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DOCOSAHEXAENOIC ACID ELICITS CYTOPROTECTIVE POTENTIAL AGAINST TOXIC MANIFESTATIONS DUE TO ARSENIC TRIOXIDE ADMINISTRATION IN H9C2 CARDIOMYOCYTES

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ABSTRACT

Docosahexaenoic acid (DHA) is an essential dietary fatty acid and is an important cell membrane constituent which participates in the regulation of cell membrane functions. Arsenic trioxide (As_2O_3), the highly effectual acute promyelocytic leukemia (APL) drug, causes toxic manifestations in cardiac cells. We evaluated the curative potential of DHA on As_2O_3 induced toxicity in H9c2 cardiomyocytes. Administration of As_2O_3 (10 µM) alone resulted in cytotoxic complications such as elevation in reactive oxygen species (ROS) levels with the reduction in levels of intracellular proteins, sodium – potassium and calcium ATPases, free radical scavenging potential and antioxidant levels which were significant ($p<0.05$) from the control group. DHA (100 µM) in combination with As_2O_3 showed significant ($p<0.05$) reduction of cytotoxicity from As_2O_3 treated group which includes replenishment of redox status, proteins, and ATPases levels. Our study findings showed that DHA combination administration can protect H9c2 cardiomyocytes from adversities due to As_2O_3 uptake.

KEYWORDS: Arsenic Trioxide; ATPases; Docosahexaenoic Acid; H9c2 Cardiomyocytes; Reactive Oxygen Species

Omega-3 (n-3) fatty acids comprise a class of polyunsaturated fatty acids (PUFA) possessing a double bond in the third carbon position from the methyl terminal. These fatty acids are essential dietary fats which are associated with an array of health benefits (Chang and Deckelbaum, 2013). Docosahexaenoic acid (DHA; 22:6) is a long chain omega-3 polyunsaturated fatty acid which is abundant in fish and vegetable oils. DHA is one of the key components of cell membranes of all the vital organs (Krauss-Etschmann et al., 2007). In the modern life scenario with the change in food habits, it becomes challenging to get the adequate intake of DHA through diet alone. It has been found that supplementation of DHA can enrich the content of this fatty acid in human system (Swanson et al., 2012).

DHA has been found to impact a broad spectrum of biological effects in both cell culture and animal models. Experimental studies have established that supplementation with DHA alters the membrane phospholipid fatty acid composition of cardiomyocytes primarily by an increase in the proportion of this fatty acid. Evidences showed that DHA posses the ability to regulate cell membrane physicochemical properties such as fluidity, organization and permeability (Leifert et al., 2000). Research findings have suggested that increased consumption of DHA can reduce the risk of coronary heart disease. DHA administration has been found to reduce the triglyceride levels, inflammation and ischemia-reperfusion injury (Lee and An, 2015).

The semimetal arsenic has ubiquitous distribution in the environment as a consequence of both anthropogenic and natural processes (Liao et al., 2004). Due to its hazardous effects, arsenic is considered as one of the predominant environmental toxicants (Mandal and Suzuki, 2002). This metalloid exists both as organic and inorganic forms in the environment (Liao et al., 2004). Arsenic is included under Group-1 carcinogens by the International Agency for Research on Cancer (IARC, 1989). On the other hand, the trivalent form of arsenic, arsenic trioxide (As_2O_3), has been used as an efficient therapeutic agent against several malignancies especially, acute promyelocytic leukemia (APL) (Jing et al., 1999).

As_2O_3 impacts its therapeutic effect in malignant cells by causing apoptosis through oxidative stress induction. However, the therapeutic application of this drug is limited by its side effects, especially cardiotoxicity (Mathews et al., 2013) and hepatotoxicity (Banerjee et al., 2009). As_2O_3 administration has been found to cause cardiac manifestations including fatal arrhythmias and sudden cardiac death (Sun et al., 2006). At cellular level, it has been found to cause morphological alterations and enhanced apoptosis. The deleterious effects of As_2O_3 in normal cells are due to the cytotoxic and genotoxic effects mediated through oxidative stress induction. Hence relieving the toxic effects on normal cells is an essentiality for improving the therapeutic efficacy of As_2O_3 (Vineetha et al., 2013). Since the toxicity due to As_2O_3 is burdened by the imbalance of redox status of normal cells, agents that can maintain the cellular redox status will be an effective choice for improving the selectivity of As_2O_3.

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Our previous *in vitro* study using H9c2 cardiomyocytes have reported the beneficial effect of DHA on As_2O_3 induced cytotoxicity which includes maintenance of cell viability, morphology and antioxidant status (reduced glutathione and catalase) near normalcy with reduction in apoptosis (Abhilash et al., 2014). The present study aims at further confirmation of the protective potential of DHA against arsenic toxicity using detection of reactive oxygen species levels, intracellular protein levels, Ca^{2+}-ATPase and Na^{+}-K^{+} ATPase levels, free radical scavenging potential and levels of the antioxidant enzymes, glutathione peroxidase and super oxide dismutase.

MATERIALS AND METHODS

Chemicals

Arsenic trioxide (As_2O_3), docosahexaenoic acid (DHA), 2′, 7′ dichlorodihydrofluorescein diacetate (DCFH-DA) and Rhodamine-123 (R-123) were obtained from Sigma (USA). 2, 2′Azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS) was purchased from Sisco Research Laboratories Pvt. Ltd. (India). Fetal Bovine Serum (FBS), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), Trypsin - EDTA solution and other chemicals were obtained from Hi Media Pvt Ltd (India).

Cell Culture

The H9c2 cell line derived from embryonic rat heart was purchased from the National Centre for Cell Science (NCCS), Pune, India. The cells were allowed to attain confluency and subsequently subcultured in tissue culture flasks in Dulbecco's Modified Eagle's medium (DMEM) having glucose (4.5 g/l), sodium bicarbonate (1.5 g/l) and sodium pyruvate (110 mg/l). The medium was supplemented with 10% Fetal Bovine Serum (FBS) and 1% 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 maintained at 37^0C in humidified atmosphere in a CO_2 incubator. On reaching 70-80% confluency, cells were seeded in well plates at a concentration of 1 X 10^4 cells/100 µL of medium for carrying out the experiments.

Drug Preparation and Experimental Design

Aqueous stock solution (10 mM) of As_2O_3 was prepared and diluted to the final working concentration of 10 µM in DMEM medium. The stock solution of DHA was prepared at a concentration of 140 mM in 99 % ethanol and was stored in cryovials at -80°C. The working standard was prepared at a concentration of 100 µM such that ethanol concentration will be less than 0.05%.

The cells were divided into various groups which included (a) Control cells (b) Cells treated with As_2O_3 (10 µM) (c) Cells treated with DHA (100 µM) (d) Combination treatment group (simultaneous administration of DHA and As_2O_3). The treatment period was 48 hours.

Estimation of Reactive Oxygen Species (ROS) Production

For the visual detection of intracellular ROS, a cell permeable fluorescent probe 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) was used. DCFH-DA can diffuse through the plasma membrane where it undergoes hydrolysis by intracellular esterases to form the non-fluorescent dichlorofluorescin. This non-fluorescent form of the dye undergoes oxidation by ROS to form the fluorescent dichlorofluoresin. Hence the colour developed is directly proportional to the concentration of ROS in cells (Wang and Joseph, 1999). H9c2 cardiomyocytes on attaining semi-confluency were incubated for 48 hours with 10 µM of As_2O_3, 100 µM of DHA and their combination at 37^0 C. After the experimental period, the cells were washed repeatedly with PBS (pH 7.4). DCFH-DA dissolved in absolute DMSO was added and the cells were incubation of in dark at 37^0 C for 30 minutes. Using FITC filter of a fluorescence microscope (Olympus 1 × 51), representative images were taken at 20 × magnification and relative fluorescence of different groups were measured using Image J 1.48 software.

Detection of Intra Cellular Protein Levels

Intracellular proteins play key roles in cells including structural and transport functions and metabolic regulation. The semi-confluent H9c2 cells of different experimental groups were incubated for 48 hours with 10 µM of As_2O_3, 100 µM of DHA and their combination. After the experimental period, cell culture media was removed and the cells were washed repeatedly with PBS (pH 7.4). Cells were then treated with cell lysis buffer with the constituents 0.1 M tris HCl, 0.25 M EDTA, 2 M NaCl and 0.5% Triton X-100. The cell lysate was used for detecting the intracellular concentration of proteins. The protein levels were estimated as per the method of Lowry

et al., (1951), using bovine serum albumin (BSA) as the standard.

Detection of the Activity of Calcium- ATPase (Ca^{2+} - ATPase)

The transport protein Ca^{2+} - ATPase is associated with the regulation of calcium transport across the cell membrane. The different groups of H9c2 cells were treated with As_2O_3 (10 µM), DHA (100 µM) and their combination for 48 hours. On completion of the treatment period, the culture media was removed. The cells were subjected to washing with PBS (pH 7.4) for twice. The washed cells were subjected to lysis with cell lysis buffer containing 0.1 M tris HCl, 0.25 M EDTA, 2 M NaCl and 0.5% Triton X-100. Ca^{2+} - ATPase activity in the cell lysates were measured by the method of Hjerton and Pan, (1983). The assay was carried out using ATP as substrate in the presence of Mg^{2+} and Ca^{2+} ions.

Detection of the Activity of Sodium-Potassium ATPase (Na^+/K^+ -ATPase)

The integral membrane protein Na^+, K^+-ATPase catalyzes the transport of three Na^+ ions out of the cell and two K^+ ions in at the expense of one molecule of ATP. This protein produces electrical and chemical gradients that are necessary for a variety of cellular/ physiological functions. The semi-confluent H9c2 cells were subjected to treatment with As_2O_3 (10 µM), DHA (100 µM) and their combination for 48 hours at 37^0 C. After the completion of experimental period, the cells were treated with cell lysis buffer (0.1 M tris HCl, 0.25 M EDTA, 2 M NaCl and 0.5% Triton X-100). The cell lysates were centrifuged at 3000 rpm for 15 minutes and the supernatant was retained. Na^+/K^+-ATPase activity in cell lysates belonging to different experimental groups were quantified according to the method of Bonting et al., (1970). This assay was done in the presence of Na^+ and K^+ ions.

Analysis of Free Radical Scavenging Potential

The H9c2 cells were seeded in well plates at a concentration of 1 X 10^4 cells/100 µL of cell culture media. On attaining semi-confluency, the cells were treated for 48 hours with As_2O_3 (10 µM), DHA (100 µM) and their combination. After the treatment period, the cells were washed twice with PBS (pH 7.4) for removing traces of medium. The free radical scavenging potential of cells belonging to different groups was analyzed by ABTS assay according to the method of Re et al. (1999). The colourless compound ABTS gets oxidized to the blue–green counterpart $ABTS^+$ by the loss of an electron of the parent compound. The ABTS stock solution was mixed with 2.45 mM potassium persulfate and the mixture was allowed to stand in dark at room temperature for 16 hours prior to use. This results in the generation of blue–green $ABTS^+$. 200 µL of $ABTS^+$ solution was added to the samples and was allowed to stand at room temperature under dark condition for 15 minutes. The absorbance of the samples was measured at 734 nm. Ascorbic acid was used as the standard.

Estimation of the Levels of Major Antioxidant Enzymes

The levels of the major antioxidants glutathione peroxidase (GPx) and superoxide dismutase (SOD) were detected in H9c2 cells after the completion of treatment period. GPx assigned with the reduction of lipid hydroperoxides to their corresponding alcohols and free hydrogen peroxide to water was assayed according to the method of Rotruck et al (1973). SOD, the enzyme that catalyzes the dismutation of the superoxide (O_{2-}) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2), thus protecting the cells from oxidative damage was measured by the method of Kakkar et al (1984).

Statistical Analysis

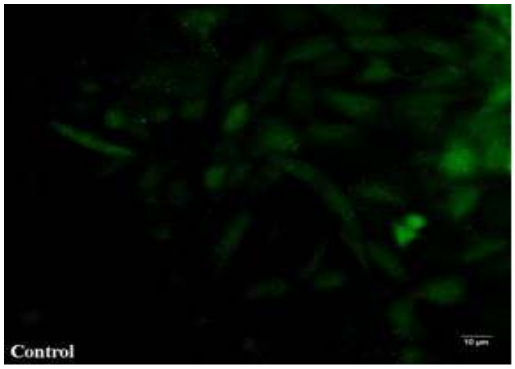
Results obtained from the experiments were expressed as mean ± standard deviation. Statistical evaluation was carried out by one-way analysis of variance (ANOVA) using the statistical software, SPSS (version 20.0, SPSS Inc, Chicago, IL, USA). Experimental values were considered as significantly different if $p<0.05$.

RESULTS

DHA Mitigates the Reactive Oxygen Species Production by As_2O_3

Detection of ROS levels gives an indication of free radical production inside the cells. We observed higher levels of ROS production in cells subjected to As_2O_3 exposure (Fig 1B). This was indicated visually by the higher levels of green fluorescence. The enhanced green fluorescence is due to the oxidation of non-fluorescent dichlorofluorescin (DCFH) to fluorescent dichlorofluoresin (DCF) by higher levels of ROS. Cells which were treated with DHA alone showed less fluorescence, indicating basal levels of ROS production (Fig. 1C). DHA combination administration was found to be effective in reducing the As_2O_3 induced ROS

production as indicated by the basal levels of fluorescence (Fig. 1D).



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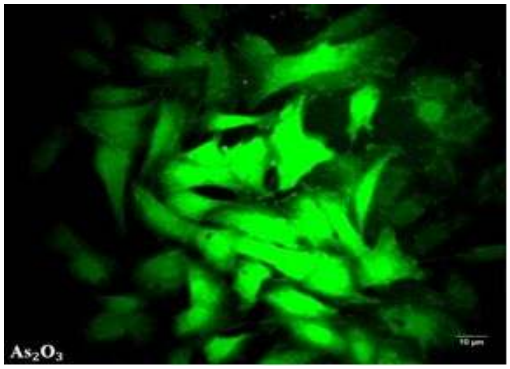

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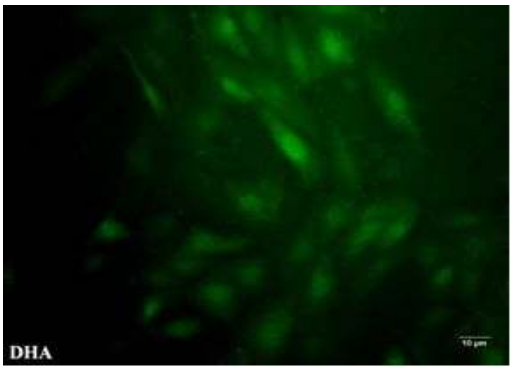

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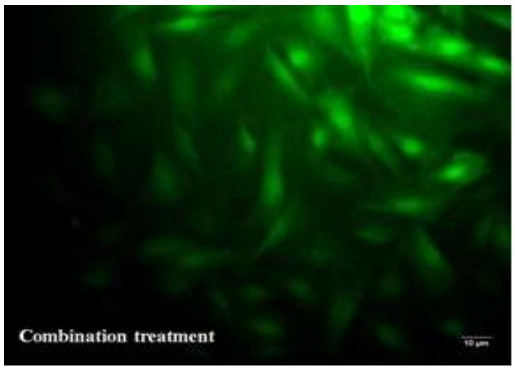

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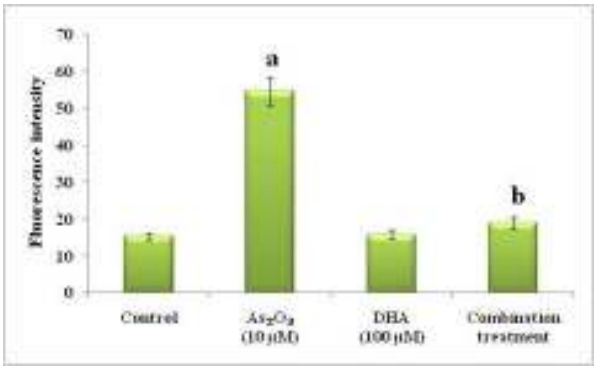

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Figure 1: Reactive oxygen species (ROS) assay of H9c2 cells belonging to different experimental groups. ROS production is detected visually by green fluorescence due to the oxidation of non-fluorescent dichlorofluorescin (DCFH) by ROS to fluorescent dichlorofluoresin (DCF) (Original magnification × 20). A Control cells; B Cells treated with As_2O_3 (10 µM); C Cells treated with DHA (100 µM); D Cells treated with the combination of As_2O_3 (10 µM) + DHA (100 µM); E Graph showing the fluorescence intensity of cells which was measured using the Image J 1.48 software. Fig. 1B (As_2O_3 treated group of cells) showed higher fluorescence, indicating higher levels of ROS production. DHA when given in combination with As_2O_3 (Fig. 1D) reduced the oxidative stress as indicated by reduction in fluorescence. Data represented as mean± SD, n=6. a (indicates significant difference between Control and As_2O_3), b (indicates significant difference between As_2O_3 and Combination treatment group). $p<0.05$was considered to be significant.

Effect of DHA administration on intracellular protein levels

Intracellular proteins are involved in carrying out the normal metabolism and signal transduction along with numerous other functions in cells. We observed a significant reduction in levels of intracellular proteins in cells subjected to As_2O_3 (10 µM) exposure for a period of 48 hours. This reduction was significantly different ($p<0.05$) from the normal control group of H9c2 cells. DHA as a single agent maintained the protein levels near normal levels. In the combination treatment group, DHA showed substantial efficacy in bringing back the intracellular protein levels near normalcy which was significantly different ($p<0.05$) from the As_2O_3 alone treated group (Fig. 2).

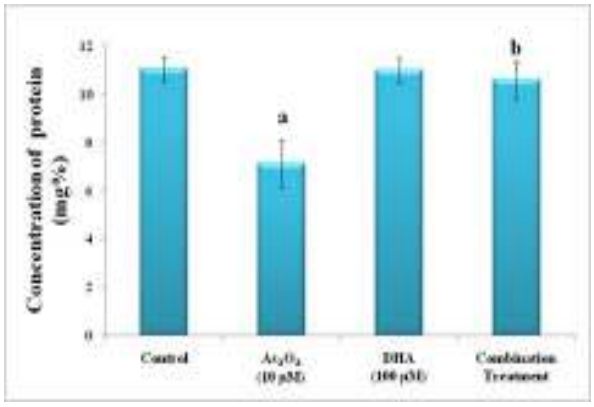

Figure 2: Assay on intracellular protein levels of H9c2 cells belonging to different experimental groups: Control; As_2O_3 -10 µM; DHA - 100 µM; Combination treatment (As_2O_3 -10 µM + DHA - 100 µM). Data represented as mean± SD, n=6. a (indicates significant difference between Control and As_2O_3), b (indicates significant difference between As_2O_3 and Combination treatment group). p<0.05was considered to be significant.

DHA was effective in regulating the normal levels of Ca^{2+} -ATPase in cardiomyocytes

Fig. 3 shows the levels of Ca^{2+}-ATPase in H9c2 cells which were categorized into different experimental groups. As_2O_3 administration resulted in a significant ($p<0.05$) reduction in levels of this transport protein when compared with the normal control group. This is an indicative of abnormal regulation of calcium transport in the cells. The combination treatment with DHA was found to be effective in maintaining the levels of this protein in cells which showed a significant difference from the As_2O_3 alone treated group of cells.

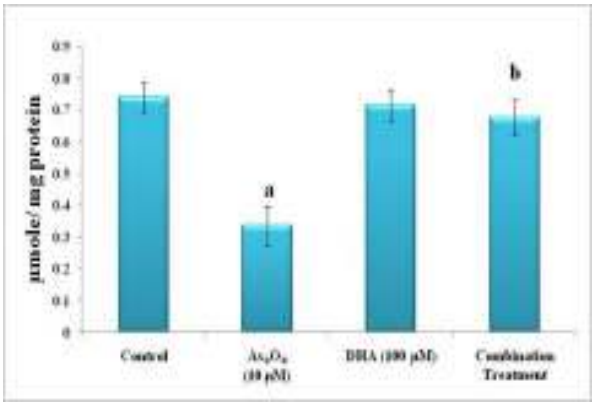

Figure 3: Assay on Ca^{2+}-ATPase levels of H9c2 cells belonging to different experimental groups: Control; As_2O_3 -10 µM; DHA - 100 µM; Combination treatment (As_2O_3 -10 µM + DHA - 100 µM). Data represented as mean± SD, n=6. a (indicates significant difference between Control and As_2O_3), b (indicates significant difference between As_2O_3 and Combination treatment group). p<0.05 was considered to be significant.

DHA combination treatment maintains the normal levels of Na^+/K^+-ATPase in H9c2 cells

As_2O_3 was found to cause a significant ($p<0.05$) reduction in levels of the integral membrane protein Na^+/K^+-ATPase in H9c2 cells, when compared with the control group. This indicates the abnormal regulation of Na^+ and K^+ ions transport across the cell membrane. DHA when administered in combination with As_2O_3 was found to maintain the levels of this transport protein near normalcy. This again signifies the cytoprotective effect of DHA against As_2O_3 (Fig 4).

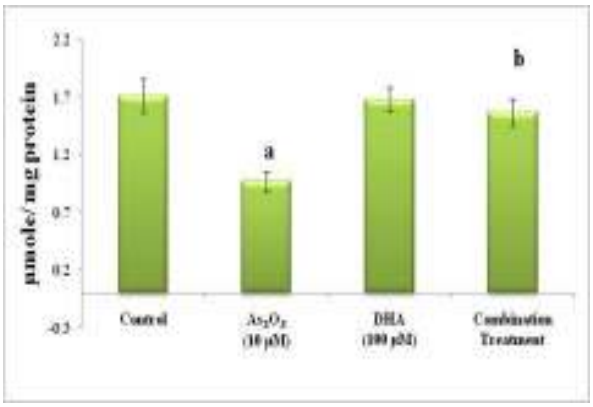

Figure 4: Assay on Na^+/K^+-ATPase levels of H9c2 cells belonging to different experimental groups: Control; As_2O_3 -10 µM; DHA - 100 µM; Combination treatment (As_2O_3 -10 µM + DHA - 100 µM). Data represented as mean± SD, n=6. a (indicates significant difference between Control and As_2O_3), b (indicates significant difference between As_2O_3 and Combination treatment group). p<0.05was considered to be significant.

DHA maintains the free radical scavenging potential of H9c2 cardiomyocytes

The free radical scavenging potential of H9c2 cardiomyocytes of different experimental groups was estimated from the decolourisation of $ABTS^{*+}$, which was detected spectrophotometrically at 734 nm. The results obtained have been expressed as percentage inhibition in comparison to the normal control. The agents which cause higher free radical production will show higher readings and hence lower inhibition/ lower percentage of scavenging. As_2O_3 was found to cause higher levels of free radical generation as indicated by the significantly

($p<0.05$) lower percentage of free radical scavenging when compared with the control group. DHA as a single agent showed higher levels of free radical scavenging. In the combination treatment group, DHA showed substantial efficacy in bringing down the free radical production by As_2O_3 (Fig. 5).

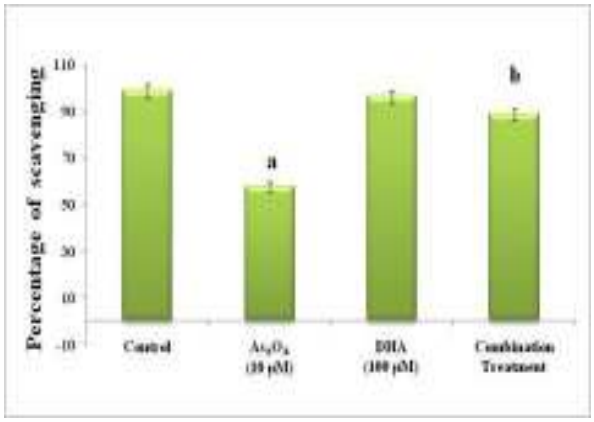

Figure 5: Free radical scavenging assay of H9c2 cells belonging to different experimental groups: Control; As_2O_3 -10 µM; DHA - 100 µM; Combination treatment (As_2O_3 -10 µM + DHA - 100 µM). Data represented as mean± SD, n=6. a (indicates significant difference between Control and As_2O_3), b (indicates significant difference between As_2O_3 and Combination treatment group). p<0.05was considered to be significant.

Effect of DHA on antioxidant defense markers

Glutathione peroxidase (GPx) is an important antioxidant which is involved in reducing organic hydroperoxides and lipoproteins in cells, thereby protecting the cells from free radical induced damages. Fig. 6 presents the levels of GPx in various groups of H9c2 cells. We can observe from the graph that As_2O_3 (10 µM) was able to reduce the levels of GPx to a significant level ($p<0.05$) from the control group of cells. DHA (100 µM) was found to impact a positive effect on this antioxidant status with levels near to that observed in control group of cells. Moreover DHA in combination with As_2O_3 was found to be effective in bringing back GPx back to levels observed in normal control, indicating the protective potential of this fatty acid.

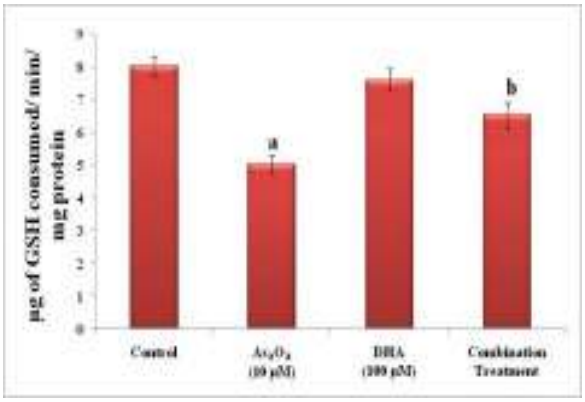

Figure 6: Assay on glutathione peroxidase levels of H9c2 cells belonging to different experimental groups: Control; As_2O_3 -10 µM; DHA - 100 µM; Combination treatment (As_2O_3 -10 µM + DHA - 100 µM). Data represented as mean± SD, n=6. a (indicates significant difference between Control and As_2O_3), b (indicates significant difference between As_2O_3 and Combination treatment group). p<0.05was considered to be significant.

Fig. 7 presents the levels of the antioxidant superoxide dismutase (SOD) as observed from our study. SOD catalyzes the dismutation of superoxide to H_2O_2 which in turn is removed by the antioxidant catalase. This results in protection of cells from further oxidative damages. We observed a significant ($p<0.05$) reduction in levels of this antioxidant in As_2O_3 alone treated group of cells. Supplementation of DHA was found to maintain the levels of SOD near levels observed in control cells, which was significantly ($p<0.05$) different from the arsenic treated group.

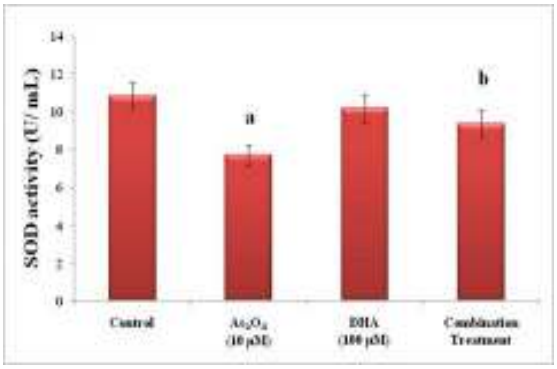

Fig. 7 Assay on superoxide dismutase levels of H9c2 cells belonging to different experimental groups: Control; As_2O_3 -10 µM; DHA - 100 µM; Combination treatment (As_2O_3 -10 µM + DHA - 100 µM). Data represented as mean± SD, n=6. a (indicates significant difference between Control and As_2O_3), b (indicates

significant difference between As_2O_3 and Combination treatment group). p<0.05was considered to be significant.

DISCUSSION

The present study described the effects of DHA on As_2O_3 –induced cytotoxicty in a cardiomyocyte cell line, the H9c2 cardiomyocytes. Our study has showed that in comparison with As_2O_3 alone treated group, the combination treatment group of DHA with As_2O_3 showed reduction of ROS production, maintenance of intracellular protein levels, cellular ATPases levels (Ca^{2+} -ATPase and Na^+/K^+-ATPase), free radical scavenging potential and antioxidant status. These results provide an idea by which DHA attenuates the cardiotoxic complications caused by As_2O_3.The concentrations of As_2O_3 (10 µM) and DHA (100 µM) were selected based on the pilot studies conducted in our research laboratory. In the pilot study we found that DHA combination strategy was effective in protecting H9c2 cardiomyocytes against arsenic toxicity by maintaining the cellular viability, morphology and certain redox parameters and apoptosis similar to that of the normal control group of cells (Abhilash et al., 2014).

The H9c2 cell line was originally derived from the embryonic rat heart tissue and it shows many similarities to primary cardiomyocytes, including membrane morphology, signalling and protein levels (Hescheler et al., 1991; Sipido and Marban, 1991). Reactive oxygen species (ROS) or free radicals are produced as the byproducts or intermediates of aerobic metabolism and also through reactions with drugs and toxins. The amount of ROS production and the activity of antioxidant defense system in cells are the determinant factors which decide whether the impacts of ROS are beneficial or harmful (Halliwell, 1996). Direct evaluation of ROS presents an accurate indication of oxidative damage in living cells (Wang and Joseph, 1999). The augmented cellular levels of free radicals cause damages to membrane lipids, nucleic acids and proteins (Halliwell, 1996). Our study results showed that As_2O_3 administration at a dose of 10 µM caused a significant elevation of ROS production in H9c2 cardiomyocytes. Our observation is in line with some previous studies indicating that arsenic has the capability to cause accumulation of ROS in cells (Chen et al., 1998). The arsenic induced ROS generation has been found to play a central role in cardiac cell damage (Das et al., 2010). On the other hand, when As_2O_3 was administered to H9c2 cells along with DHA, ROS levels were found to be significantly reduced in comparison with As_2O_3 alone treated group. This showed the efficacy of DHA in reducing As_2O_3 induced intracellular ROS generation in H9c2 cells. Richard et al., (2009) reported that DHA can down regulate the intracellular production of NADPH oxidases, the major enzyme behind ROS production in cells. This may be the underlying reason behind the reduction in ROS production by DHA in combination treatment as observed in our study.

Proteins are major intracellular agents assigned with the function of carrying out the duties as per the information encoded in genes (Lodish et al., 2004). Proteins have the capability to bind other proteins, lipids as well as small-molecule substrates specifically and tightly (Sankaranarayanan and Moras, 2000). Through this binding, proteins regulate enzymatic activity, cell cycle progression, cell membrane characteristics and other biological functions (Mathews and Van Holde, 1990). Since proteins constitute approximately 68% of the dry weight of cells, they are potentially the major targets for oxidative damage. The damage caused by oxidative agents has been found to be non-repairable and has deleterious consequences on protein structure and function (Davies, 2003). Enhanced levels of oxidative stress impacted by As_2O_3 can be one of the reasons for the reduction in levels of intracellular proteins as observed in our study. Trivalent forms of arsenic have natural affinity for sulfhydryl groups and can bind to reduced cysteine residues in peptides and proteins. The binding of arsenic to a specific protein alters the protein conformation, bringing about loss of its function, and can inhibit its interaction with other proteins and DNA (Shen et al., 2013). Hence along with oxidative stress, direct binding of arsenic to proteins can also contribute to the reduction in levels of intracellular proteins. DHA in combination with As_2O_3 showed substantial efficacy in maintaining the protein levels similar to that of the control group of cells. Ghule et al. (2015) reported that the fatty acids containing DHA may offer protection to thiol groups through inhibition of the generation of peoxidative products of oxidative damage. Since arsenic attacks mainly the thiol groups of proteins, protection of these groups by DHA may be the underlying reason for the protective effect of DHA on intracellular proteins.

The detection of membrane associated enzyme activities such as ATPases gives an indication about membrane modifications under pathological circumstances in cells. The ATPases such as Na^+/K^+-ATPase and Ca^{2+}-ATPase are integral membrane proteins which are

associated with regulation of the transport of major ions such as Na^+, K^+ and Ca^{2+} across the cell membranes. Proper regulation of intracellular ionic balance is necessary for maintaining the normal homeostasis in cardiomyocytes (Dessi et al., 2013). In the present study we observed a significant decrease in the activity of the ATPases, Na^+/K^+-ATPase and Ca^{2+}-ATPase, in As_2O_3 treated group of cells. Decline in activity of Na^+/K^+-ATPase has been reported to cause a decrease in sodium efflux. The reduction in sodium efflux can alter membrane permeability. The major adverse effect associated with alteration of membrane permeability or membrane fragmentation is the leakage of ions such as Ca^{2+} into cells. The higher levels of Ca^{2+} ions in cells may lead to reduction in activity of Ca^{2+}-ATPase (Prabu and Muthumani, 2012). Along with this, enhanced Ca^{2+} levels may result in irreversible cell damage by potentiating the action of phospholipases and accumulation of lipid metabolites (Leifert et al., 1999).

The ATPases, Ca^{2+}-ATPase and Na^+/K^+-ATPase are thiol roup containing and lipid dependent enzymes. Enhanced oxidative stress due to higher levels of ROS can inhibit the activity of these enzymes (Ghule et al. 2015). In addition to this, the fascination of arsenic to the thiol groups of these enzymes can also inhibit their functioning. Supplementation of cells with fatty acids such as DHA has been found to modify the cell membrane composition and enhance the membrane fluidity (Jahangiri et al., 2006). The enhancement of membrane fluidity results in better regulation of the activity of transmembrane proteins, including receptors, enzymes, and ion channels along with reduction in stress induced damages (Dessi et al., 2013). DHA has also been found to posses the ability to control the rate of Ca^{2+} uptake as well as its release from the sarcoplasmic reticulum of cells (Jahangiri et al., 2006). Hence maintenance of the activities of ATPases as observed in our study may primarily be due to the ability of DHA to affect cell membrane properties.

Antioxidants are agents that posses the ability to hinder free radical generation, scavenge free radicals, and/or diminish the oxidation and damage due to these radicals. The antioxidant defense system of cells comprise several components such as enzymes, free radical scavengers, inhibitors to free radical generating enzymes and metal chelators (Shi et al., 2004). Free radical scavenging assay or total antioxidant assay refers to detection of the cumulative effect of entire antioxidant defense system of cells. ABTS method is the widely accepted method and it is based on the capability of entire antioxidant molecules in cells to reduce the radical cation of ABTS (Re et al., 1999). As_2O_3 administration was found to cause a significant decline in the free radical scavenging potential of H9c2 cells. This showed that the free radical production induced by arsenic is much more than the capability of the cellular defense systems to neutralize them. However the combination treatment with DHA was found to be effective in replenishing the cellular free radical scavenging potential. Our observation agrees with the findings of Saw et al., (2013) that DHA can induce antioxidant activity in cells.

Among the various antioxidant enzymes in cells, superoxide dismutase (SOD) catalyzes the dismutation of superoxide to H_2O_2. The H_2O_2 so formed is in turn removed by catalase (Usoh et al., 2005). Hence SOD act as a primary defense agent against superoxide anions in cells and this prevents further generation of intracellular free radicals (Prabhu and muthumani, 2012). In our study As_2O_3 as a single agent significantly ($p< 0.05$) reduced the levels of SOD in cells. It has been pointed out by researchers that abnormally higher levels of superoxide radical anions can inhibit SOD activity (Prabhu and muthumani, 2012). The combination of As_2O_3 with DHA was found to be effective in maintaining SOD levels almost similar to that observed in normal cells. Richard et al., (2009) has been reported that DHA has the potential to reduce superoxide radical production against various stimuli in cells. This reduction in superoxide levels by DHA may be the major reason for safe guarding the normal functioning of SOD in combination treatment group.

Glutathione peroxidase (GPx) is a reduced glutathione (GSH) dependant antioxidant enzyme. GPx catalyzes the reduction of organic hydroperoxides within membranes and lipoproteins in the presence of reduced glutathione (GSH) (Messarah et al., 2012). As_2O_3 administration has been found to cause a significant reduction ($p<0.05$) in levels of GPx compared with the normal control cells in the present study. It was previously reported that arsenic can form inactive As- Selenium complex by the interaction with selenocysteine moiety of GPx. This can both inhibit the activity as well as production of GPx in cells (Prabu and Muthumani, 2012). Along with this, the excess consumption of GSH by arsenic and free radicals can also contribute to GPx depletion in cells. The protective potential elicited by combination treatment group may be due to the ability of

DHA to reduce free radical production as well as its ability to offer protection to thiol groups in cells.

CONCLUSION

The observations from our study indicated the cardiotoxic complications of As_2O_3 which include higher ROS production with reduction in free radical scavenging potential, antioxidant status, proteins and ATPases levels. On the other hand, DHA showed sufficient potential in maintaining the redox status, radical scavenging potential, antioxidant status as well as intracellular proteins and ATPase levels. This showed the potential of DHA to counteract As_2O_3 induced toxic manifestations in cardiac cells. Research is going on in our laboratory for elucidating the exact molecular mechanisms behind the protective potential of DHA against As_2O_3 induced cardiotoxicity.

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