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Vitamin C pretreatment mitigates the genotoxic effects of sodium arsenite in human lymphocytes *in vitro*

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ABSTRACT

The present study was aimed to evaluate Vitamin C (VC) as a potent natural antioxidant to reduce the genotoxic effects of sodium arsenite in human lymphocytes. The study was design to assess the role of VC (10 µg/ml), upon sodium arsenite induced *in vitro* genotoxicity by using alkaline comet assay technique. Lymphocytes were incubated for 3h at 37ºC in RPMI-1640 medium with different concentrations of sodium arsenite (12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml) and also in combined treatment of sodium arsenite with VC (100 µg/ml + 10 µg/ml).Our findings suggest that treatment with sodium arsenite increased the primary DNA damage and pretreatment with VC reduces the genotoxic effects of sodium arsenite effectively and efficiently at the highest concentration *in vitro*, proving its mitigating effect.

1. INTRODUCTION

Arsenic is well known water contaminant raising several health issues throughout the world [1]. Exposure to arsenic occurs via the ingestion, inhalation, dermal contact and the parenteral route to some extent. Humans can be exposed to arsenic through the intake of air, food and water [2]. Arsenic has been considered as a potent toxicant and carcinogenic to humans [3]. Long-term exposure to arsenic-contaminated water causes a wide range of adverse health effects, including skin pigmentations, keratosis, vascular diseases, and conjunctivitis in the eyes, neuropathy, lung diseases and different types of cancer [4]. Earlier works on arsenic clearly indicate the genotoxic potential of arsenic both *in vivo* and *in vitro* [5, 6].*In vitro* exposure of arsenic caused primary DNA damage in human peripheral blood lymphocytes [7]. The toxicity of arsenic depends on its chemical state. Arsenic can occur in the environment in several oxidation states (-3, 0, +3 and +5) but in natural water mostly found in organic form as oxyanions of trivalent arsenite (AS III) or pentavalent arsenic (ASV) [8]. Inorganic arsenic in its trivalent form is more toxic than penatvalent arsenic. Sodium arsenite is pentavalent form of inorganic arsenic species. Most studies show that inorganic arsenic is genotoxic and cytotoxic *in vitro* and *in vivo* [9]. The property of VC to increase the activity of glutathione (GSH) reductase, superoxide dismutase (SOD) and catalase (CAT) in swiss albino mice has been implicated to possibly play a protective role against chemical toxicity [10].VC is the most widely used antioxidant and prevents oxidative damage to the cell membrane induced by radicals [11].

VC can protect the body from damage by ROS that can be generated during normal metabolism and other exposure like toxins and carcinogens. Previous work has reported the use of VC to combat arsenic-induced genotoxicity [12, 13].The present study was designed to confirm the genotoxic potential of inorganic arsenic *in vitro* and vitamin c was selected as the most potential natural antioxidant to prevail over arsenite induced genotoxicity

2. MATERIALS AND METHODS

2.1 Chemicals

Sodium arsenite (CAS No. 7784-465), Phosphate buffered saline (PBS), trizma base were obtained from Hi-Media Ltd., Mumbai, India. Histopaque was purchased from Sigma-Aldrich Co. (USA).Vitamin C (CAS No.50-81-7), Normal melting point (NMP) agarose, low melting point (LMP) agarose, di-sodium salt of ethylene diamine tetra acetic acid (EDTA), Tris buffer, ethidium bromide (EtBr), thiobarbituric acid (TBA), trichloroacetic acid (TCA),Triton X-100, DMSO, sodium hydroxide, sodium chloride, acetic acid and methanol were obtained from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India).

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2.2 Sample Collection and Isolation of lymphocytes

Heparinised vacutainers were used for collection of human peripheral blood from healthy volunteers. Lymphocytes were isolated from fresh blood by using Histopaque [14].The cells were washed with PBS and resuspended in RPMI-1640 media at a concentration of 10^6 cell mL^1 for further use. This experimental study was approved by the Ethics Committee of Calcutta University.

2.3 Experimental design

The study included seven groups and presented in Table 1. In all the groups the exposure time for test chemical was 3 hr.

2.4 Treatment of the lymphocytes

Based on the results of the viability test 100 µg/ml the concentration of sodium arsenite was selected as the LD_{50}; as at this concentration approximately 50% of the cells died during the 3 hours of treatment. Therefore further all investigations were carried out using a concentration of 100 µg/ml sodium arsenite.

Incubation of freshly isolated lymphocytes was done at 37 ºC for 3h in RPMI-1640 media with different concentrations of sodium arsenite (12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml). Following treatment, the lymphocytes were processed for detection of DNA damage by alkaline comet assay.

2.5 Trypan blue dye exclusion method

Cytotoxicity was determined by trypan blue dye exclusion method [15].Viable cells were counted in a Neubauer chamber under a light microscope.

2.6 Comet assay *in vitro*

The DNA damage were studied by alkaline comet assay [16, 17]. Slides were prepared in triplicates per concentration. Slides were immersed in cold lysis solution at p^H 10.The lysis solution consisted of 2.5M NaCl, 100mM Na_2EDTA,10mM Trizma base, 1% TritonX 100, 10% DMSO and kept at 4ºC for 60 min. After lysis the DNA was allowed to unwind in the electrophoresis buffer (300 mM NaOH: 1mM Na_2EDTA at p^H 13.5) for 20 min. This was followed by electrophoresis conducted at a constant voltage of 25V and 300 mA at 4ºC. Slides were neutralized in 0.4M Tris (p^H 7.5) for 5 min and finally rinsed in water. Each experiment was repeated thrice.

2.7 Scoring of slides

EtBr (20 μgmL^{-1}) was used for staining the slides and rinsing the slides in water to wash off excess stain. Slides were scored using image analysis system (Kinetic imaging; Andor Technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany). The microscope was connected to a computer through a charge coupled device (CCD) camera to transport images to software (Komet 5.5) for analysis. The final magnification was 100X. Among the comet parameters we report the percent tail DNA, tail extent moment and olive tail moment. This will give us a clear indication of the extent of DNA damage induced by the test chemical. Images of 150 (50X3) cells per concentration were analysed for human lymphocytes. The median values of each concentration with respect to the comet parameter were calculated.

2.8 Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the % Tail DNA, Tail Extent Moment and Olive Tail Moment data at different concentration treatment. All the data were expressed as the mean ± standard deviation (SD). Pair wise comparison between the study groups was done using Tukey's multiple comparison tests. The level of significance was taken as $P<0.05$.

3. Result and Discussion

3.1 Cell Viability by Trypan blue dye exclusion method

Human lymphocytes were treated with different concentrations of Sodium arsenite (12.5, 25, 50 and 100µg/ml), VC (10µg/ml) and highest dose of sodium arsenite with VC (100µg/ml + 10µg/ml) as described earlier (Table 1).The results of cell viability assays after incubation of lymphocytes with the sodium arsenite at different concentrations are displayed in Table 2. The cutoff point was 70% [18]. Viability was measured both before and after treatment and range of viable cells was within 75–90% in all experiments. The viability varied between 95% in control set to 81-86% at different treatment (12.5, 25, 50 and 100µg/ml) of sodium arsenite. The cell viability decreased with increase in the concentration of sodium arsenite as compared with control ($P<0.001$). In the combined treatment of VC at concentration of 10 µg/ml and highest concentration of sodium arsenite 100 µg/ml in the culture increased the cell viability ($P<0.001$) and proving the ameliorative effect of VC presented in (Table 2).

Table. 1: Description of the experimental design.

Experimental groups	Treatment	Doses
Group I	Control	
Group II	Sodium arsenite (NA1)	NA1 (12.5µg/ml)
Group III	Sodium arsenite (NA2)	NA2 (25µg/ml)
Group IV	Sodium arsenite (NA3)	NA3 (50µg/ml)
Group V	Sodium arsenite (NA4)	NA4 (100µg/ml)
Group VI	Vitamin C (VC)	VC (10µg/ml)
Group VII	Sodium arsenite + Vitamin C (NA4+VC)	NA4 (100µg/ml) + VC (10µg/ml)

Table. 2: Effect of the sodium arsenite and mitigating effects on viability of human lymphocytes measured by the trypan blue dye exclusion method.

[Values are mean ± SD]		
Groups	Treatment	Viability (%)
Group I	Control	95.63 ± 1.97
Group II	NA1	95.05 ± 1.34^{abc}
Group III	NA2	$79.78 \pm 2.03^{***a}$
Group IV	NA3	$77.00 \pm 1.63^{***b}$
Group V	NA4	$56.34 \pm 0.73^{***cd}$
Group VI	VC	96.01 ± 0.41
Group VII	NA4 + VC	$87.05 \pm 4.88^{***d}$

P Values: $^{***}P<0.001$ compared to control group, $^{a,b,c,d}<0.001$ having similar superscripts in the respective columns are significantly different from each other.

3.2 Analysis of DNA damage by using comet assay

This study includes seven groups presented in Table 1.To study the dose dependent genotoxic effect induced by Sodium arsenite, four increasing doses were selected and comet assay was performed as endpoint of genotoxicity assessment. To study the beneficial effect of VC, the highest dose of sodium arsenite was given in VC pretreated lymphocytes as summarized in Table 3. A dose dependent increase in % tail DNA, Comet length and olive tail moment was observed, all of which were statistically significant when compared to untreated control lymphocytes. Increased percent DNA in comet tail ($P<0.01$; $P<0.001$), tail extent moment ($P<0.01$), olive tail moment ($P<0.01$; $P<0.001$) visibly indicated sodium arsenite induced DNA damage at the given treatment. This DNA damage by sodium arsenite escorted with the decreased percent DNA in comet tail ($P<0.05$) and olive tail moment ($P<0.01$) when supplemented with VC (10 µg/ml) in cultures. These findings visibly indicated protective action of VC against sodium arsenite induced DNA damage (Table 3).

Table. 3: Comet parameter: % DNA in tail, tail extent moment and olive tail moment in human lymphocytes of control, sodium arsenite, vitamin C alone, and combined treatment of both sodium arsenite and vitamin c as measured by comet assay.

[Values are mean ± SD]

Treatment	% Tail DNA	Tail Extent Moment	Olive Tail Moment
Control	1.38 ± 0.30	0.03 ± 0.01	0.06 ± 0.02
NA1	2.73 ± 0.89^{a}	0.07 ± 0.03^{b}	0.10 ± 0.02^{a}
NA2	4.23 ± 1.55**	0.16 ± 0.02	0.14 ± 0.05**
NA3	5.13 ± 1.08**	0.25 ± 0.05	0.19 ± 0.01**
NA4	8.49 ± 1.17^{***ab}	0.32 ± 0.04^{**b}	0.27 ± 0.03^{***a}
VC	1.55 ± 0.05	0.02 ± 0.01	0.04 ± 0.01
NA4 + VC	6.28 ± 0.39^{*b}	0.26 ± 0.06	0.25 ± 0.03^{**a}

P Values: $^{***}<0.001$; $^{**}<0.01$; $^{*}P<0.05$ compared to control group, $^{b}<0.05$; $^{a}<0.01$ values having similar superscripts in the respective columns are significantly different from each other.

4. DISCUSSION

The present study focuses on sodium arsenite induced genotoxicity and also the protective effect of VC on the lymphocytes *in vitro*. The enigma associated with arsenic is that it is point mutations and shows clastogenic effects in both animals and humans [19].Epidemiological data gathered for more than a century have shown that arsenic and its species is a potent human carcinogen. Animal bioassays have failed to show any evidence of carcinogenesis. Arsenic is documented as a paradoxical human carcinogen that interacts indirectly with DNA [19].

In the current study, inorganic arsenic apparently caused dose-dependent increase in genotoxic indices and also cellular injury.VC is known to be an antioxidant and is able to scavenge free radicals. Earlier work suggests that VC and Vitamin E (VE) has proved evident as efficient drug against arsenic mediated toxicity [20].Previous study confirmed the protective role of VE and VC on nickel induced liver damage, through alteration of liver marker enzymes and both vitamins have attenuated the toxic effects of nickel [21].VC also helps to prevent atherosclerosis through the synthesis of collagen in the arterial wall and prevent undesirable adhesion of leukocytes to the damaged artery [22]. Oral co-administration of rutin and ascorbic acid attenuates hypercholesterolemia-induced hepatic toxicity in female rats by decreasing liver enzymatic and lipid peroxidative markers as well as increasing the antioxidative cascade [23]. VE is an important lipid soluble antioxidant present in cells, as it is the major chain terminating antioxidant in biological membranes [24]. Previous *in vivo* and *in vitro* studies indicate that curcumin possesses antioxidant and anticarcinogenic properties [25, 26]. Earlier work indicates towards the protection of lymphocytes from toxic effects of arsenic by melatonin *in vitro* [27]. A recent study revealed that curcumin supplementation protects from *in vitro* genotoxic effects of arsenic in human lymphocytes [28].

Although how inorganic arsenic induces genetic injury is not fully explained, oxidative damage is likely involved in arsenic-induced DNA strand breaks, MN and even apoptosis in a variety of cell systems [29]. Ameliorative action of VC was observed against toxic effects of arsenic and Sodium fluoride, by reducing oxidative stress [30]. Arsenic induces reactive oxygen species (ROS) leading to oxidative stress and carcinogenesis [31]. It has been reported that arsenic exposure can result in increased cellular reactive oxygen species and oxidative DNA damage [32]. It has not been proven that inorganic arsenic reacts directly with DNA, but it has been suggested that arsenite induces oxidative damage causing single and double-strand breaks [33], although oxidative stress is only one of the possible mechanisms involved in arsenic genotoxicity.

Our study reveals the *in vitro* cytotoxicity of sodium arsenite in human lymphocytes. The Trypan blue dye exclusion test showed cytotoxic response in sodium arsenite treated human lymphocytes *in vitro* and indicating possible induction of mitochondrial damage by sodium arsenite. In the present study percent of cell viability decreased in all the doses of sodium arsenite treated lymphocytes. In the combined treatment of VC (10µg/ml) and sodium arsenite (100µg/ml) in the culture increased the cell viability and proving the positive effect of VC.

We found that VC at 10µg/ml significantly protected DNA of normal human lymphocytes against primary DNA damage induced by sodium arsenite at highest concentration (100 µg/ml).In this study,VC supplementation caused radical amelioration in the genotoxic effects brought about by sodium arsenite.This data thus signifies that VC has a definitive protective action against inorganic arsenic exerted genotoxicity (Table 3).

A recent study revealed the ability of arsenic and its metabolites to cause cytotoxic effects and primary DNA damage in human lymphocytes, when exposed *in vitro* for 24h, assessed by single cell gel electrophoresis as analyzed in the our study [34].VC has antioxidant and free radical scavenging activities, which suggests that this vitamin may modulate oxidative DNA damage in mammalial cells [35]. Earlier finding shows that VC supplementation exhibits anti-hyperuricemic, anti-oxidation and nephroprotective activity in rat model of hyperuricemia-induced oxidative damage and renal injury [36].It is observed that VC may

have hepatoprotective effect [37]. Arsenic contamination in ground water has become a major problem in many countries along with India. The problem is increasing day by day because there is no medicine that can prevent or cure arsenicosis. There is only measure to combat with arsenic related problems by providing arsenic-free water. Vitamins with anti-oxidant property viz., ascorbic acid found mainly in fresh vegetables can also be thought of as a dietary supplement. Our study has shown that sodium arsenite is a potent genotoxic agent capable of inducing DNA damage and also supported the beneficial effect of vitamin C in human lymphocytes *in vitro*.

5. CONCLUSION

Thus in view of present findings and previous works; we conclude that VC successfully ameliorates the *in vitro* genotoxic effects of sodium arsenite in human lymphocytes and clearly indicating its antioxidant property.

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