

Synergistic action of L-Ascorbic acid and α -Tocopherol on arsenic trioxide mediated oxidative stress in HL-60 cells

Vineetha R C¹, Binu P¹, Arathi P¹, Harikumanan Nair R¹

(¹School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India.)

Corresponding author: Harikumanan Nair R

Abstract: Acute promyelocytic leukemia (APL) is a distinctive subtype of acute myeloid leukemia (AML), which accounts for approximately 10% of all acute myeloid leukemia cases. It has been reported that arsenic trioxide (As_2O_3) is a very effective therapeutic agent against APL. Recent studies showed that oxidative stress plays a key role in As_2O_3 -induced cytotoxicity. The present study aims to evaluate the synergistic potential of L-Ascorbic acid and α -Tocopherol in arsenic trioxide chemotherapy. The APL cell line HL-60 was used for the study. Cells were subjected to morphological analysis, cell viability assay, LDH assay, Total antioxidant capacity assay, lipid peroxidation assay, nitrate assay and fluorescent double staining. On the basis of these direct *in vitro* findings, our studies provide evidence that HL-60 cells became more susceptible to As_2O_3 in the presence of L-Ascorbic acid and α -Tocopherol which may extend the therapeutic spectrum of arsenic trioxide.

Keywords - Acute promyelocytic leukemia, Arsenic trioxide, α -Tocopherol, L-Ascorbic acid, oxidative stress

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I. INTRODUCTION

Arsenic is one of the most toxic metals widely distributed in the nature. Human exposure to arsenic pollution occurs mainly through drinking water. India and Bangladesh are the two worst hit nations [1]. Higher content of arsenic is found in certain areas of Argentina, Chile, Finland, Greece, Hungary, Mexico, USA and many other countries. Arsenic exposure can cause cancer of various organs such as skin, liver and lung, bladder tumors and neurological diseases. Oral intake of arsenic through drinking water for a prolonged time results in skin lesions, and skin, lung, bladder and kidney cancer [2]. Conversely, arsenic is also used in the treatment of various cancers.

Arsenic trioxide (As_2O_3), the trivalent form of arsenic, is reported to be an effective therapeutic agent against acute promyelocytic leukemia (APL) [3]. APL is a subtype of acute myeloid leukemia (AML), which affects all age groups of people. It is a blood cancer that is formed by specific chromosome translocation t (15; 17), which results in the rearrangement of promyelocytic leukemia (PML) gene and retinoic acid receptor α (RAR α) gene resulting in the expression of PML-RAR α chimeric protein [4]. However, the administration of As_2O_3 is a matter of concern in the clinical community since it may result in various harmful side effects commonly associated with dose-dependent cardiotoxicity and hepatotoxicity [5,6]. The mechanisms of action of As_2O_3 include induction of apoptosis mediated by the generation of reactive oxygen species (ROS)/ nitrogen species (RNS) resulting in oxidative stress [7]. Notably, the combined effect of As_2O_3 and vitamins was specific for leukemic cells, since no apoptotic effect was observed in normal cells [8]. It has therefore been suggested that antioxidants when administered together with arsenic trioxide could decrease the toxic side effects without affecting its therapeutic efficacy will be a suitable choice. Studies are making progress to ease arsenic trioxide induced damage in normal cells, and also magnify the same in leukemic cells.

In the present study, antioxidant vitamins such as L-Ascorbic acid (L- AA) or vitamin C and α -Tocopherol (α -TOC) or Vitamin E have been tried and tested with promising results with regards to their effect on arsenic trioxide induced oxidative stress using HL-60 cell line as an *in vitro* model.

II. MATERIALS AND METHODS

Reagents

Arsenic trioxide, L-Ascorbic acid, α -Tocopherol, and Trypsin - EDTA solution were purchased from Sigma (USA). Fetal Bovine Serum (FBS), Roswell Park Memorial Institute (RPMI) 1640 medium, antibiotic-antimycotic solution and other chemicals were purchased from Himedia Pvt Ltd (Mumbai, India).

Cell culture and Treatment

The APL cell line used in this study was HL-60, acquired from National Centre for Cell Sciences (NCCS), Pune. HL-60 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium

supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution and incubated at 37°C maintained in an atmosphere of 5% CO₂ and 95% air according to the standard procedures. Cells were sub cultured to 80% confluence before the experiments. The experimental group consists of (a) Control cells, (b) Cells treated with 0.2% ethanol (Negative control) for 48 hours, (c) Cells treated with 10 μ M As₂O₃ for 48 hours, (d) Cells treated with 50 μ M α -Tocopherol for 48 hours, (e) Cells treated with 100 μ M L-Ascorbic acid for 48 hours and (f) Cells treated with 10 μ M As₂O₃, 100 μ M L-Ascorbic acid and 50 μ M α -Tocopherol for 48 hours.

Morphological Analysis

After 48 hours of treatment with As₂O₃, L-AA and α -TOC, the morphology of HL-60 cells was observed using an inverted phase contrast microscope at 20 x magnification.

Cell viability Assay

Cell viability of various experimental groups was determined after 48 hours of incubation by MTT assay kit according to the manufacturer's instruction (Himedia).

Lactate dehydrogenase release

The release of cytoplasmic lactate dehydrogenase (LDH) is used as a quantitative marker enzyme for the intact cell. Measurement of lactate dehydrogenase release is an important and frequently applied test for cell damage. LDH leakage assay was performed with cell free supernatant mixed with potassium phosphate buffer, 6mM NADH solution and sodium pyruvate solution. The OD was recorded at 340nm in a spectrophotometer [9].

Lipid peroxidation Assay

Lipid peroxidation in cell cultures was estimated by measuring the formation of malondialdehyde (MDA). The treated cells were centrifuged at 4000 rpm for 10 minutes. Cell lysis buffer was added to the pellet and kept for 30 minutes incubation at 4°C. 70% alcohol and 1% TBA were added to each of the tubes and were kept in a boiling water bath for 20 minutes. Acetone was added to all the test tubes after cooling to room temperature and the absorbance was read at 535 nm [10].

Evaluation of total antioxidant capacity (TAC)

Total antioxidant assay is a spectroscopic method used for the quantitative determination of antioxidant capacity, through the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH. 100 μ L cell free supernatant was combined with 1 mL of reagent (0.6 M H₂SO₄, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tube was capped and incubated in a boiling water bath at 95°C for 90 minutes. After cooling the sample to room temperature, the absorbance was measured at 695 nm against a blank [11].

Estimation of Nitrate by Griess method

HL-60 cells were incubated with appropriate concentrations of As₂O₃, L-AA and α -TOC independently and in combination for 48 hours. The concentration of nitrate was determined using Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 2% phosphoric acid). The amount of nitrate present in various samples was measured at 540nm [12]

Fluorescent microscopy measurements

Cells were labeled with Acridine Orange/ Ethidium Bromide (AO/EB) to detect apoptosis. A mixture of AO (100 μ g/ml) and EB (100 μ g/ml) were added and incubated at room temperature for 10min. After washing with PBS the cells were observed under a fluorescent microscope [13].

Statistical Analysis

Data were collected from repeated experiments and the results were presented as mean ($\pm$ Standard deviation). Data were subjected to one-way analysis of variance (ANOVA) using Origin, version 7, Origin Lab Corporation, Northampton, USA. P<0.05 was considered to be statistically significant.

III. RESULTS

Effect of Arsenic trioxide and antioxidant vitamins on cell morphology

We examined the morphology of HL-60 cells following treatment with As_2O_3 and treatment with L-Ascorbic acid and α -Tocopherol alone and in combination. As shown in Figure 1, untreated control cells were relatively larger in size and have showed round or oval shapes. Following As_2O_3 treatment, the cell size was decreased. In addition, the combination treatment group of As_2O_3 , L-AA and α -TOC, the cell size was greatly decreased when compared with all the other groups.

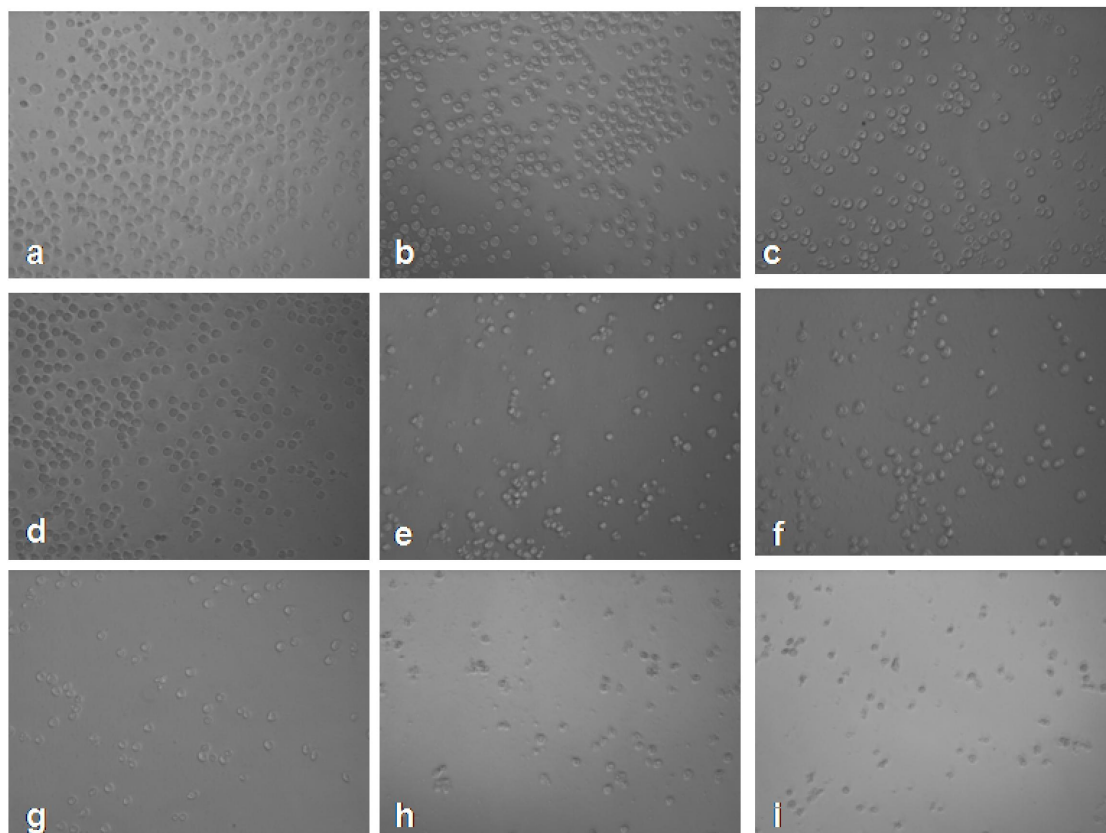


Figure 1: Morphological Analysis of HL-60 cells after 48 Hours of treatment (a) Control cells (b) Cells treated with vehicle control (c) Cells treated with 10 μM As_2O_3 (d) Cells treated with 100 μM L-AA (e) Cells treated with 50 μM α -TOC (f) Cells treated with L-AA and α -TOC (g) Cells treated with 10 μM As_2O_3 and 100 μM L-AA (h) Cells treated with 10 μM As_2O_3 and 50 μM α -TOC (i) Cells treated with 10 μM As_2O_3 , 100 μM L-AA and 50 μM α -TOC. (Original magnification x 20)

L-Ascorbic acid and α -Tocopherol markedly reduces the viability of HL-60 cells when combined with As_2O_3

Cell viability assay was performed to evaluate the cytotoxic effect of arsenic trioxide and vitamins towards the leukemic cells. We tested the effects of As_2O_3 and vitamins (L-AA and α -TOC), separately and in combination. Figure 2 shows that HL-60 cells when treated with 10 μM As_2O_3 , alone for 48 hours, the cell viability was moderately reduced to 52.21%. Various combinations of arsenic trioxide and vitamins were evaluated for their cytotoxic effect. However, if the cells were treated with 10 μM As_2O_3 and 100 μM L-AA in combination, a nearly 48.43% reduction of cell viability was observed and in cells treated with a combination of 10 μM As_2O_3 and 50 μM α -TOC, 49.12% cell death was observed when compared with the untreated cells. A marked reduction of viability (42.02%) was observed after 48 hours of incubation of As_2O_3 with L-Ascorbic acid and α -Tocopherol and was found to induce synergic cytotoxicity.

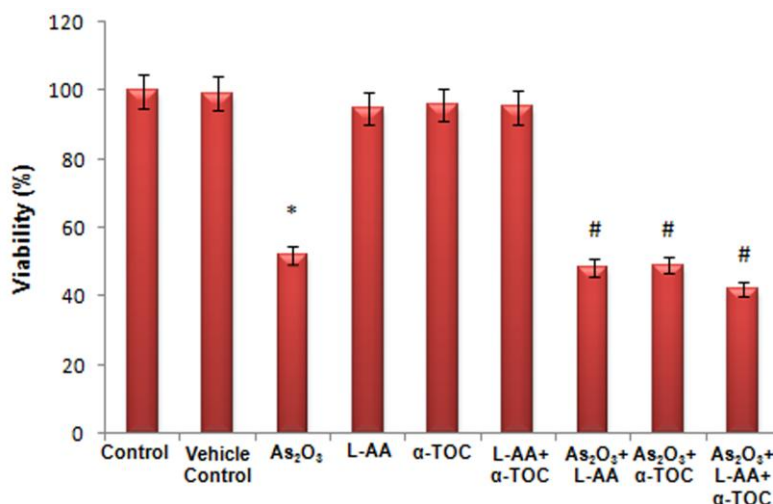


Figure 2: MTT Assay after 48 hours of treatment. Data represented as mean $\pm$ SD, * p <0.05 versus normal control and # p <0.05 versus As₂O₃ treated groups.

Effects of L- Ascorbic acid and α -Tocopherol on Arsenic trioxide induced cytotoxicity

In order to evaluate membrane integrity of HL-60 cells, LDH leakage of various experimental groups was measured. 10 μ M concentration of As₂O₃ caused a significant increase (P <0.05) in LDH leakage when compared with the untreated cells while cotreatment with vitamins (100 μ M L-Ascorbic acid and 50 μ M α -Tocopherol) markedly augmented LDH leakage caused by arsenic trioxide.

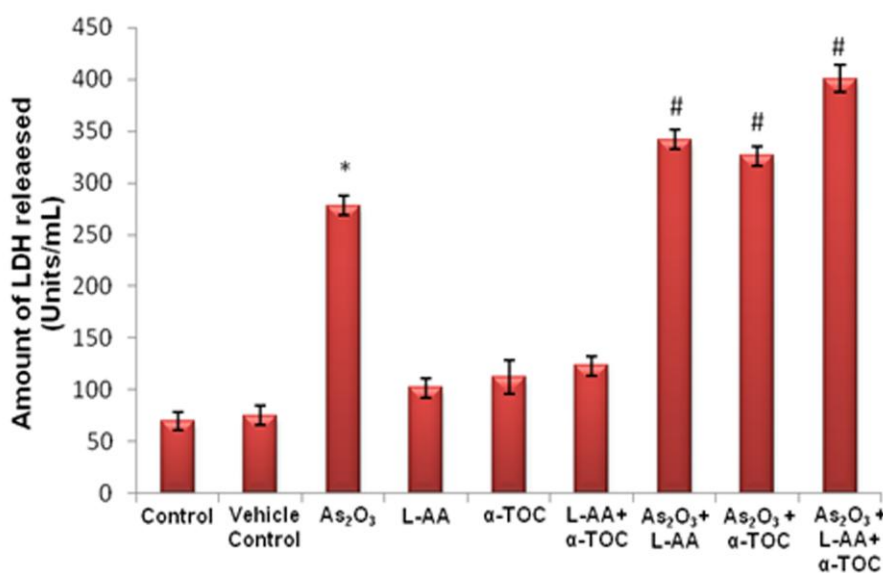


Figure 3: LDH leakage. Data represented as mean $\pm$ SD, * p <0.05 versus normal control and # p <0.05 versus As₂O₃ treated groups.

Effect of arsenic trioxide and vitamins on total antioxidant capacity

The quantitative determination of antioxidant capacity was illustrated by the total antioxidant assay. There is a significant reduction in the total antioxidant capacity in cells treated with arsenic trioxide when compared with the normal cells. Also the combination treatment of arsenic trioxide with vitamins showed a significant decline in the level of total antioxidant capacity when compared with the arsenic trioxide treated groups (Fig. 4).

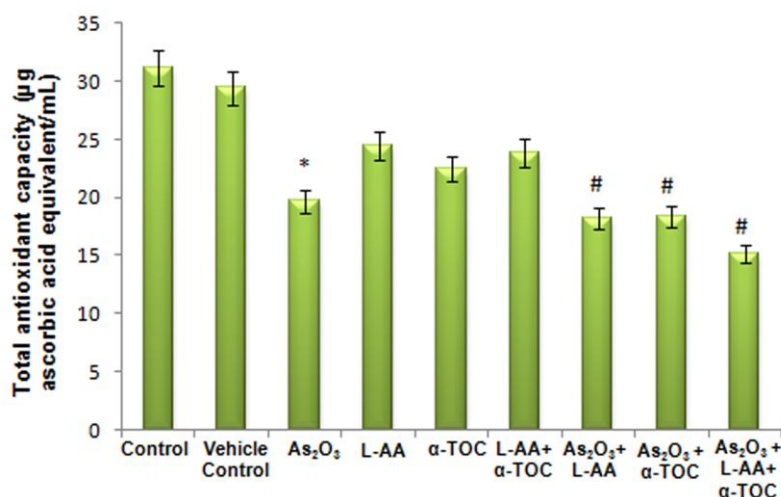


Figure 4: Determination of total antioxidant capacity. Data represented as mean $\pm$ SD, * p <0.05 versus normal control and # p < 0.05 versus As₂O₃ treated groups.

Determination of Nitrate levels

The nitrate levels of all the experimental groups were estimated using Griess reagent. The amount of nitrate released was found to be significantly higher in arsenic trioxide treated cells when compared with the untreated cells. Fig 5 showed that the combination of L-AA and α -TOC with arsenic trioxide significantly increased the level of nitrate released in HL-60 cells.

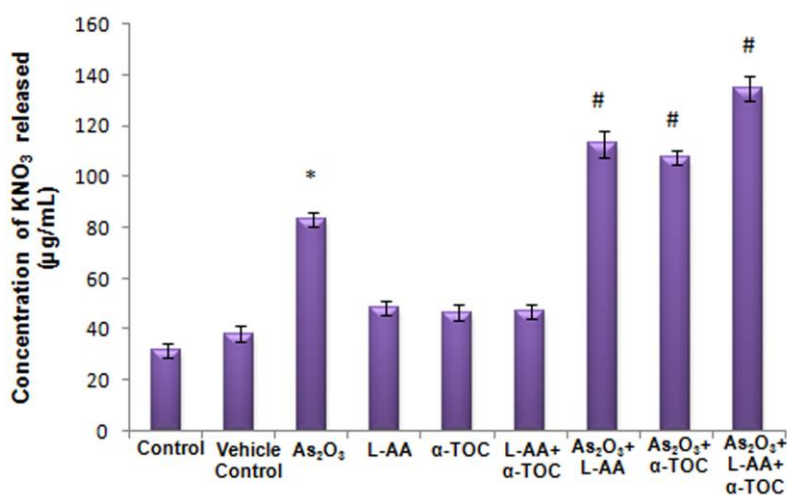


Figure 5: Effect of arsenic trioxide, L-Ascorbic acid and α -Tocopherol on the nitrate level in HL-60 cells. Data represented as mean $\pm$ SD, * p <0.05 versus normal control and # p <0.05 versus As₂O₃ treated groups.

Effects of L-Ascorbic acid and α -Tocopherol on arsenic trioxide-induced apoptosis

AO/EB double staining illustrated that exposure to 10 μ M of arsenic trioxide reduced the viability of HL-60 cells. The cells which are stained green in color distinguish viable cells whereas red color is a characteristic of non-viable cells. Co- administration of L-AA and α -TOC with arsenic trioxide showed significant increase in the number of non-viable cells compared to the cells treated with 10 μ M arsenic trioxide alone. This indicates the cytotoxicity nature exerted by the combination of L-Ascorbic acid and α -Tocopherol with arsenic trioxide.

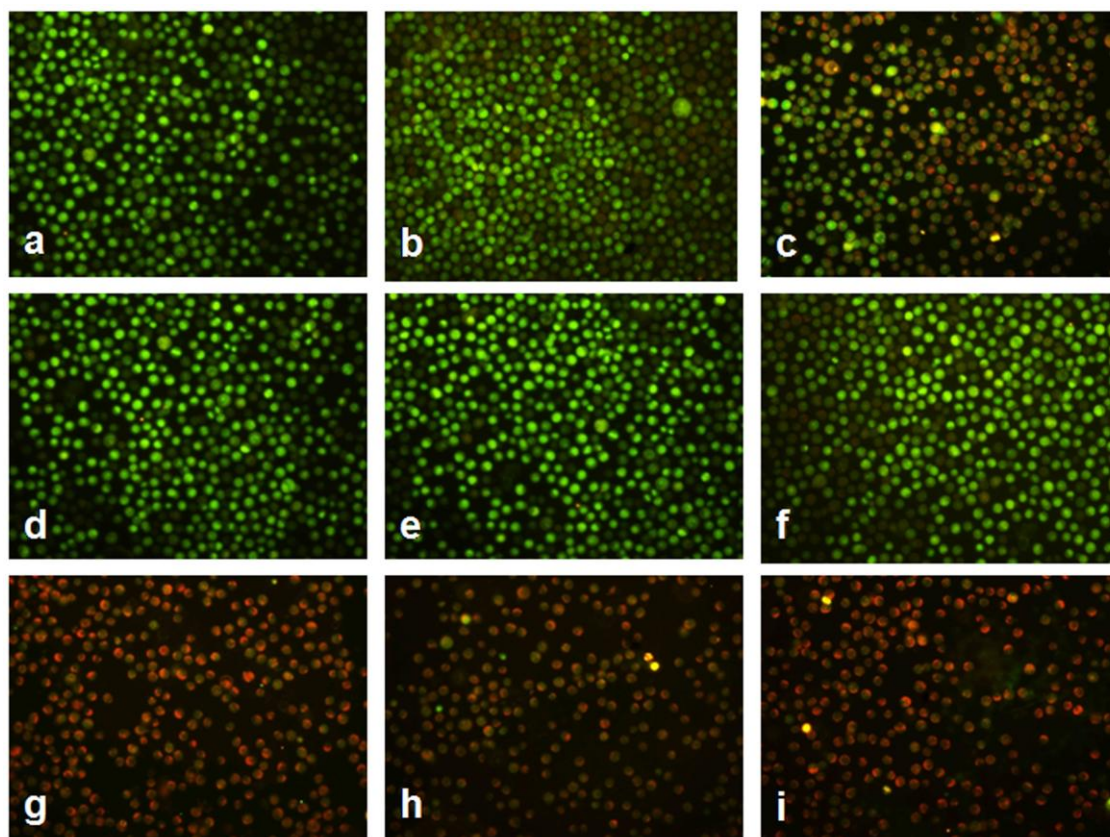


Figure 6: Morphological observation of HL 60 cells by AO/EB staining showing (a) Control cells (b) Cells treated with vehicle control (c) Cells treated with 10 μ M As_2O_3 (d) Cells treated with 100 μ M L-AA (e) Cells treated with 50 μ M α -TOC (f) Cells treated with 100 μ M L-AA and 50 μ M α -TOC (g) Cells treated with 10 μ M As_2O_3 and 100 μ M L-AA (h) Cells treated with 10 μ M As_2O_3 and 50 μ M α -TOC (i) Cells treated with 10 μ M As_2O_3 , 100 μ M L-AA and 50 μ M α -TOC. (Original magnification x 20)

IV. DISCUSSION

Arsenic exposure can cause cancer of various organs such as skin, liver and lung, bladder tumors and neurological diseases. But it is also used as an effective drug in a number of traditional Chinese remedies and is reported as a very potent chemotherapeutic agent against acute promyelocytic leukemia (APL). In the current study, we assessed the prospect of whether the L-Ascorbic acid and α -Tocopherol could augment the sensitivity of As_2O_3 therapy. Ascorbic acid treatment alone was not cytotoxic, suggesting that L-AA and α -TOC has the potential to be a safe and effective chemosensitizing agent in arsenic trioxide-based chemotherapy. Our results validate the rationale involved in selecting the combination of these drugs, by revealing the efficiency of vitamins in augmenting the apoptotic cell death in HL-60 cells induced by arsenic trioxide.

The cell killing property of a chemical compound independent from the mechanism of death is its cytotoxicity. The toxicity of a compound to various cell types can be judged using *in vitro* cytotoxicity tests and its inclusion in early discovery efforts provides an important advantage in identifying potentially cytotoxic compounds [14]. Here, the experimental outcome noticeably demonstrated that 100 μ M L-AA and 50 μ M α -TOC with 10 μ M As_2O_3 remarkably enhanced the cytotoxicity in HL-60 cells, showing that the clinically attainable concentration of As_2O_3 cause reduced cell viability of HL-60 cells. Likewise, As_2O_3 increased the release of Lactate dehydrogenase, indicating the induction of apoptosis. LDH is an enzyme released during cell or tissue damage and is a clinically important marker of injury and disease. The LDH leakage assay evaluates the outflow of the soluble cytoplasmic LDH enzyme into the extracellular medium by means of cellular lysis. Although the mechanism by which antioxidants enhances arsenic trioxide-mediated cytotoxicity in HL-60 cells remains unknown, here we provide evidence that antioxidant vitamins, L-AA and α -TOC potentiates arsenic trioxide-induced oxidative stress in human leukemia cells.

As_2O_3 cotreatment with vitamins reduced the total antioxidant capacity in HL-60 cells. The total antioxidant capacity may detect a possible synergism between known antioxidants and the contribution of unknown or rarely estimated antioxidants. In the current study, we could show a statistically significant increase of intracellular lipid peroxidation products (TBARSs) in HL-60 cells sensitive to the combination of As_2O_3 with L-AA and α -TOC. Free radicals attack the double bonds of polyunsaturated fatty acids and thereby initiating a

chain reaction which affect membrane integrity and cellular function. Studies have reported that arsenic trioxide diffuses through cell membrane into the cytoplasm and produces cytotoxic effect by generating reactive oxygen species. It has also been reported that arsenic trioxide causes oxidative stress and cell death in a variety of cells including acute promyelocytic leukemia (APL), acute myeloid leukemia and chronic myeloid leukemia as well as solid tumor cells *in vitro* [15]. Our study showed an increase in the nitrate level in As₂O₃ treated cells. The nitrates level was further enhanced on cotreatment of arsenic trioxide with L-Ascorbic acid and α -Tocopherol. Nitrate, a stable product of Nitric Oxide (NO) plays a major role in As₂O₃ induced apoptosis in HL-60 cells. NO is a highly unstable free radical produced by the enzyme Nitric Oxide Synthases (NOS) that binds to cytochrome oxidase and can block the mitochondrial respiratory chain thereby induces apoptosis [16]. It was remarkable that HL-60 cells were observed under fluorescent microscope after acridine orange/ethidium bromide staining. More number of red coloured cells was observed in the group cotreated with arsenic trioxide and vitamins when compared to the cells treated with arsenic trioxide alone. However, the administration of As₂O₃ is a matter of concern in the clinical community since it may result in various harmful side effects commonly associated with cardiotoxicity [17] and hepatotoxicity [6]. Our findings suggest that the combination of As₂O₃ and vitamins like L-Ascorbic acid and α -Tocopherol may be especially efficient in APL cells and further investigations on this combination in acute promyelocytic leukemia are therefore clearly warranted.

V. CONCLUSION

In summary, our data showed that antioxidant vitamins enhance As₂O₃-mediated apoptosis in APL cell line. Although the exact molecular mechanism of arsenic trioxide in chemotherapy is not well understood, we have investigated in the present study its mechanism of oxidative stress-induced intrinsic pathway of apoptosis in HL-60 cells. Enhanced intracellular lipid peroxidation seems to constitute a key event contributing to the synergistic effect of As₂O₃ and antioxidant vitamins. From the present study, we suggest that vitamins not only play an important role in reducing the cytotoxicity in normal cells due to arsenic exposure but also act synergistically with arsenic trioxide in leukemic cells. The characterization of enhancing effect of L-AA and α -TOC on As₂O₃-induced apoptosis at its molecular level may help to provide new insights into its therapeutic aspects rather than as an environmental pollutant.

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