).

DNA damage

DNA strand breaks caused by As induced free radicals analyzed through comet assay in bone marrow cells revealed a significant ($p<0.05$) increase in tail moment and tail length when compared with control rats, shown in Fig. 3 and 4. DATS had a significant ($p<0.05$) inhibitory effect on As induced DNA damage in bone marrow cells. DATS at a dose of 80 mg/kg body weight exhibited significant inhibitory effect ($p<0.05$) on As induced DNA damage. There was a significant different between control and DATS (80 mg/kg B.W) alone treated rats without any damage to DNA of bone marrow cells.

Fig. 3: Effect of As and DATS on DNA damage (tail moment)

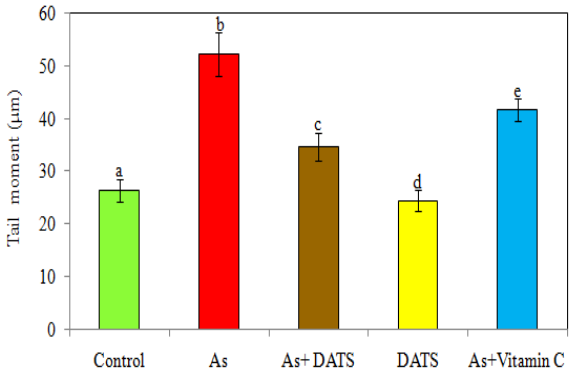

Fig. 3: Effect of As and DATS on DNA damage (tail moment) for bone marrow cells of control and experimental rats. Values are mean ± SD for 6 rats in each group. Values not sharing a common superscript letter (a–e) differ significantly at $p<0.05$ (DMRT).

Fig. 4: Effect of As and DATS on DNA damage (tail length)

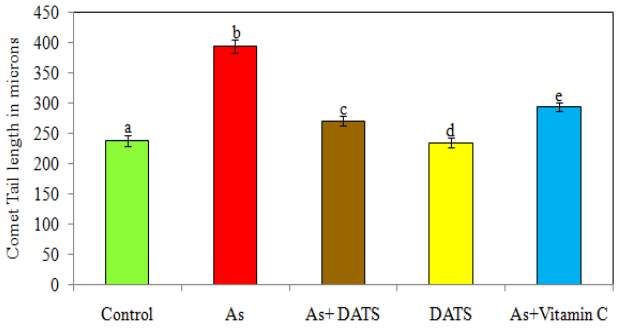

Fig. 4: Effect of As and DATS on DNA damage (tail length) for bone marrow cells of control and experimental rats. Values are mean ± SD for 6 rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p<0.05 (DMRT).

8-OHdG level

In plasma the oxidative stress marker of DNA, 8-OHdG level was significantly (p<0.05) increased in As treated rats when compared to control. Supplementation of DATS significantly reduced the level of 8-OHdG in plasma when compared with As exposed rats. There was no significant difference in the levels of 8-OHdG between DATS alone treated rats and control rats (Fig.5).

Fig. 5: Effect of As and DATS on changes in the levels of plasma 8-OHdG level

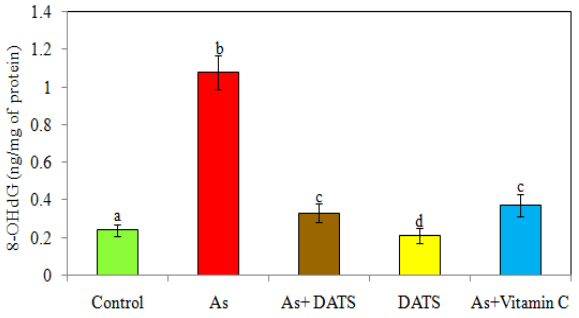

Fig. 5: Effect of As and DATS on changes in the levels of plasma 8-OHdG level of control and experimental rats. Values are mean ± SD for 6 rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p<.05 (DMRT).

Discussion

Although multiple pathways such as inhibition of DNA repair, methylation status and co-carcinogenesis with other environment toxicants have been proposed, one common theme that has emerged is the role of reactive oxygen species (ROS) in the pathogenesis of arsenic induced diseases. These free radicals generated as a consequence of arsenic exposure are linked to cell signaling, apoptosis and mutagenesis.

Cytogenetically, data generated from this study clearly indicate a significant increase of cytogenetic damage in the bone marrow cells, due to As exposure induced chromosomal aberrations in the present work; deletion, ring, centric separation and dicentric. Also, a significant increase (P<0.05) in micronuclei induction and reduced mitotic index was observed in arsenic-treated treated rats cells compared to the controls rats. The types of cytogenetic damage observed in this study have been attributed to several modes of arsenic-induced toxicity including the inhibition of various enzymes involved in DNA repair and expression [40], the induction of reactive oxygen species capable of inflicting DNA damage [41] or the induction of gene expression of a number of stress response proteins leading to alteration in DNA repair mechanism causing DNA damage [42]. As had already been reported to have adverse effects on chromosomes and As burden in the body has been reported to be directly correlated with chromosomal aberrations [43]. Patlolla [43] observed that in animals administered with As there was a significant increase in the number of chromosomal aberrations and a decline in mitotic index. Free-radicals can originate from exposure to various environmental toxicants, As being one of them, resulting in disturbed homoeostasis and the induction of biological stress as manifested by a sharp decline in mitotic index and an elevation of chromosomal aberrations. The types of cytogenetic damage observed in this study have been attributed to several modes of arsenic-induced toxicity including the inhibition of various enzymes involved in DNA repair and expression, the induction of reactive oxygen species capable of inflicting DNA damage or the induction of gene expression of a number of stress response proteins leading to alteration in DNA repair mechanism causing DNA damage [43]. Cytogenetic effects of arsenic compounds have also been studied in different rodents using CA and MN assays. These genotoxic effects have been observed in *invivo* experiments with mouse fetal chromosome [44] as well as with mouse fibroblasts [45]. Arsenic-induced SCAs have also been demonstrated in *invitro* studies with CHO cells [46–48], V79 cells [49], and SHE cells [24,50]. Interestingly in our study supplementation of DATS to As treated rats significantly reduced the number of chromosomal aberrations, micro nuclei induction with an increase in mitotic index assay. The -SH

group present in DAS has been implicated in its antimutagenic effects. The proposed mechanism of protection involves scavenging of potentially toxic and mutagenic electrophiles and free radical modification of phase I and II enzymes and thus enhances the detoxification pathways [51]. Already the *invivo* and *invitro* free radicals scavenging potentiality of DATS against As induced ROS has been reported by Sumedha and Milton [33].

DATS, a lipid soluble antioxidant, is a powerful reducing agent capable of rapidly scavenging a number of ROS including $\dot{O}_2$ induced by As in rats and it is an effective antioxidant. In its action as an antioxidant, DATS can activate Nrf2 mechanisms which help to boost up the secretion of antioxidant enzymes in the As exposed rat [52]. Therefore rats supplemented with DATS show reduced DNA damage as visualized as comet tail moment and tail length due its free radical scavenging effects when compared with As exposed rats and the result is accordance with our earlier lab reports.

8-OHdG is novel biomarkers for As-induced genotoxicity and oxidative stress. Nain and Smits reported that the increased levels of 8-OHdG levels in plasma can lead to genetic damage with time in As exposed rats. In our study also we found the increased levels of 8-OHdG in plasma of As treated rats and it is similar with the reports of Nain and Smits [53]. Increased in the levels of 8-OHdG is due to the oxidative stress induced by the As [42]. Surprisingly DATS supplementation to As exposed rats significantly reduced the 8-OHdG level in rats plasma when compare with As alone exposed rats and it is due to its antioxidant activities and ability to ameliorates oxidative stress induced by As [34].

Conclusions

The organosulfur compound, DATS have acted as ameliorating agents against As induced genotoxicity. DATS when administered prior to As treatment show positive impacts by reducing chromosomal aberration, micronucleic induction, DNA damaged and an incline mitotic index. Our study suggested that DATS can be an effective agent for the treatment of As induced oxidative stress because of its ability to counter the genotoxicity of As and it is supported with the decreased level of genotoxicity marker 8-OHdG after it supplementation.

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