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Docosahexaenoic acid relieves cellular damage due to oxidative stress induced by the chemotherapeutic agent arsenic trioxide in H9c2 cardiomyocytes

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ABSTRACT

The therapeutic efficacy of arsenic trioxide, the highly effective cancer drug against acute promyelocytic leukemia (APL), is hindered by its cardiotoxicity due to oxidative stress generation. Effective regulation of oxidant-antioxidant status is essential for the proper proliferation and maintenance of cells. An imbalance in the antioxidant response mechanism may lead to defect in cellular proliferation coupled with enhanced apoptosis. The present study aimed to evaluate the protective potential of the omega-3 poly unsaturated fatty acid, docosahexaenoic acid, against adversities of arsenic trioxide - induced toxicity in H9c2 cardiomyocytes. The effect of the therapeutic concentration of arsenic trioxide (10 µM) on H9c2 cardiomyocytes was evaluated. Arsenic trioxide causes severe damage in cardiomyocytes due to enhanced oxidative stress. Significant ($p \leq 0.05$) reduction in cell viability and antioxidant enzyme status along with elevation in levels of lipid peroxidation, LDH release, nitric oxide (NO) and apoptosis coupled with altered cell morphology were observed. Co-treatment with docosahexaenoic acid (100 µM) corrected the toxic effects of arsenic trioxide, as evident from reduced levels of LDH, lipid peroxidation, apoptosis and nitric oxide coupled with rise in antioxidant enzyme levels and enhanced cell viability. In conclusion, our experimental results pointed out to the underlying effects of oxidative stress induction in cardiomyocytes by arsenic trioxide exposure along with the protective potential of docosahexaenoic acid against these adversities.

I. INTRODUCTION

Cellular proliferation is an indispensable step for relieving the damage caused by oxidants and toxicants. The de-regulation of cell growth and proliferation by oxidative stress can lead to the development of various diseases affecting the organ system [1]. Antioxidant proteins including catalase and reduced glutathione (GSH) are important agents in maintaining the 'redox homeostasis of the cell. They suppress endogenous reactive oxygen species (ROS) and reactive nitrogen species (RNS) by inhibiting the reactive species-initiated reactions induced by external insults. The varied balance between oxidant load and the endogenous cellular antioxidant defense system can guide to oxidative stress, a deleterious process that can be a vital mediator of damage to cell structures, including lipids and membranes, proteins, and DNA [2].

Arsenic (As) is a highly toxic metalloid that is widely distributed in the environment [3]. The trivalent form, arsenic trioxide, is a highly effectual anticancer agent against acute promyelocytic leukemia (APL) [4]. APL constitutes a subtype of acute myeloid leukemia which is predominated by the distinctive chromosomal translocation, t (15, 17), that results in the PML-RARα fusion protein. Arsenic trioxide causes apoptosis in cancer cells mainly by the generation of reactive oxygen species (ROS) and oxidative stress [5]. The clinical effectiveness of arsenic trioxide is hindered by its toxicity profile that mainly includes cardiotoxicity along with other organ toxicities. Arsenic trioxide is supposed to cause widespread organ toxicity by combining to protein thiol groups. Multiple adverse effects associated with cardiotoxicity of arsenic trioxide include QT prolongation, torsades de pointes (TdP) and sudden cardiac death [6]. The precise mechanism for arsenic trioxide induced cardiotoxicity is still unclear. Emerging research has pointed out that apoptosis of terminally differentiated cardiomyocyte is the causative factor for the development of myocardial infarction and congestive heart failure [7]. Hence the attenuation of arsenic trioxide mediated toxicity on the heart is expected to be an effective measure in elucidating the complete therapeutic potential of this cancer drug.

Omega-3 polyunsaturated fatty acids (PUFAs) are used as food supplements due to the beneficial effects of these fatty acids on human body. The two major omega-3 PUFAs are docosahexaenoic acid and eicosapentaenoic acid. Even though both docosahexaenoic acid and eicosapentaenoic acid bestow protective effects on the cardiovascular system, docosahexaenoic acid is considered to be comparatively more important. Docosahexaenoic acid is more abundant than eicosapentaenoic acid in the myocardium and is highly effective than eicosapentaenoic acid against cardiac arrhythmias, hypertension [8], [9] and inflammation [10]. Docosahexaenoic acid was found to positively regulate the antioxidant defense system of the body thereby relieving oxidative stress [11]. The ability of this fatty acid to incorporate into the damaged cell membranes is expected to be one of the mechanisms behind its protective effects [12]. However more studies are required to elucidate the exact mechanism.

The present study aims to evaluate whether docosahexaenoic acid could offer protection to H9c2 cardiomyocytes against diverse impacts of the oxidative insult caused by action of the cancer drug, arsenic trioxide.

II. MATERIALS AND METHODS

Chemicals

Arsenic trioxide, docosahexaenoic acid, acridine orange, ethidium bromide and 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Sigma (USA). Fetal Bovine Serum (FBS) was purchased from Invitrogen (India). 3-(4, 5, dimethylthiazol-2-yl)-2, 5, diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), Trypsin - EDTA solution and other chemicals were obtained from HiMedia Pvt Ltd (Mumbai, India).

Cell line and drug treatment

H9c2 cell line was sourced from the cell repository of National Centre for Cell Science (NCCS), Pune, India, and was maintained in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS). The cells were allowed to attain confluency at 37ºC in presence of 5% CO_2 in humidified atmosphere in a CO_2 incubator (NBS, Ependorf, Germany). Cells attained 80% confluence before the experiments.

The experimental group consists of (a) Control cells; (b) Cells treated with 10 μM arsenic trioxide for 48 hours; (c) Cells treated with 100 μM docosahexaenoic acid for 48 hours; (d) Cells treated with 10 μM arsenic trioxide and 100μM docosahexaenoic acid for 48 hours.

Morphological Observation

Morphological observation of the H9c2 cardiomyoblast cells was done after incubation for 48 hours with the chemicals using an inverted phase-contrast microscope (Olympus CKX41 with Optika Pro5 camera).

Analysis of cardiomyocyte viability

The viability of cells belonging to various experimental groups was determined colorimetrically after 48 hours of incubation by the MTT assay. This assay measures the reduction of yellow 3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters and passes into the mitochondria of the cells, where it is reduced to an insoluble, coloured formazan product. Using the organic solvent Dimethyl sulfoxide, the cells were then solubilised. The released formazan product was measured using an ELISA plate reader (Erba Manheim, Germany) at 540 nm [13].

Estimation of Lactate Dehydrogenase Release

The release of cytoplasmic lactate dehydrogenase (LDH), the quantitative enzyme marker of intact cell, into the culture medium was estimated by the method of Renner et al., (2003) [14]. LDH release assay was performed with cell free supernatant mixed with potassium phosphate buffer, 6mM NADH solution and sodium pyruvate solution. The reduction in optical density was recorded at 340nm.

Apoptosis detection by fluorescent microscopy

The H9c2 cells after adequate treatment were stained with the fluorescent dyes acridine orange/ ethidium bromide (AO/EB) for detecting apoptosis according to the method of Zhang et al., (1998) [15] and subjected to fluorescent microscopic observation (Olympus CKX41 with Optika Pro5 camera).

Estimation of lipid peroxidation

The extent of membrane lipid peroxidation, an indicator of membrane damage, was estimated by measuring the formation of malondialdehyde. Cells after the required treatment were centrifuged at 4000 rpm for 10 minutes. Cell lysis buffer was added followed by incubation at 4°C for 30 minutes. After the addition of 70% alcohol and 1% TBA, the tubes were kept in boiling water bath for 20 minutes. Acetone was added to all the tubes and the absorbance was read at 535 nm in a spectrophotometer [16].

Nitric oxide Assay

The concentration of nitric oxide in the form 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 [17].

Estimation of antioxidant enzyme status

The levels of the key antioxidant enzymes, Catalase (CAT) and reduced glutathione (GSH), were estimated according to the methods of Sinha et al (1972) [18] and Moron et al (1979) [19] respectively.

Statistical Analysis

Data were obtained from repeated experiments and the results were represented as mean (±Standard deviation). The results obtained from the experiments were analyzed using the statistical program Origin, version 7 (OriginLab Corporation, Northampton, USA). Comparison of different groups was done using the One-way analysis of variance. $p \leq 0.05$ was considered significant.

III. RESULTS

Protective effect of docosahexaenoic acid on cardiomyocyte morphology

Observation using the inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 camera) showed that cells subjected to arsenic trioxide administration followed by incubation for 48 hours had undergone distinct morphological variations such as shrinkage, rounding up and detachment from the surface of the plate (Fig.1b). In the combination treated group (Fig 1d), there was no such morphological alteration, indicating the protective potential of docosahexaenoic acid.

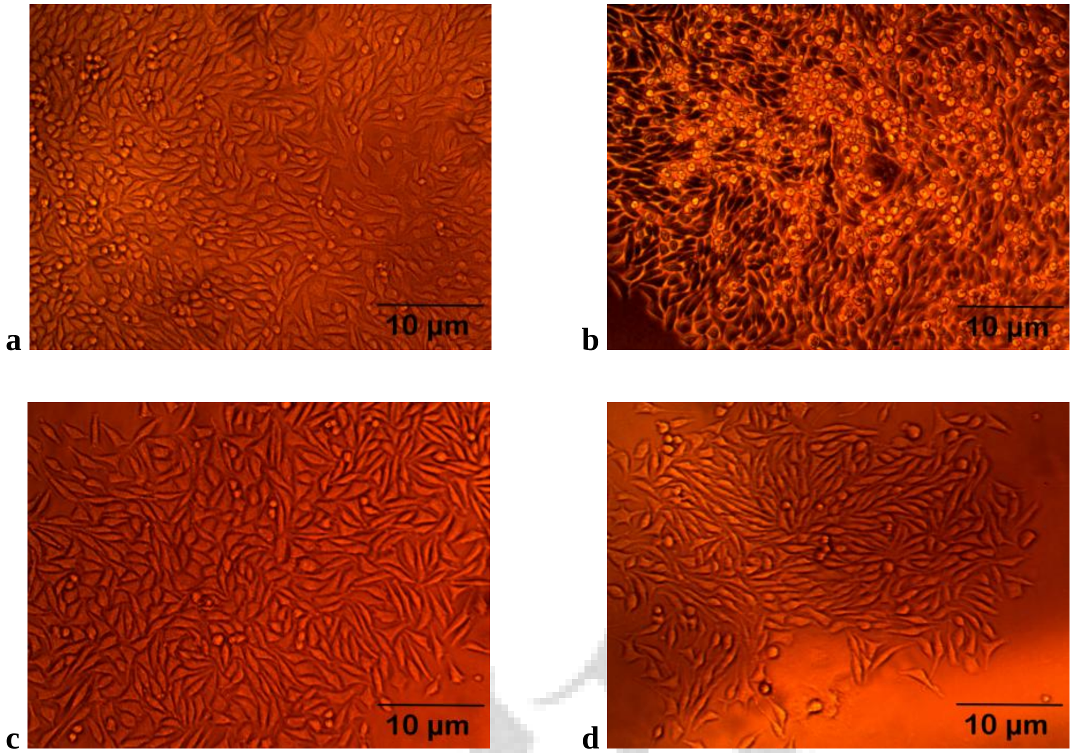

Fig. 1 Morphological analysis of H9c2 cells as observed after 48 hours of incubation. Images as obtained from inverted phase contrast microscope (Original magnification X 10). **a** Control cells; **b** Cells treated with 10 µM arsenic trioxide; **c** Cells treated with 100 µM docosahexaenoic acid; **d** Cells treated with 10 µM arsenic trioxide + 100 µM docosahexaenoic acid.

Ameliorative potential of docosahexaenoic acid against arsenic trioxide – induced cytotoxicity

Arsenic trioxide (10 µM) administration was found to significantly reduce ($p \leq 0.05$) the cardiomyocyte viability after 48 hours of exposure as evidenced from the MTT assay (Fig.2a). Docosahexaenoic acid co-treatment on the other hand acts as a protective measure against arsenic trioxide – induced cytotoxicity. The combination treatment of arsenic trioxide with docosahexaenoic acid was found to be effective in reducing the leakage of LDH enzyme from cardiomyocytes which again showed the protective potential of docosahexaenoic acid against arsenic trioxide – induced cytotoxicity (Fig.2b).

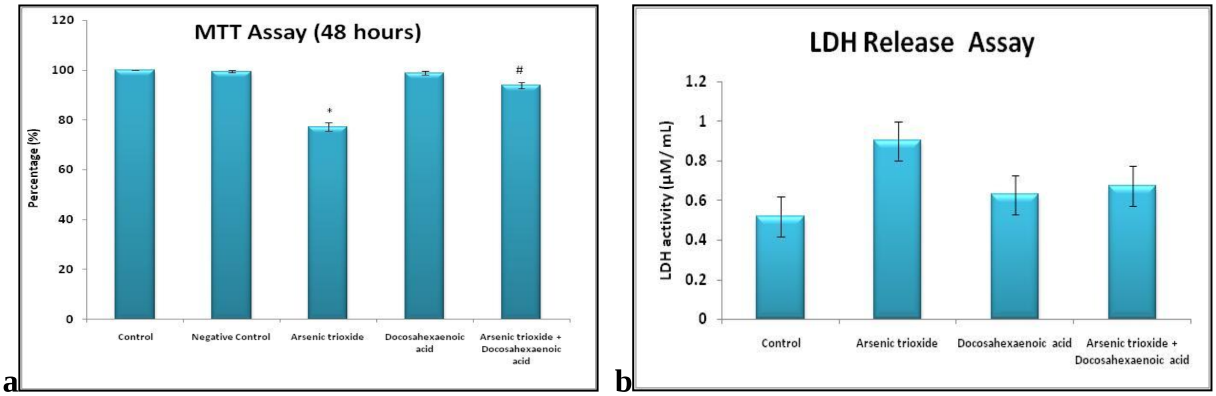

Fig. 2 Cytotoxicity assays of H9c2 cells. **a** MTT Assay (24 hours); 0.2% ethanol is used as negative control. **b** LDH releasing assay. Data represented as mean± SD,* p≤0.05 versus normal control and # p≤0.05 versus arsenic trioxide treated groups.

Apoptotic detection using fluorescent microscopy analysis

Apoptosis might be the contributing factor for the reduction of cell viability due to arsenic trioxide. AO/EB staining (Fig. 3b) showed increased levels of apoptotic cells, as indicated by red coloured nuclei in arsenic trioxide treated H9c2 cells. Docosahexaenoic acid administration (Fig. 3d) effectively reduced apoptosis as indicated by the green coloured nuclei, as a result of staining with AO which can enter even live cells and stains the nuclei green.

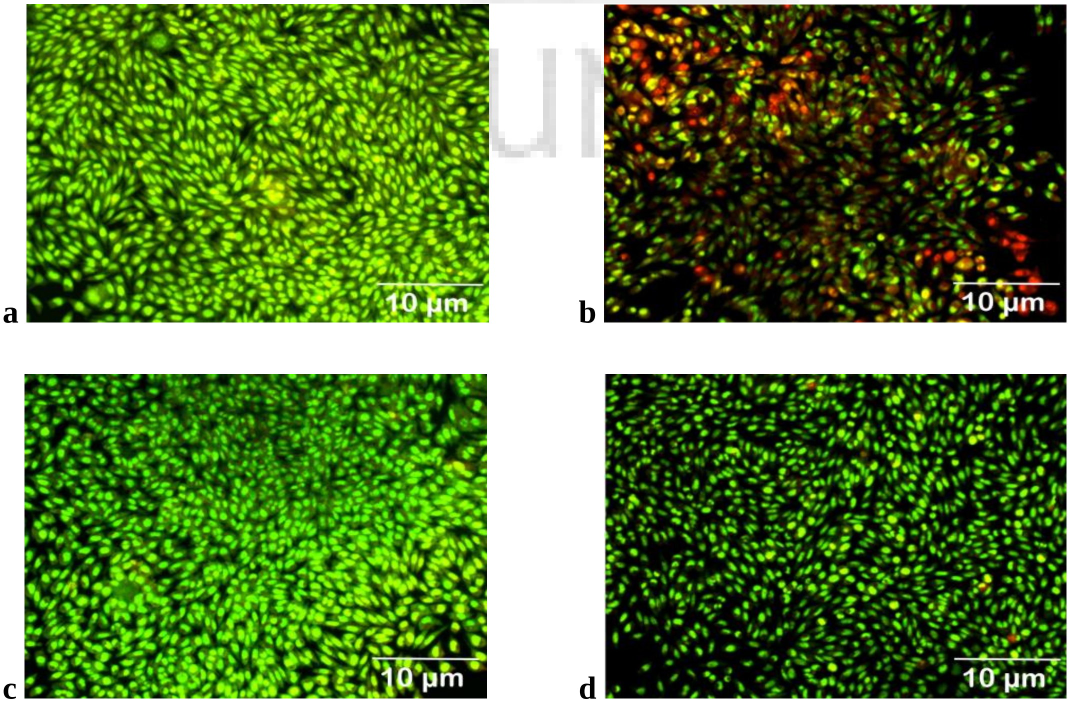

Fig. 3 Fluorescent microscopic images of H9c2 cells stained with Acridine Orange/ Ethidium Bromide (AO/ EB) dye (Original magnification X 10). **a** Control cells; **b** Cells treated with 10 µM arsenic trioxide; **c** Cells treated with 100 µM docosahexaenoic acid; **d** Cells treated with 10 µM arsenic trioxide + 100 µM docosahexaenoic acid.

Reduction in arsenic trioxide – induced lipid peroxidation by DHA

Docosahexaenoic acid when used along with arsenic trioxide (Fig. 4) was found to significantly reduce ($p \leq 0.05$) the lipid peroxidation rate as indicated by reduction in the levels of malondialdehyde.

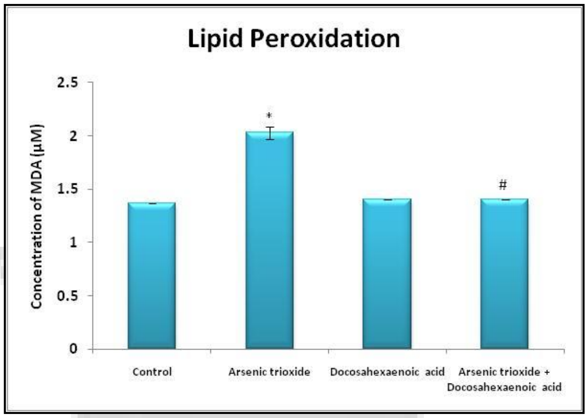

Fig. 4 Lipid peroxidation assay of H9c2 cells. Data represented as mean ± SD,* $p \leq 0.05$ versus normal control and # $p \leq 0.05$ versus As_2O_3 treated groups.

Docosahexaenoic acid protects H9c2 cells against the reactive nitrogen species (RNS) inducer- nitric oxide production by arsenic trioxide

Arsenic trioxide was found to significantly ($p \leq 0.05$) enhance the levels of the reactive nitrogen species inducer- nitric oxide in H9c2 cardiomyocytes after 48 hours of incubation with the drug. However, arsenic trioxide when used in combination with docosahexaenoic acid, the levels of nitric oxide were near normalcy, indicating the protective effect of this fatty acid (Fig. 5).

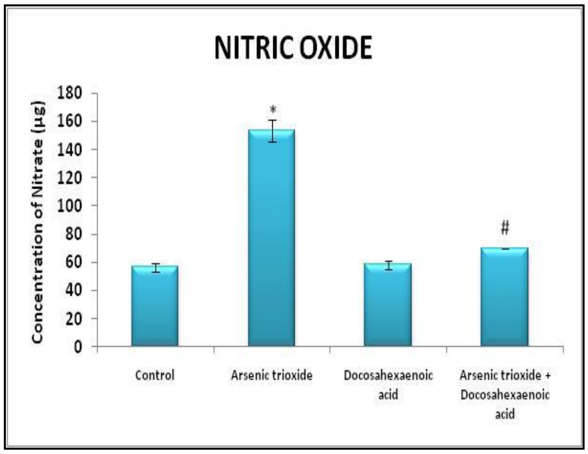

Fig.5 Nitric oxide assay of H9c2 cells. Data represented as mean± SD,* $p \leq 0.05$ versus normal control and # $p \leq 0.05$ versus arsenic trioxide treated groups.

Docosahexaenoic acid helps to restore the antioxidant status in cardiomyocytes

Docosahexaenoic acid was found to be an effective agent in restoring the cellular levels of the key antioxidant enzymes- catalase and GSH in cardiomyoctes against the oxidative stress induced by the chemotherapeutic drug arsenic trioxide. Docosahexaenoic acid co-treatment was found to be significant ($p \leq 0.05$) in restoring the cellular antioxidant status as shown in Fig. 6.

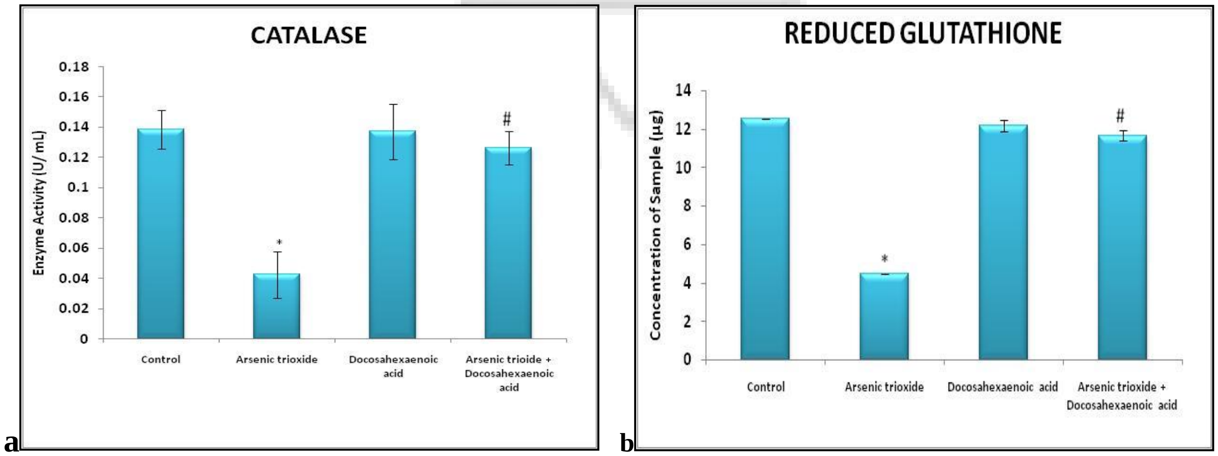

Fig. 6 Effect of DHA on antioxidant enzyme status of H9c2 cardiomyoctes. Data represented as mean± SD,* $p \leq 0.05$ versus normal control and # $p \leq 0.05$ versus arsenic trioxide treated groups.

IV. DISCUSSION

The widely used chemotherapeutic against APL, arsenic trioxide, is also known for its side effects, which is a major hindrance for elucidating its therapeutic potential. The major side effect, cardiotoxicity, occurs mainly due to alterations in membrane permeability of cardiac cells, mainly due to enhanced oxidative stress [6]. Our study was aimed at investigating the diverse effects of arsenic trioxide -induced toxicity and the ameliorative potential of docosahexaenoic acid on these adversities in cardiomyocytes at the molecular level. The cell line model used for the study was H9c2 cardiomyocytes. The H9c2 cell line is derived from embryonic rat heart and it maintains adult cardiomyocyte like features and hence used as an experimental model to explore the molecular mechanism of cardiomyocyte pathophysiologies [20].

Docosahexaenoic acid is the longest and most unsaturated form of omega-3 PUFA. This fatty acid is an essential constituent of cell membranes especially in the brain, retina and heart and it also represents the precursor of signaling molecules called docosanoids [21]. It has been reported that docosahexaenoic acid has anti-inflammatory, anti-thrombotic, vasodilatory, hypolipidemic and anti-arrhythmic properties and hence exerts favourable effects on cardiovascular function [22]. Hence our study is directed towards the protective efficacy of docosahexaenoic acid against arsenic trioxide -induced cardiotoxicity.

The present study showed that arsenic trioxide at a concentration of 10 µM causes morphological alterations in H9c2 cardiomyocytes which include shrinkage, rounding up and detachment from the surface of the tissue culture plate. This shows the typical apoptotic death of the cardiomyocytes due to arsenic toxicity. Reduced cell viability as indicated by MTT assay followed by enhanced LDH leakage showed the cytotoxicity of arsenic trioxide. AO/EB staining showed higher numbers of red coloured nuclear DNA in arsenic trioxide treated group of cells. This is due to the induction of higher rate of apoptosis by arsenic trioxide in cardiomyocytes. Apoptosis of cardiomyocytes is an important contributing factor for heart failure [23].

Enhanced lipid peroxidation as indicated by elevated levels of malondialdehyde was observed in cells subjected to arsenic trioxide exposure. Studies had reported that the increased lipid peroxidation due to arsenic trioxide is due to reactive species induction by this chemotherapeutic agent [24], [25]. We found a significantly higher level of nitric oxide in cells subjected to arsenic trioxide treatment. Nitric oxide is a strong reactive isomer of nitrate anion (NO^{3-}) and at abnormally higher concentrations, is highly toxic. Nitric oxide can lead to the formation of peroxynitrites thereby resulting in DNA damage. This acts as a stimulating factor for cardiac damage by promoting apoptosis [26], [27].

The modulation of the apoptotic process might attenuate arsenic trioxide -induced cardiotoxicity. In our study docosahexaenoic acid at a concentration of 100 µM was found to safe guard the normal morphology and viability of cardiomyocytes along with reduction in LDH leakage and apoptosis. Docosahexaenoic acid has been found to have the capability to incorporate itself into the phospholipids of cardiac cell membranes [28], [29]. The incorporated docosahexaenoic acid was found to protect the cardiac cells from injury and thereby maintains normal cellular structure. This helps in inhibiting the leakage of various cellular constituents as a result of cardiac cell membrane damage [30].

Docosahexaenoic acid has been reported to have free radical scavenging activity [31]. The maintenance of normal cardiomyocyte morphology along with reduced levels of LDH leakage and lipid peroxidation in the combination treatment group as observed in our study may be due to the membrane safe guarding effect of docosahexaenoic acid. The capability to remove free radicals by this fatty acid may be a contributing factor for the reduction in levels of nitric oxide thereby a corresponding reduction in the lipid peroxidation rate. Hence the membrane stabilizing effect coupled with free radical scavenging activity of docosahexaenoic acid may be the underlying reason for the protection of cells from apoptosis.

The antioxidant defence system must function in an effective manner so as to reduce the oxidative stress induced cell damage. We observed significantly lower levels of the major antioxidants- catalase and GSH in arsenic trioxide treated group. Catalase, an important antioxidant enzyme, protects the cells from oxidative damage by reactive species. This enzyme catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. The generation of reactive species especially H_2O_2, is of high relevance in the induction of arsenic-mediated cell death. The reduction in levels of catalase enzyme results in accumulation of H_2O_2 thereby promoting apoptosis of cells by the activation of the caspase cascade [32].

GSH in cells has the capacity to conjugate with arsenic resulting in the formation of $As(GS)_3$ complexes or to sequester the reactive species induced by arsenic [24]. However, when the concentration of reactive species builds up to a much higher level, they were found to be inhibited. This could either be due to direct binding of arsenic trioxide due to thiol preference or due to the utilization of GSH as electron donor for arsenic metabolism [2], [33], [34]. The results of our study showed that the oxidative stress induced by arsenic trioxide is much higher than that could be neutralized by the antioxidant defense system of the body. However when arsenic trioxide in combination with docosahexaenoic acid is administered to the cells, the antioxidant levels were found to be maintained near normalcy. Our results are in accordance with the previous reports [11], [31], [35] that docosahexaenoic acid acts as a stimulator of the antioxidant defence system of the body.

V. CONCLUSION

In conclusion, our study results showed the protective efficacy of docosahexaenoic acid against arsenic trioxide induced toxicity in H9c2 cardiomyocytes. Arsenic trioxide caused oxidative stress in cardiomyocytes along with suppression of the antioxidant defense mechanism. Oxidative stress results in alterations in morphology, reduced cell viability and enhanced apoptosis. Docosahexaenoic acid co-treatment protected the cells from arsenic trioxide toxicity resulting in enhanced cell viability and proliferation coupled with proper regulation of the antioxidant systems. These findings may promote the development of protective strategies to prevent or modify arsenic trioxide -induced myocardial damage.

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