



Evaluation of the cardioprotective effect of docosahexaenoic acid against arsenic trioxide induced toxicity in H9c2 cardiomyocytes by preliminary dose standardization assays

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Abstract

The acute promyelocytic leukemia drug, arsenic trioxide, causes cardiac side effects including arrhythmias and cardiomyocytes degeneration. Our aim was to evaluate the protective potential of docosahexaenoic acid, the biologically active omega-3 polyunsaturated fatty acid, against arsenic trioxide induced toxicity in H9c2 cardiomyocytes. The preliminary dose standardization assays, cell viability and lactate dehydrogenase release assays, were performed as Pre-treatment, Co-treatment and Post treatment experiments for a period of 24 hours. Arsenic trioxide administration (2.5, 5, 7.5, 10, 12.5 and 15 μ M) alone resulted in a significant ($p < 0.05$) dose dependant reduction in cell viability along with a dose dependant enhancement of lactate dehydrogenase release. However, in combination with varying concentrations of docosahexaenoic acid (50, 75, 100, 125 and 150 μ M), the cells were safe guarded in Pre-treatment and Co-treatment experiments, but not in Post treatment. The best dose for combination was 10 μ M arsenic trioxide with 100 μ M of docosahexaenoic acid in both Pre-treatment and Co- treatment experiments. Thus our study showed that docosahexaenoic acid administration as Pre-treatment or Co-treatment can reduce arsenic trioxide induced cardiotoxicity. More in depth studies are required to reveal the mechanisms behind the protective effects.

Keywords: Arsenic trioxide, cardiotoxicity, docosahexaenoic acid.

Introduction

The toxic metalloid, arsenic, has received worldwide attention due to its potential to act as a toxicant and a human carcinogen (Mandal et al., 2002). The trivalent form of this metalloid, arsenic trioxide (As_2O_3), is used as an effective medicine against acute promyelocytic leukemia (APL). The drawback of this treatment is the toxic effects of arsenic which includes cardiotoxicity and certain other organ toxicity. Due to this the clinical use of As_2O_3 is limited in almost all the nations (Jing et al., 1999; Zhao et al., 2008). Arsenic has sufficient potential to obstruct different body functions and organs including the heart, central nervous system, liver and kidney. Arsenic trioxide is suggested to cause organ toxicity by reacting with protein thiol groups. The adversities due to arsenic

trioxide toxicity include QT prolongation, torsades de pointes (TdP) and sudden cardiac death (Ficker et al., 2004). The exact mechanism behind cardiotoxicity by arsenic trioxide is still unclear. Research findings have indicated that the programmed cell death of terminally differentiated cardiomyocyte is the underlying reason for the development of myocardial infarction and congestive heart failure (Zhao et al., 2008). Hence suitable adjuvants that can attenuate arsenic trioxide mediated toxicity in heart is expected to be the effective measure in elucidating the therapeutic potential of this cancer drug to the maximum.

Omega-3 polyunsaturated fatty acids (PUFAs) are generally prescribed for regular use due to the

favourable effects of these fatty acids on human system. The two major forms of omega-3 PUFA are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Among the omega-3 PUFAs, the most biologically active form is docosahexaenoic acid and therefore it is considered to be comparatively more important to the body. DHA has been found in fairly good levels at healthy individuals. It has been suggested to have good curative efficacy against organ abnormalities. Moreover, the remarkable ability of this fatty acid to up regulate the antioxidant defense mechanism of the body by relieving oxidative stress makes it an effective natural product (Stillwell et al., 2005; Saw et al., 2013; Capó et al., 2014). Hence this study was designed to assess the protective effect of DHA against As_2O_3 induced toxicity in cardiomyocytes.

The metabolic conversion of yellow methyl tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) into blue formazan crystals, by mitochondrial dehydrogenases of live cells is a commonly used biologic assay for cytotoxicity screening. The estimation of viability by MTT assay based on the reduction of MTT by the cells that remain alive after exposure and incubation with a test chemical serves an excellent indicator method of cellular metabolism. The yellow water-soluble form of the salt is converted to an insoluble, intracellular purple formazon metabolite by the mitochondrial dehydrogenases at the cytochrome b and c sites of live cells. The so formed formazon is solubilized by dimethyl sulfoxide (DMSO) which can be quantified spectrophotometrically yielding results related to the proportion of live cells. The detection of cell growth by MTT reduction correlates well with indices of cellular protein and viable cell number (Murray et al., 2000; Furukawa et al., 2004; Cetin and Bullerman., 2005; Ozdemir et al., 2009). The enzyme, lactate dehydrogenase, acts as a marker for intact cell. This intracellular enzyme gets accumulated at extracellular spaces in damaged or dead cells due to cell membrane damage. Unlike most other released enzymes, LDH has more stability in medium for longer periods and thus this enzyme levels gives an idea about the cellular damage coupled with cell viability (Legrand et al., 1992; Haslam et al., 2000; Fischer et al., 2003; Zhang et al., 2014).

Our study employed the above mentioned methods as preliminary dose standardization assays. The effects of As_2O_3 , DHA and their combinations were studied in H9c2 cardiomyocytes through MTT and LDH release assays. The assays were performed with time duration

of 24 hours as Pre-treatment, Co-treatment and Post treatment experiments..

Materials and Methods

Chemicals: Arsenic trioxide and docosahexaenoic acid were purchased from Sigma (USA). Antibiotic-antimycotic solution, Fetal Bovine Serum (FBS), 3-(4, 5, dimethylthiazol-2-yl)-2, 5, diphenyl tetrazolium bromide (MTT) assay kit, dimethyl sulfoxide (DMSO), Minimum Essential Medium Eagle (MEM), Trypsin - EDTA solution and other chemicals were purchased from Hi Media (Mumbai, India).

Cell line maintenance: H9c2 cardiomyocyte cell line was sourced from the cell repository of National Centre for Cell Science (NCCS), Pune, India. The cell line was grown in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 10 ml/l 100 × antibiotic – antimycotic solution containing 10,000 units of penicillin and 10 mg/ml streptomycin in 0.9% normal saline. The cell line was subcultured in tissue culture flasks and was allowed to attain confluency at 37°C in presence of 5% CO_2 in humidified atmosphere in a CO_2 incubator. The cells were divided into various experimental groups which include (a) Control cells; (b) Negative control (Ethanol which was used for dissolving DHA); (c) Cells treated with varying concentration of As_2O_3 for 24 hours; (d) Cells treated with varying concentration of DHA for 24 hours; (e) Cells treated with varying combinations of As_2O_3 and DHA for 24 hours.

Experimental pattern: For carrying out the experiments, three types of treatment patterns were adopted: the Pre-treatment, Co-treatment and Post treatment patterns. The Pre-treatment method comprises DHA administration followed by incubation for a period of 24 hours at 37°C in presence of 5% CO_2 in humidified atmosphere in a CO_2 incubator before the administration of As_2O_3 . In Co-treatment pattern, the cells were administered with both As_2O_3 and DHA simultaneously and then incubated for 24 hours. The treatment of As_2O_3 was followed by DHA in the Post treatment method.

Detection of cell viability: The cardiomyocytes were allowed to attain 80% confluency in well plates. Different concentrations of As_2O_3 , DHA and their combinations were administered as Pre-treatment, Co-treatment and Post treatment patterns on attaining confluency. The viability analysis of cells under different experimental groups was detected

colorimetrically after 24 hours of incubation by the MTT assay. In this assay, the reduction of yellow 3-(4, 5, dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase is detected which also indicates the ability of cells to carry out metabolic activities. The cells were washed twice with 1 X PBS. MTT solution at a concentration of 5 mg of MTT / L of PBS was then added to the cells. The cardiomyocytes were then incubated for 3 hours in a CO₂ incubator thermostated at 37°C and having a supply of 5% CO₂. On completion of the incubation period, the cells were washed with 1 X PBS followed by solubilization with the organic solvent dimethyl sulfoxide. The MTT taken up by the cells passes into the mitochondria, where it gets reduced to an insoluble, coloured formazan product. The formazan product released was measured colorimetrically using an ELISA plate reader (Erba Manheim, Germany) at 540 nm. Since reduction of MTT reagent can only be facilitated by metabolically active cells, the level of activity is a measure of the viability of the cells (Cetin and Bullerman., 2005). MTT assay was performed using commercially available kit [Hi Media Pvt Ltd (Mumbai, India)].

Evaluation of cell damage by lactate dehydrogenase release: The endogenous enzyme lactate dehydrogenase (LDH), constitutes an important member of the glycolytic pathway of cells. This enzyme catalyses the conversion of pyruvate to lactate inside the cells. Damage to the cellular membranes results in the leakage of this enzyme into the extracellular spaces of cells. Thus LDH acts as an indicator of irreversible cell membrane damage leading to apoptosis. The level of LDH in the culture medium was detected according to the method of Renner et al (2003). For performing the assay, the cell free supernatant was mixed with potassium phosphate buffer, 6mM NADH solution and sodium pyruvate solution. The alteration in optical density was recorded at 340nm.

Statistical Analysis: The results were obtained from repeated experiments. The experimental data were represented as mean (\pm SD). The results were analyzed statistically using the statistical software Origin, version 7 (OriginLab Corporation, Northampton, USA). $p < 0.05$ was considered as significant in all the experiments.

Results

Cardiomyocyte viability was safe guarded by docosahexaenoic acid

As₂O₃ administration resulted in significant ($p < 0.05$) dose dependant reduction of H9c2 cardiomyocyte viability after 24 hours of incubation with this chemical. The higher level of viability was observed with 2.5 μ M which steadily gets reduced with increasing dose of As₂O₃ to reach the lowest viability with 15 μ M. This indicates the cytotoxic potential of this chemical agent in cardiomyocytes (Fig 1a). On the other hand, the administration of DHA was found not to cause any significant reduction in viability of cells. This was the indication of non-toxic effect of DHA on H9c2 cardiomyocytes (Fig 1b). Hence the combination of As₂O₃ along with DHA was administered to test the effect of their combination in cardiomyocyte viability. The three combination treatment patterns adopted were Pre-treatment, Co-treatment and Post treatment methods. The Pre-treatment and Co treatment patterns were found to be effective in H9c2 cells against As₂O₃ induced toxicity. The most effective dose of DHA was found to be 100 μ M and it was found to protect H9c2 cells against As₂O₃ at a dose of 10 μ M in Pre-treatment and Co-treatment methods. Beyond 10 μ M, the protective effect of DHA was found to be reduced. The administration of DHA as Pre-Treatment pattern was found to offer maximum protection. However DHA administration was found not to impart any protective effect in Post treatment method (Fig 1c – 1h).

DHA administration reduced the release of lactate dehydrogenase from H9c2 cardiomyocytes

As₂O₃ has been found to cause enhanced leakage of LDH from H9c2 cells at significant levels ($p < 0.05$) after 24 hours of incubation. The release of LDH was found to be based on a dose dependant manner with the highest level of LDH release with 15 μ M of As₂O₃. This once again signifies the cytotoxic effect of As₂O₃ to the H9c2 cells. The elevated release of LDH indicated that the cellular membranes subjected to As₂O₃ administration had undergone irreversible damage, resulting in the release of endogenous LDH to the extracellular spaces. The DHA control groups showed no significant alteration in LDH release from the normal control, again indicating that DHA was not toxic to H9c2 cells. DHA in combination with As₂O₃ as Pre-treatment and Co-treatment methods resulted in

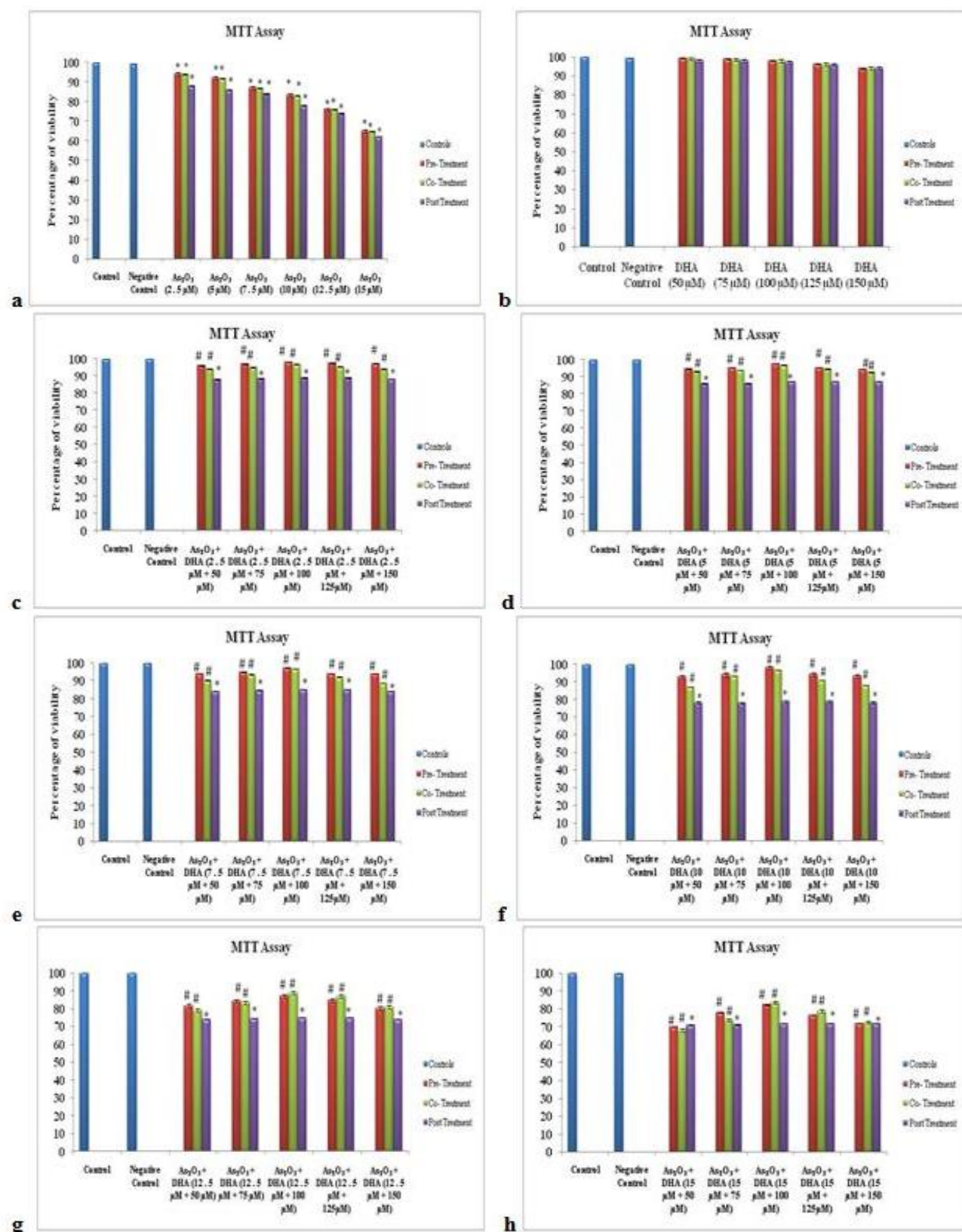


Fig. 1 MTT Assay (24 hours) of H9c2 cells. (As₂O₃ - Arsenic trioxide; DHA - Docosahexaenoic acid); 0.2% Ethanol is used as negative control. Data represented as mean±SD,* p 0.05 versus normal control group. The negative control used was ethanol which was used for dissolving DHA.

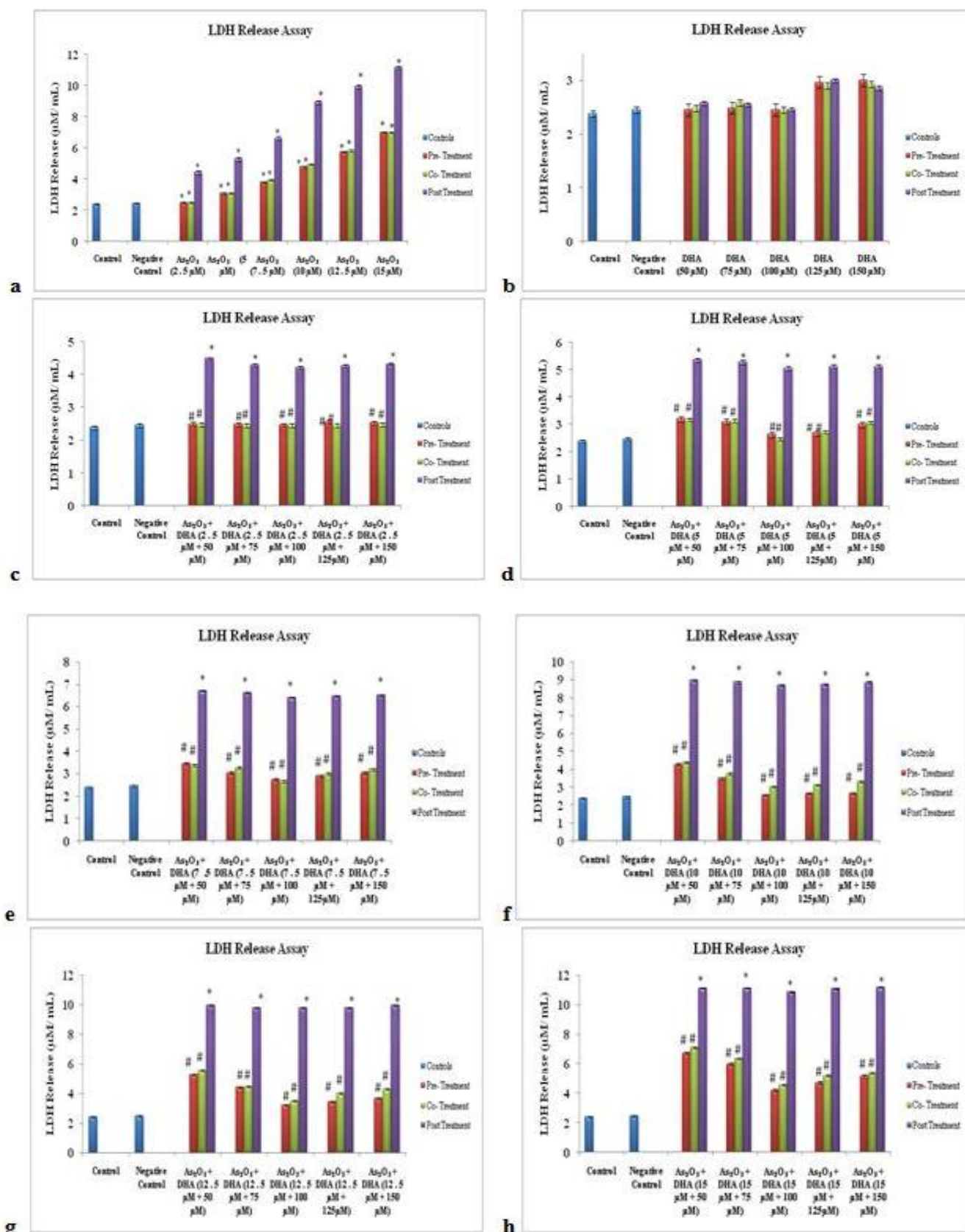


Fig. 2 LDH releasing assay of H9c2 cells; 24 hours. (As₂O₃ - Arsenic trioxide; DHA - Docosahexaenoic acid); 0.2% ethanol is used as negative control. Data represented as mean± SD,* p 0.05 versus normal control group. The negative control used was ethanol which was used for dissolving DHA.

bringing down the LDH levels which showed significant ($p < 0.05$) difference from the As_2O_3 alone treated groups. This indicated the protective capability of DHA signifying that DHA has membrane protecting effect. The highly effective dose of DHA was found to be $100 \mu M$ and it showed its maximum efficacy with $10 \mu M$ of As_2O_3 in both Pre-treatment and Co-treatment experiments. The Pre-treatment method was found to be the most effective treatment pattern. The Post treatment method on the other hand failed to produce any protective effect in H9c2 cells (Fig 2c – 2h).

Discussion

The cascades of molecular events that obstruct macromolecular synthesis, leading to unequivocal functional and structural damage in cells are generally represented by the term 'cytotoxicity' (Ozdemir et al., 2009). The effective cancer drug against APL, arsenic trioxide, is also known to cause side effects in humans. This harmful characteristic of arsenic trioxide therapy offers the major obstacle for elucidating its therapeutic potential. The major side effect, cardiotoxicity, is caused mainly by enhanced oxidative stress resulting in alterations in membrane permeability of cardiac cells (Ficker et al., 2004). The cell line model adopted for the study was H9c2 cardiomyocytes. This cell line is derived from embryonic rat heart and it has almost adult cardiomyocyte like features. Hence H9c2 cells are widely used as an experimental model to explore the molecular mechanism of cardiomyocyte pathophysiology (Sipido et al., 1991). Studies have suggested that arsenic is normally transported by aquaglyceroporins, which are the main transporters of water. Due to the resemblance with the substrates of these transporting proteins, it becomes easy for the inorganic arsenic compounds to enter the cardiac cells (Smith et al., 2000; Liu et al., 2004; Rahman et al., 2005; Zhao et al., 2008; Zhang et al., 2013). Studies have reported that arsenic has the potential to accumulate in organs including heart (Zhang et al., 2013; Mathews et al., 2014). Any damage at cellular level may affect the heart resulting in abnormal responses like arrhythmias which may ultimately affect the entire human system. Hence the study on arsenic trioxide induced toxicity in cardiac cells deserves special attention.

The viability analysis using MTT method is widely considered as a sensitive indicator to assess the cytotoxicity of various chemicals. This assay reflects not only the viable cell number but also the cellular metabolic status. The advantages of this method

include effortless, quickness, repeatability and it does not require radioisotopes (Ozdemir et al., 2009; Stockert et al., 2012). The results obtained from our study showed a significant dose dependent reduction in viability of As_2O_3 treated groups of H9c2 cells from the MTT assay. This showed the adverse effects of increasing concentration of As_2O_3 on the cellular metabolic status along with cell viability. The extracellular concentration of LDH was found to increase with the elevation in concentration of As_2O_3 . Studies have suggested that up regulated leakage of LDH may be due to the damage of cell membranes. The change in normal cellular architecture due to damage of membranes may result in the enhanced leakage of cellular constituents to the extracellular spaces thereby facilitating apoptosis (Legrand et al., 1992; Haslam et al., 2000; Fischer et al., 2003; Zhang et al., 2014). Results obtained from LDH release assay agrees with the observations from the MTT assay. Hence altogether, these two assays indicated the cytotoxic potential of As_2O_3 .

DHA constitutes the biologically active and most unsaturated form of omega-3 fatty acids. This fatty acid acts as an important component of cell membranes especially in the brain, retina, liver and heart. DHA also acts as the precursor of signaling molecules called docosanoids. Studies have reported that docosahexaenoic acid has been equipped with anti-inflammatory, hypolipidemic and antioxidant properties and thereby exerts favourable effects on organ function (Stillwell et al., 2005; Saw et al., 2013; Capó et al., 2014; Richard et al., 2014). On the basis of these facts, our study was aimed at investigating the toxic potential of As_2O_3 and the ameliorative potential of DHA on this toxicity.

Our study results indicated that DHA administration as Pre-treatment and Co-treatment patterns along with As_2O_3 can ameliorate the cytotoxicity due to As_2O_3 by maintaining the cellular viability along with reduction in LDH release from cells. The Post treatment administration of DHA was found not to be effective in producing any protective effect. Our study results showed that the regular uptake of DHA can save the human system from the side effects caused by arsenic chemotherapy. Studies have pointed out that DHA may possess membrane stabilizing effect. It has been found that supply of DHA results in increased uptake of this fatty acid by the cells. The DHA thus entered the cells has been suggested to incorporate itself into the phospholipids of cell membranes (Pepe et al., 1996; Pepe et al., 2002; González-Peñáz et al., 2006; Chapkin et al., 2008). The reduced leakage of LDH

indicating protection of cells from irreversible damage as observed from our study may be due to the efficacy of DHA in safeguarding the normal cellular architecture by its suggested incorporation in membranes. The failure of the Post treatment method to offer any protection to the cardiomyocytes indicates that DHA administration cannot impart any protective effect once cellular damage has been caused by arsenic.

Conclusion

The results obtained from our study lead us to the conclusion that arsenic trioxide causes toxic side effects in H9c2 cardiomyocytes which was indicated by the reduction in cell viability coupled with enhanced leakage of LDH from the cells. The results from the assays also indicated that the metabolic status in cells was affected along with cell membrane damage. The administration of DHA as an adjuvant with As₂O₃ as Pre- treatment and Co- treatment patterns were found to be effective in protecting the cardiomyocytes from toxicity. However the Post treatment pattern with DHA failed to offer any protection to the cells. Thus the preliminary assays of our study showed that DHA can be used as an effective adjuvant in cancer treatment using As₂O₃. However more in depth studies at molecular levels are mandatory to investigate the mechanisms behind the protective effect of combined administration of As₂O₃ with DHA.

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