

## Alpha-Tocopherol ameliorates arsenic induced nephrotoxicity in Wistar rats

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$\alpha$ -Tocopherol's (Vitamin E) antioxidant and anti-inflammatory properties may help reduce the progression of fibrosis in kidney by limiting tissue damage and inflammation induced by arsenic. Knowledge of the mechanisms of action of natural medicinal substances in arsenic toxicity will be improved by the analysis of the ameliorative effects of  $\alpha$ -tocopherol. The goal of the current investigation was to determine whether Vitamin E can protect rats from nephrotoxicity caused by sodium arsenite ( $\text{NaAsO}_2$ ). Twenty-five Wistar rats were split into five groups viz: Group I with distilled water as control; Group II-IV with 8.4 (Low dose)/12.3 (Moderate dose)/16.4 mg/kg  $\text{NaAsO}_2$  (High dose); and Group V as in Gr. IV + 50 mg/kg  $\alpha$ -Tocopherol. Both the doses were administered orally to rats for 60 days.  $\alpha$ -tocopherol decreased the concentration of serum parameters like urea nitrogen (UN) and creatinine (CRT) whereas increased the concentration of albumin (ALB), acid phosphatase (ACP), alkaline phosphatase (ALP) and succinic dehydrogenase (SDH) ( $P < 0.05$ ). In comparison to control group, the transcript levels of p53 were significantly higher in the LDG, MDG, and HDG rats, respectively, by  $\sim 0.7$  fold,  $\sim 0.4$  fold, and  $\sim 0.5$  fold. Similar to this, p21 transcript levels were higher in LDG, MDG and HDG groups than in those from the control group by  $\sim 0.2$  fold,  $\sim 0.4$  fold and  $\sim 0.6$  fold, respectively. Additionally, as compared to rats in the control group, the levels of p27 transcripts were decreased by  $\sim 0.5$  fold,  $\sim 0.5$  fold, and  $\sim 0.4$  fold in the LDG, MDG, and HDG rat populations, respectively. Co-administration of  $\alpha$ -tocopherol with  $\text{NaAsO}_2$  showed decreased mRNA expression of p53 and p21 followed by increased mRNA expression of p27. In this investigation, it was discovered that  $\alpha$ -tocopherol had a protective effect against renal damage brought on by  $\text{NaAsO}_2$ .

**Keywords:** DNA damage, Heavy metal contamination, Kidney, Oxidative stress, Soil pollution, Water pollution, Vitamin E

The World Health Organization (WHO) has designated Arsenic, an element in group-15 of the periodic table, as one of the ten substances of most public health concern<sup>1</sup>. The maximum tolerated value for arsenic in drinking water is  $10 \mu\text{g}/\text{L}^2$ . Mortality rates caused by drinking groundwater containing arsenic are very high, particularly in Bangladesh, China and India<sup>3</sup>. Increases in oxidative radicals ( $\text{HNO}_3$ ,  $\text{O}_2$ ,  $\text{OH}$ ) which alter cellular architecture, permeability, and cell viability, have been linked to arsenic exposure<sup>4</sup>. Bladder cancer risk is enhanced as a result of arsenic's promotion of tumor suppressor gene mutations, including those involving tumor proteins like P53 (TP53)<sup>5</sup>. The kidney is the main organ targeted by arsenic-mediated toxicity since it eliminates arsenic through urine (60%)<sup>6</sup>. Chronic arsenic exposure in the kidney has been associated with glomerular hypertrophy, interstitial nephritis, and tubular cell vacuolation<sup>7</sup>. A study claimed that the

development of arsenic-induced nephrosis and death of tubular cells of kidney, which results in weight loss, depend heavily on oxidative stress<sup>8</sup>. Experimental studies have identified indicators of oxidative stress in patients with renal failure<sup>9,10</sup>. Arsenic exposure caused mice to have significantly lower amounts of D-serine, reduced Serine Racemase expression, and elevated D-amino acid oxidase (DAAO) protein expression<sup>11</sup>. Albuminuria, Chronic kidney disease (CKD) and proteinuria are caused due to DNA damage and blockage of blood vessels brought on by arsenic<sup>12</sup>. The proximal convoluted tubule and bowman's capsule of mice exposed to arsenic showed histopathological abnormalities, an increase in DNA strand breakage and an increase in 8-hydroxy-2-deoxyguanosine (8-OHdG) expression<sup>13</sup>.

In a laboratory experiment, antioxidants were found to reduce arsenic-induced DNA damage (ROS)<sup>14</sup>. A class of eight structurally similar molecules, including alpha, beta, gamma, and delta tocopherol and its equivalent tocotrienols, is referred to as Vitamin E<sup>15</sup>. Research indicates that alpha and gamma tocopherol have significant antioxidative and

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anti-inflammatory qualities and may be able to prevent chronic disease states linked to elevated oxidative stress and inflammation<sup>16</sup>. Vitamin E or  $\alpha$ -tocopherol, aids in the conversion of GSSG (oxidized glutathione) to GSH (reduced glutathione), which in turn aids in the conversion of mono and dehydroascorbic acid to ascorbic acid. Additionally, it improves the cell's capacity for antioxidants and inhibits the production of disulfide from unbound or protein-bound sulfhydryls<sup>17</sup>. Because vitamin E donates hydrogen, it inactivates free radicals and acts as the most effective antioxidant on the market for preventing the oxidative damage caused by heavy metals<sup>18</sup>. Arsenic-induced damage was greatly reduced when antioxidants like Ascorbic acid and  $\alpha$ -tocopherol were given together with arsenic<sup>19</sup>.

In the kidney of male albino rats, ascorbate and  $\alpha$ -tocopherol specifically modulated the degree of DNA damage by lowering TNF- $\alpha$  and prevented the caspase cascade from activation<sup>20</sup>. In adult male rats, co-administration of Vitamin C and Vitamin E effectively reduced hematological and hepatic changes induced by arsenic<sup>21</sup>. Rats receiving vitamin E supplements had more active spermatogenesis<sup>22</sup>. Vitamin E and selenium increased intracellular glutathione and super oxide dismutase which reduced free radical-induced lethal damage and checked upon lipid peroxidation<sup>23</sup>. A 250 IU/kg dose of vitamin E supplementation decreased the tissue deposition of arsenic and shielded kids from the oxidative stress caused by arsenic<sup>24</sup>. Supplementation with  $\alpha$ -tocopherol reduced the harmful effects of sodium arsenite exposure on ovarian tissue in rats<sup>25</sup>. Co-administration of Vitamin C and Vitamin E caused increased levels of luteinizing hormone, follicle-stimulating hormone, and estradiol in mice treated with NaAsO<sub>2</sub>. The antioxidants also decreased DNA damage and oxidative stress in the ovarian tissue<sup>26</sup>. In rats with contrast-induced nephropathy, renal function and malondialdehyde levels were significantly reduced in alpha-tocopherol group, while renal SOD was significantly increased. Remarkably, this group showed protection of tubular damage and both a rise in total antioxidant capacity when compared to the control group<sup>27</sup>. The effects of sodium arsenite on sperm quality were effectively mitigated by selenium and vitamin E, while also preserving heart and respiratory rates and enhancing odor in male goats<sup>28</sup>. Therefore, here, we investigated the effects of sodium arsenite (NaAsO<sub>2</sub>) on p53, p21 and p27 levels and the

ameliorative role of  $\alpha$ -tocopherol in rectifying the kidney damage caused by arsenic in a rat model.

## Materials and Methods

### Chemicals

Triton X-100,  $\alpha$ -tocopherol, EDTA and sodium arsenite (NaAsO<sub>2</sub>) were purchased from Merck (Germany). Agarose, Tris-HCl, thiobarbituric acid (TBA) and sodium dodecyl sulphate (SDS) were procured from Sigma (St Louis, MO, USA). Accurex Biomedical Pvt. Ltd. provided the kits for the kidney function test. All additional chemicals and reagents were purchased from BDH chemicals (Mumbai), Sigma (USA) and Merck (Germany). To prevent metal contamination, the experiment employed ultra-pure water made by Millipore which was also used to make the reagents and buffers needed for the study of different biochemical assays.

### Animal maintenance

Female Wistar rats (3-4 months old) were purchased from Bilwal Medchem and Research Laboratory Pvt. Ltd., H-9, SKS, RIICO industrial area, Reengus, Sikar, Rajasthan, and maintained in the Animal House facility at the Department of Zoology, University of Rajasthan, Jaipur. The period of acclimatization was for 14 days prior to use. The animals were kept in polypropylene cages at 25°C temperature and 50-65% of relative humidity. Rats were kept hydrated at all times during the trial and fed regular diet from Hindustan Lever Limited, Delhi. The Institutional Animal Ethics Committee (IAEC), Department of Zoology, University of Rajasthan, India (UDZ/IAEC/II/01; Dated: 18/12/2021), gave its approval to the current study project.

### Experimental design

A total of twenty-five adult and healthy female Wistar rats (age: 3-4 months, body weight: 80-120 g) were used for the study. After 14 days of acclimatization, twenty-five animals were randomly divided into five groups of five rats each. These groups were designated as Control, low-dose, medium-dose, high-dose and high-dose +  $\alpha$ -tocopherol. When administered orally to Wistar rats, sodium arsenite's estimated LD<sub>50</sub> is 41g/kg body weight<sup>29</sup>. Animals in group V received oral administration of  $\alpha$ -tocopherol at 50 mg/kg body wt. dose level. The rats received dosages via oral gavage throughout the experiment. Rats in the Control group received distilled water. Rats were given their corresponding doses orally every day for 60 days and were

subdivided into 5 groups: Gr. I: Control rats treated with distilled water (vehicle); Gr. II-IV: Low/Moderate/High dose (LDG/MDG/HDG) treated with 20, 30 or 40% of LD<sub>50</sub> i.e. 8.2 or 12.3 or 16.4 mg/kg/day; and Gr. V: as in Gr. IV +  $\alpha$ -tocopherol 50 mg/kg/day.

Animals were sacrificed by cervical dislocation after receiving the prescribed dose for two months and measurements of body weight and different organs were taken. For serum and biochemical analysis, blood was taken through cardiac puncture and collected in tubes. After being centrifuged at 1000×g for 10 min, serum was collected and kept in aliquots at -20°C. Kidneys were utilised for metal analysis after being cleaned in cold saline, blotted and weighed. Each rat's kidney was split in half, with half processed right away for genotoxic parameters and the other half stored at -20°C for biochemical assays & histological examination.

#### DNA damage parameters

##### *DNA fragmentation assay*

DNA fragmentation assay in renal tissue was performed using the method as described<sup>30</sup>. The tissue sample was homogenized in 10 mL of lysis buffer containing 10 mM Tris-HCl, 1 mM EDTA, 0.2% Triton X-100 at pH of 8. The homogenate was maintained in a pre-warmed water bath for 2.5 h at 50°C. At 4°C for 15 min, the cell lysate was centrifuged at 13000×g. DNA was extracted from one portion of the supernatant (S) and electrophoresed for 1 h on 1.8% agarose gel with ethidium bromide (0.5 g/mL) to examine DNA fragmentation in the control and experimental samples. With 10% TCA, the second portion of the supernatant was precipitated. In a lysis buffer, the original 13000×g pellet (P) was disintegrated. Supernatant (S) and pellet (P) fractions received 10% TCA solution. Both were held at 35°C for 10 min followed by centrifugation at 2000×g for 10 min. DNA was extracted from pellets using 5% TCA at 90°C for 15 min followed by centrifugation at 2000×g for 15 min. By doubling the amount of the diphenyl amine (0.088 mole/mL DPA in 98% (v/v), glacial acetic acid with 1.5% conc. H<sub>2</sub>SO<sub>4</sub>) reagent, DNA concentration in supernatants was calculated. The samples were stored in the dark for 20 h, and absorbance at 600 nm was noted. DNA fragmentation was expressed as a percentage using the formula (S/S+P) × 100.

##### *Alkaline comet assay*

One mL of ice cold phosphate buffer saline (50 mM, pH 7.4) was used to mince 10 mg of kidney tissue,

and the cell suspension was then filtered over a 100  $\mu$ m nylon membrane. At 37°C, 20 mL of cell suspension and 80 mL of 0.75% low melting agarose were combined, and the resulting mixture was pipetted onto a slide covered with 1% normal melting agarose. Slides without cover slip were placed in a cold lysis solution composed of 100mM EDTA, 10 mM Tris-HCl, 10% DMSO, 1% Triton X-100, 2.5 M NaCl at 4°C (pH=10) for 2.5 h after the agarose layer had solidified. To unwind the DNA and express the alkali-labile sites, the slides were dipped in cold electrophoresis buffer composed of 1 mM EDTA, 0.3 M NaOH at pH of 13 for 20 min. At 20 V and 300 mA for 20 min at 4°C, electrophoresis was done on a horizontal gel electrophoresis platform. A buffering agent was used to neutralize the slides (0.4 M Tris-HCl, pH 7.5). The slides were then covered with cover slip after being stained with 20 mg of ethidium bromide (2 mg/mL). The assay was carried out in total darkness<sup>31</sup>.

##### *Comet capture and analysis*

Slides were examined at 400X magnification under fluorescent microscope equipped with dual excitation filters. The images were then analyzed using Comet Score Software (Tri Tek Corp., Sumerduck, US). The DNA content, tail length and moment were examined to gauge the degree of DNA damage. The tail length, which is measured in pixels, represents the distance that DNA migrates out from the nucleus and is inversely proportional to the size of the DNA fragment. The amount of DNA that has migrated from the nucleus to the tail is represented by intensity of tail. Tail moment is the product of the percentage of the total DNA that makes up the tail and length of the tail.

#### Measurement of lipid peroxidation

Free radicals have the ability to oxidize poly-unsaturated fatty acids (PUFA), producing lipid peroxides as a result. The most prevalent aldehyde by-product of PUFA lipid peroxidation is malondialdehyde (MDA). On the basis of how it reacts with thiobarbituric acid (TBA) in an acidic media, it is often quantified. Butylated hydroxytoluene (BHT), 1% (w/v) was added to samples to prevent auto-oxidation during heating. A sample comprising 500 g of proteins and 50-100 mL was obtained for the LPx assay. Next, 950 mL of the LPx cocktail (9.3 mL of autoclaved DD water, 16.2 mL of 8.1% SDS, 37.5 mL of 20 % Acetic acid, 30 mL of 1% TBA and 2 mL of 1% BHT) were added to it. The mixture was

thoroughly combined before being heated for one hour at 95°C in a pre-warmed water bath. The tube was heated and then centrifuged for 15 min at 1000×g. In a spectrophotometer, the supernatant's (reddish-pink colour) absorbance was measured against an appropriate blank at 532 nm. The concentration of Thiobarbituric acid reactive substance (TBARS) was determined using its extinction coefficient ( $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ). The result was expressed in terms of nmol TBARS produced per mg protein<sup>32</sup>.

#### **Estimation of arsenic in renal tissue using atomic absorption spectroscopy**

All glasswares were cleaned with 10% HNO<sub>3</sub> followed by washing with distilled water. A well-mixed 1g test amount was weighed and transferred to a 250 mL beaker. It was then heated to 95°C on a hot plate with the addition of HNO<sub>3</sub> (210 mL). The beaker was then covered with watch glass. The digestion continued until there were no brown fumes present and the solution was clear and colourless. To reach room temperature, the beaker was cooled. The solution was poured into a volumetric flask, diluted with double the amount of distilled water, and then analyzed by PG Instruments' AA500 spectrophotometer.

#### **Biochemical analysis of kidney function (Renal function test)**

Measurement of Urea, Creatinine, Albumin, acid phosphatase (ACP), alkaline phosphatase (ALP) and Succinic dehydrogenase (SDH) was performed using commercially available kits from Accurex biomedical Pvt Ltd, Chandigarh, India. Multiskan GO Microplate Spectrophotometer by Thermofischer Scientific was used for spectrophotometric analysis.

#### **Histological analysis**

In brief, kidney tissue was cut into small pieces and put in fixative (sublimate formol) for 24 hours. After fixation, it was kept under running water for 24 hours and dehydrated in 30%, 50% and 70% alcohol for 30 minutes. It was kept in iodine ethanol for 30 min to remove traces of fixative and transferred to 70 % and 90% alcohol. After this, tissue was kept for 2 hours in absolute alcohol with two changes (one change/1 hour). After dehydration, alcohol was cleared from tissue following two changes of xylene for 10 minutes each and then kept for 3 hours (one change/1 hour) in melted wax at 60°C. 'L-mould' paraffin blocks were prepared using melted wax-paraffin mixture. Tissue sections were cut at 5 µm with microtome and spread on slides coated with albumin. The slides were kept in xylene for de-paraffinization and rehydrated in a

downgraded series of ethanol (absolute, 90%, 70%, 50% and 30%) for 10 minutes each. After washing with double distilled water, sections were stained with hematoxylin for 2 minutes and washed carefully. Sections were then dehydrated with an up-graded series of alcohol (30%, 50, 70 and 90%) for 5 min each, stained with eosin for 15 minutes and dehydrated in 2 changes of absolute alcohol. Slides were cleared with two changes of xylene. Finally, sections were mounted in DPX and covered with glass cover slip and left for drying under light. Photographs of the sections were captured in Light microscope (DM 1000, Leica Microsystems, Germany) and photographed by attached digital camera (DFC 450C).

#### **RT-PCR for determination of p53, p21 and p27 gene expression**

Utilizing the Trizol reagent, total RNA was extracted from the kidney. To investigate the mRNA expression of p53, p21, and p27, the extracted RNA was utilized for RT-PCR. The standard RT-PCR procedure was used on 7500 Real Time PCR system (Applied Biosystems) & Aria MX Real Time PCR System with 96 well block module (Agilent Technologies, Santa Clara, USA). The primer sequences with reaction conditions used were as follows: p53- forward 'CTACTAAGG TCGTGAGACGCTGCC' reverse 'TCAGCATAACAGG TTTCTTCCACC' p21- forward 'TAGGACTTCGG GGTCTCCTT' reverse 'GCTCTGGACGGTACGCT TAG' p27- forward 'GAGGGCAGATACGAGTGG CAG' reverse 'CTGGACACTGCTCCGCTAACC'. The reaction composed of an initial 15 min of enzyme activation (95°C), 40 cycles of denaturation (95°C), annealing (59°C) and a final extension (72°C) for 5 min. The Cycle Threshold method was used to estimate the expression levels in three PCR replicates of five animals<sup>33</sup>.

#### **Statistical analysis**

The data is depicted as the mean and standard deviation for five animals. A one-way ANOVA was used to establish the degree of significance, along with the Tukey HSD test. At  $P < 0.01$ , a difference was deemed statistically significant.

#### **Results**

##### **Effects on body weight & organ weight (Initial body weight, Final body weight, and Relative kidney weight)**

For arsenic-treated rats, the body and organ weights were significantly reduced. There was no significant difference in the weights of arsenic +

$\alpha$ -tocopherol treated rats with the rats of control group. Body weight and relative kidney weight of rats of five Groups are presented in Table 1. In contrast with Group I, the final body weights of the rats in Groups II, III, and IV were significantly lowered by 27, 32 and 31.8%, respectively. Rats in Gr. V displayed a less significant difference (19%) from Gr. I, respectively. When compared to Gr. I, Gr. II & III, and the relative kidney weights of Gr. IV were all significantly lowered by 20, 34 and 33%, respectively.

#### Protective effect of $\alpha$ -tocopherol on clinical serum parameters and renal function biomarkers

In relation with control rats, the levels of kidney markers significantly increased indicating arsenic-induced kidney damage. In contrast to the arsenic-treated group, the serum levels of albumin were significantly reduced in arsenic +  $\alpha$ -tocopherol group. The levels of blood urea, creatinine and albumin are presented in Table 2. In contrast to Group I, the blood urea levels of Group II, Group III, and Group IV all considerably increased by 45, 20 and 63%,

respectively. In relation with Gr. I, Groups II, III and IV had considerably higher amounts of creatinine (59, 70 and 71%, respectively). Also, Groups II, III and IV had significantly lower albumin levels, decreasing by 6, 12 and 40%, respectively. Groups II, III and IV all had considerably lower levels of alkaline phosphatase than Gr. I, with differences of 16, 28 and 37%, respectively. In contrast to Gr. I, the levels of acid phosphatase in Groups II, III and IV were significantly lower by 17, 39 and 44%, respectively. There was no significant difference between the serum levels of BUN, CRT, ALB, ACP, ALP and SDH of Gr. I (control) rats and Gr. V (arsenic +  $\alpha$ -tocopherol) rats.

#### $\alpha$ -Tocopherol reduced NaAsO<sub>2</sub>-induced DNA damage in nephrocytes

Renal DNA damage of five groups is presented in Table 3. When compared to control rats, the comet assay parameters significantly increased in arsenic-treated groups (Gr. II, III & IV) indicating arsenic-induced kidney damage as shown in Table 3. When compared to the arsenic-treated group, the comet

Table 1 — Effect of NaAsO<sub>2</sub> and  $\alpha$ -tocopherol on body and organ weight

Parameter	Group I	Group II	Group III	Group IV	Group V
Initial body wt.	112±0.35 <sup>a</sup>	109.2±0.27 <sup>b</sup>	120±0.34 <sup>c</sup>	128.4±0.28 <sup>d</sup>	116±0.36 <sup>a</sup>
Initial body wt.	135±2.80 <sup>a</sup>	98±3.36 <sup>b</sup>	92±3.72 <sup>b</sup>	89±4.61 <sup>c</sup>	109±2.72 <sup>a</sup>
Relative kidney wt.	0.90±0.04 <sup>a</sup>	0.72±0.05 <sup>b</sup>	0.66±0.04 <sup>c</sup>	0.59±0.04 <sup>d</sup>	0.86±0.04 <sup>a</sup>

[Effect of NaAsO<sub>2</sub> on body weight in Wistar rats. Gr. I: Control rats (90 days old); Gr. II/III/IV: Rats treated with 8.2/12.3/16.4 mg/kg NaAsO<sub>2</sub>; Gr. V: Rats treated with 16.4 mg/kg NaAsO<sub>2</sub> along with 50 mg/kg  $\alpha$ -tocopherol. Duration of treatment was 60 days. Data are expressed as Mean ± SD with N=5. Data having different superscripts (a-d) in the same row differ significantly ( $P < 0.05$ ) from the control group]

Table 2 — Effect of NaAsO<sub>2</sub> and  $\alpha$ -tocopherol on serum & biochemical parameters

Parameters	Group I	Group II	Group III	Group IV	Group V
BUN (mg/mL)	12.56±1.48 <sup>a</sup>	22.70±2.36 <sup>b</sup>	28.50±1.18 <sup>c</sup>	34.10±1.92 <sup>d</sup>	13.70±2.11 <sup>a</sup>
CRT (mg/mL)	0.57±0.03 <sup>a</sup>	1.42±0.08 <sup>b</sup>	1.94±0.22 <sup>c</sup>	1.98±0.34 <sup>d</sup>	0.62±0.40 <sup>a</sup>
ALB (mg/mL)	4.03±0.20 <sup>a</sup>	3.89±0.15 <sup>b</sup>	3.69±0.48 <sup>c</sup>	2.68±0.31 <sup>d</sup>	4.13±0.50 <sup>a</sup>
ACP ( $\mu$ IU/mL)	134.60±3.42 <sup>a</sup>	112.32±6.98 <sup>b</sup>	96.26±5.10 <sup>c</sup>	84±8.42 <sup>d</sup>	130.23±7.11 <sup>a</sup>
ALP ( $\mu$ IU/mL)	203.05±6.31 <sup>a</sup>	167.92±8.90 <sup>b</sup>	122.83±9.34 <sup>c</sup>	114.24±8.87 <sup>d</sup>	201.36±0.03 <sup>a</sup>
SDH ( $\mu$ IU/mL)	6.02±0.78 <sup>a</sup>	5.29±0.44 <sup>b</sup>	4.83±0.23 <sup>c</sup>	3.1±0.12 <sup>d</sup>	6.36±0.03 <sup>c</sup>

[Effect of NaAsO<sub>2</sub> on renal function biomarkers in Wistar rats and its amelioration by  $\alpha$ -tocopherol. Gr. I: Control rats (90 days old); Gr. II/III/IV: Rats treated with 8.2/12.3/16.4 mg/kg NaAsO<sub>2</sub>; Gr. V: Rats treated with 16.4 mg/kg NaAsO<sub>2</sub> along with 50 mg/kg  $\alpha$ -tocopherol. Duration of treatment was 60 days. BUN: Blood urea nitrogen; CRT: Creatinine; ALB: Albumin; ACP: Acid Phosphatase; ALP: Alkaline Phosphatase. Data are expressed as Mean ± SD with N=5. Data having different superscripts (a-d) in the same row differ significantly ( $P < 0.05$ ) from the control group]

Table 3 — Genotoxic effects of NaAsO<sub>2</sub> and its amelioration by  $\alpha$ -tocopherol

Parameter	Group I	Group II	Group III	Group IV	Group V
DNA fragmentation	2.44±0.27 <sup>a</sup>	2.57±0.07 <sup>b</sup>	1.58±0.04 <sup>c</sup>	3.12±0.64 <sup>d</sup>	2.3±0.04 <sup>a</sup>
Tail length (pixel)	2.6±0.33 <sup>a</sup>	3.04±0.4 <sup>b</sup>	3.84±0.55 <sup>c</sup>	3.86±0.75 <sup>c</sup>	2.76±0.75 <sup>a</sup>
%DNA in tail	1.65±0.1 <sup>a</sup>	1.77±0.05 <sup>b</sup>	1.8±0.08 <sup>b</sup>	2.92±0.55 <sup>d</sup>	1.61±0.1 <sup>a</sup>
Tail moment	0.06±0.02 <sup>a</sup>	0.17±0.01 <sup>b</sup>	1.58±0.07 <sup>c</sup>	0.18±0.02 <sup>b</sup>	0.07±0.02 <sup>a</sup>

[Effect of NaAsO<sub>2</sub> on DNA fragmentation and Comet assay parameters in Wistar rats and its amelioration by  $\alpha$ -tocopherol. Gr. I: Control rats (90 days old); Gr. II/III/IV: Rats treated with 8.2/12.3/16.4 mg/kg NaAsO<sub>2</sub>; Gr. V: Rats treated with 16.4 mg/kg NaAsO<sub>2</sub> along with 50 mg/kg  $\alpha$ -tocopherol. Duration of treatment was 60 days. Data are expressed as Mean ± SD with N=5. Data having different superscripts (a-d) in the same row differ significantly ( $P < 0.05$ ) from the control group]

assay parameters were significantly reduced in arsenic +  $\alpha$ -tocopherol group (Gr. V) in contrast with Gr. I (control) rats indicating that  $\alpha$ -tocopherol greatly reduced arsenic induced DNA damage in the renal tissue. DNA fragmentation of nephrocytes is shown in Table 3. In contrast to Gr. I (2.44  $\pm$  0.27<sup>a</sup> AU), Gr. III had significantly more DNA damage (3.12  $\pm$  0.64<sup>d</sup> AU) ( $P < 0.05$ ). Additionally, it was discovered that rats in groups II, IV, and V had DNA damage levels of 2.57  $\pm$  0.07<sup>b</sup>, 1.58  $\pm$  0.04<sup>c</sup>, and 2.3  $\pm$  0.04<sup>a</sup>, respectively. In relation with the As-treated group,  $\alpha$ -tocopherol did not reduce DNA damage when exposed to As ( $P < 0.05$ ).

#### Effect of $\alpha$ -tocopherol on Lipid peroxidation in NaAsO<sub>2</sub>-treated cells

Lipid peroxidation (LPx) levels of five groups are presented in Table 4. In contrast to control rats, the LPx levels significantly increased in arsenic-treated groups (II, III & IV) indicating arsenic-induced kidney damage. In relation with the arsenic-treated group, the LPx levels were significantly reduced by 67% in arsenic +  $\alpha$ -tocopherol group (Gr. V) than Gr. I (control) rats indicating that  $\alpha$ -tocopherol greatly reduced lipid peroxidation in the renal tissue. LPx levels in kidney samples of Gr. IV rats was significantly higher by 78% in comparison with Gr. I rats. Similarly, rats in Gr. III had lipid peroxidation levels that were 65% greater than those in Gr. I (Table 4).

#### Effect of $\alpha$ -tocopherol on the histology of NaAsO<sub>2</sub>-treated cells

The histological evaluation of epithelial and tubular cell morphology was normal in the kidney of Gr. I rats. Glomerular enlargement (GS), tubular necrosis (TN), congestion of nephrocytes (CN) and a decrease in interstitial space (IS) were observed in NaAsO<sub>2</sub>-treated groups (Gr. II, III and IV). The morphology of

kidney sections in Gr. V rats was similar to Gr. I rats indicating that  $\alpha$ -tocopherol reduced sodium arsenite induced renal damage. Nephrocytes infiltration, dilation of sinusoids and degeneration effects were observed in the kidney. Fig. 1 (B-D) shows shrink in Bowman's capsule and Hydropic degenerations in the renal tubule. Rat tissues exposed to  $\alpha$ -tocopherol showed modest histopathological alterations, as shown in Fig. 1E. Wistar rat tissues from the control group showed no discernible histological alterations (Fig. 1A).

#### $\alpha$ -Tocopherol alters NaAsO<sub>2</sub> concentration in nephrocytes

Arsenic concentration in tissues was determined using the wet digestion process in Atomic Absorption Spectrophotometer (AAS). Group II exhibited the lowest arsenic concentration followed by Gr. III and the highest concentration occurred in Gr. IV in relation with Gr. I. Arsenic concentration was reduced in Gr. V due to the ameliorative effects of  $\alpha$ -tocopherol (Table 4).

#### $\alpha$ -Tocopherol down regulates expression of p53 and p21 gene expression and up regulates p27 gene expression

The increased mRNA expressions of p53 and p21 while decreased expression of p27 in the kidney were reported in the rats given NaAsO<sub>2</sub> treatment. Co-administration of  $\alpha$ -tocopherol with arsenic caused a significant decrease in the expression of p53 and p21 while an increase in the expression of p27 was noted in contrast to NaAsO<sub>2</sub>-treated rats (Table 5).

## Discussion

Humans are susceptible to the millions of tonnes of heavy metal pollutants that are created annually. Metal contaminants are among the compounds that

Table 4 — Effect of NaAsO<sub>2</sub> and  $\alpha$ -tocopherol on lipid peroxidation; and on arsenic concentration in kidney

Parameter	Group I	Group II	Group III	Group IV	Group V
LPx (nmol TBARS/mg protein)	1.6 $\pm$ 0.33 <sup>a</sup>	2.82 $\pm$ 0.52 <sup>b</sup>	4.54 $\pm$ 0.41 <sup>c</sup>	7.28 $\pm$ 0.63 <sup>d</sup>	4.86 $\pm$ 0.43 <sup>c</sup>
Arsenic conc (mg/kg)	3.47 $\pm$ 0.06	63.7 $\pm$ 2.23 <sup>a</sup>	88.48 $\pm$ 0.82 <sup>a</sup>	368.38 $\pm$ 1.36 <sup>b</sup>	143.97 $\pm$ 1.30 <sup>c</sup>

[Effect of NaAsO<sub>2</sub> on lipid peroxidation in the kidney of Wistar rats and its amelioration by  $\alpha$ -tocopherol. Gr. I: Control rats (90 days old); Gr. II/III/IV: Rats treated with 8.2/12.3/16.4 mg/kg NaAsO<sub>2</sub>; Gr. V: Rats treated with 16.4 mg/kg NaAsO<sub>2</sub> along with 50 mg/kg  $\alpha$ -tocopherol. Duration of treatment was 60 days. Data are expressed as Mean  $\pm$  SD with N=5. Data having different superscripts (a-d) in the same row differ significantly ( $P < 0.05$ ) from the control group]

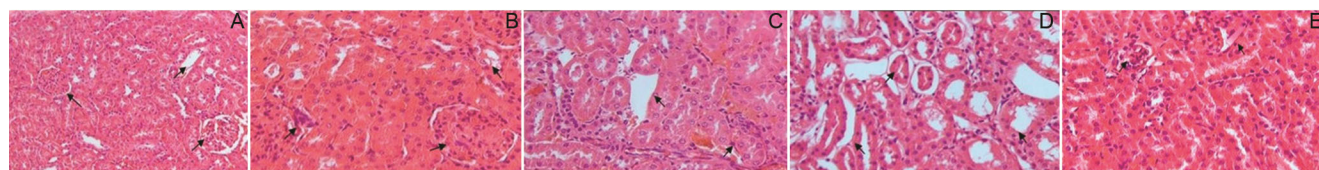


Fig. 1 — (A-E) — Effect of NaAsO<sub>2</sub> and  $\alpha$ -tocopherol treatment on transverse sections of kidney of wistar rats with hematoxylin staining. Photographs are given with 40X magnification. [(A) Group I Control rats (90 days old); (B-D) Gr. II – IV rats treated with 8.2/ 12.3 16.4 mg/kg NaAsO<sub>2</sub>, respectively; and (E) Group V rats treated with 16.4 mg/kg NaAsO<sub>2</sub> along with 50 mg/kg  $\alpha$ -tocopherol. Duration of treatment was 60 days]

Table 5 — Effect of  $\alpha$ -tocopherol on the relative mRNA expression of apoptotic genes in rats treated with NaAsO<sub>2</sub>

Gene exp.	Groups				
	I	II	III	IV	V
p53	1.0±0.0	1.51±0.10 <sup>b</sup>	2.59±0.11 <sup>c</sup>	3.3±0.02 <sup>d</sup>	1.48±0.11 <sup>a</sup>
p21	1.0±0.0	1.56±0.05 <sup>a</sup>	2.87±0.06 <sup>b</sup>	3.93±0.03 <sup>c</sup>	1.56±0.09 <sup>a</sup>
p27	1.0±0.0	2.77±0.02 <sup>b</sup>	2.43±0.09 <sup>c</sup>	2.10±0.13 <sup>d</sup>	1.45±0.10 <sup>a</sup>

[Relative mRNA expression of p53, p21 and p27 in rats treated with NaAsO<sub>2</sub> and  $\alpha$ -tocopherol. Gr. I: Control rats (90 days old); Gr. II/III/IV: Rats treated with 8.2/12.3/16.4 mg/kg NaAsO<sub>2</sub>; Gr. V: Rats treated with 16.4 mg/kg NaAsO<sub>2</sub> along with 50 mg/kg  $\alpha$ -tocopherol. Duration of treatment was 60 days. Data are expressed as Mean  $\pm$  SD with N=5. Data having different superscripts (a-d) in the same row differ significantly ( $P < 0.05$ ) from the control group]

are considered to be the most dangerous by the Agency for Toxic Substances and Disease Registry<sup>34</sup>. Heavy metal exposure is a substantial environmental health despite significant technological and governmental initiatives<sup>35</sup>. Arsenic is the primary component of about 200 different mineral species like arsenates (60%), sulphide and sulfosalts (20%) and the remaining are made up of arsenites, oxides, and elemental arsenic (20%)<sup>36</sup>. Inorganic arsenates (AsO<sub>4</sub><sup>3-</sup>) or arsenites (AsO<sub>2</sub><sup>-</sup>) are common component of air, water, food, and soil<sup>37</sup>. Metal smelting, the use of pesticides, and the burning of fuel, particularly low-grade brown coal and mineral processing, all result in occupational exposure to arsenic<sup>38</sup>. Exposure to arsenic causes lipid peroxidation which leads to DNA damage<sup>39</sup>. Several cell communication pathways are activated by arsenic that control cell growth, proliferation, and DNA repair mechanisms<sup>40</sup>. Arsenic exposure also causes an increase in DNA and protein damage as well as changes in cellular architecture, permeability and cell viability<sup>41</sup>.

Arsenic exposure may raise the likelihood of developing chronic kidney disease (CKD), which may be brought on by nephron death and compensatory alterations in healthy nephrons<sup>42</sup>. The increased levels of creatinine, BUN and lowered enzyme activities showed that sodium arsenite significantly damaged the kidney both structurally and functionally on complete levels<sup>43</sup>. It has been noted that arsenic raises the level of p53 at high doses<sup>44</sup>. Arsenic treatment caused increased expression of p53 and p21 followed by decreased expression of p27 genes in cancer cell lines<sup>45,46</sup>. These modifications were followed by significant structural abnormalities in the renal tissue, which were demonstrated by the earlier described glomerular spacing, tubular degeneration, necrosis and vacuolization<sup>47</sup>.

Treatment with arsenic caused a significant decline in total protein and creatinine and in the activities of acid and alkaline phosphatase (ACP and ALP), which are indicative of altered membrane permeability, disturbed cell functions, and probably tissue damage in the kidney of mice<sup>48</sup>. Arsenic significantly increased the production of ROS, damaged the outer membrane of mitochondria in kidney, as well as decreased the levels of ATP and membrane potential of mitochondria<sup>49</sup>. Proteins like PCNA and DNMT1 are affected by exposure to arsenic. PCNA and DNMT1, components of a gene expression repressor complex, were changed in terms of their protein levels when kidney HEK293 cells were exposed to arsenic *in vitro*<sup>50</sup>. Sodium arsenite (SA) reduced antioxidant enzyme activity and increased the levels of Interleukin-1 (IL-1), Caspase-3, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the liver and kidney of Sprague-Dawley rats<sup>51</sup>. Uptake of arsenic led to the development of autophagosomes in kidney tissue and the upregulation of genes encoding proteins related to autophagy<sup>52</sup>. There is an independent correlation between increased risk of late kidney transplant failure and circulating arsenic levels in kidney transplant recipients<sup>53</sup>. The current findings indicate that sodium arsenite treatment causes nephrotoxicity and severe structural damage to DNA and cells of the renal proximal tubules as shown by histological and biochemical data. Exposure to arsenic dramatically increased DNA damage in HaCaT cells and negatively affected the DNA repair systems and cell cycle regulatory mechanisms<sup>54</sup>.

In the present study, arsenic-exposed rats showed a significant accumulation of arsenic in kidney as compared to controls leading to the observed nephrotoxic alterations. Gr. II (LDG) exhibited the lowest arsenic concentration followed by Gr. III (MDG) and the highest concentration was observed in Gr. IV (HDG) as compared to Gr. I (Control).  $\alpha$ -Tocopherol (Vit. E) activates a cellular defence mechanism that detoxifies drugs and environmental contaminants. When combined with 16.4 mg/kg NaAsO<sub>2</sub>, it was shown that  $\alpha$ -tocopherol reduced the arsenic-induced damage in the kidney of Wistar rats. The administration of  $\alpha$ -tocopherol caused significant elevation in body and kidney weights of Gr. V rats. In groups treated with sodium arsenite, p53 and p21 gene expression is upregulated and p27 expression is down regulated. Co-treatment of cells with  $\alpha$ -tocopherol followed by NaAsO<sub>2</sub> administration

resulted in the increased expression of p27 whereas decreased expression of p21 and p53. The results showed that co-treatment of antioxidant vitamins protects against NaAsO<sub>2</sub>-induced carcinogenesis in nephrocytes. Vitamin E have significant effect on body weight, hematological and postmortem and histo-pathological changes<sup>55</sup>. Consecutively,  $\alpha$ -tocopherol (Vit. E) reduced lipid peroxidation in the kidney and elevated the enzymatic antioxidant system. Our study also showed that  $\alpha$ -tocopherol had ameliorative effects on NaAsO<sub>2</sub>-induced DNA damage, elevated cellular arsenic concentration, p53 and p21 down-regulation and cell proliferation in nephrocytes.

Overall, the current investigation has demonstrated that  $\alpha$ -tocopherol ameliorates NaAsO<sub>2</sub> induced DNA damage and lipid peroxidation in Wistar rats. Furthermore,  $\alpha$ -tocopherol was found to be efficient in an *in vitro* system for preventing sodium arsenite-mediated cell proliferation and restoring the histoarchitecture of renal cells<sup>56,57</sup>. When vit. E was given before arsenic treatment, the tissues of adult mice had lower As concentrations<sup>58</sup>. Vitamin E cotreatment significant increase in the activities of ACP, ALP and SDH enzymes<sup>59</sup>. Brain alterations caused by As-induced cholinergic and behavioural disturbances were significantly reversed by vit. E administration in rats<sup>60</sup>. Vitamin E (250 IU/kg) supplementation, either by alone or in combination with *S. cerevisiae*, was found to be partially successful in improving the growth, voluntary feed intake, and arsenic load in the faeces, urine, and hair of Black Bengal children exposed to sodium arsenite (50 mg/kg)<sup>61</sup>. Collectively, tocopherol is more effective in reducing the nephrotoxicity caused by arsenic. Tocopherol treatment decreased the serum RFT levels in arsenic-treated Sprague-Dawley rats<sup>62</sup>. The results of this study may provide light on the protective and stabilizing abilities of antioxidant vitamin E against damage caused by arsenic.

## Conclusion

The above results have demonstrative potential effects of tocopherol on arsenic toxicity in the kidney. TBARS and DNA damage levels were significantly increased in the 8.2 mg/kg NaAsO<sub>2</sub> induced group II and significantly decreased in the 50 mg/kg tocopherol dose group V, which played a protective role against 16.4 mg/kg induction of NaAsO<sub>2</sub>. While inflammation and histological damage were increased in the groups II, III and IV, the healing effects of

tocopherol administration on inflammation and renal histoarchitecture could be observed. The p53 and p21 levels were increased significantly in group IV and decreased in the 50 mg/kg curcumin dose group V. In contrast to the p53 levels, the kidney p27 levels were significantly decreased in group IV and significantly increased in the 16.4 mg/kg NaAsO<sub>2</sub> and 50 mg/kg tocopherol dose groups. For arsenic-treated rats, the body and organ weights were significantly reduced. There was no significant difference in the weights of arsenic +  $\alpha$ -tocopherol treated rats with the rats of control group. NaAsO<sub>2</sub> caused significantly more DNA damage to kidney cells of the experimental group than the control group.  $\alpha$ -tocopherol greatly reduced arsenic induced DNA damage in the renal tissue. Co-administration of  $\alpha$ -tocopherol with NaAsO<sub>2</sub> showed increased mRNA expression of p53 and p27 genes followed by decreased mRNA expression of p21 genes.  $\alpha$ -tocopherol greatly reduced lipid peroxidation in the renal tissue. It also restored the serum levels of Urea, Creatinine & Albumin proteins followed by restoring the catalytic activities of Acid phosphatase, Succinic dehydrogenase and Alkaline phosphatase enzymes. Arsenic concentration was reduced in Group V due to the ameliorative effects of  $\alpha$ -tocopherol.  $\alpha$ -tocopherol-treated rats exhibited normal morphology of epithelial and tubular cells. These findings support the notion that vitamin E supplementation lowers the amount of arsenic absorbed by the kidneys and hence has protective effects against arsenic-induced nephrotoxicity. As a result, vitamin E administration generated a sizable reversal effect and was considered to be a more effective ameliorative agent against As-induced DNA damage.

## Conflict of Interest

Authors declare no competing interests.

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