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Evaluation of the Effectiveness of Docosahexaenoic acid in protecting Liver cells against Arsenic trioxide induced Toxicity by Preliminary dose Standardization assays

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ABSTRACT- The anticancer drug arsenic trioxide is effective for acute promyelocytic leukemia. But the clinical trials are restricted due to its potential side effects. Since the major part of arsenic metabolism and detoxification occurs in the liver, this organ faces a major threat. The hepatic side effects include fatty liver, fibrosis, and inflammation and hepatocyte degeneration. Our study aimed to evaluate the protective potential of the fatty acid, docosahexaenoic acid, against adversities of arsenic trioxide in an *in vitro* model, the chang liver cells. Two preliminary dose standardization assays, cell viability, and lactate dehydrogenase release assays, were employed. The assays were performed as Pre-treatment, co-treatment, and Post-treatment experiments for a period of 24 hours. Arsenic trioxide at various doses (2.5, 5, 7.5, 10, 12.5 and 15 µM) showed a significant ($p \leq 0.05$) dose-dependent reduction in cell viability along with a dose-dependent enhancement of lactate dehydrogenase release. However when the cells were treated with a combination of docosahexaenoic acid at varying concentrations (50, 75, 100, 125 and 150 µM), the above-mentioned conditions were found to be reversed in pre-treatment and co-treatment experiments, but not in post-treatment. The most effective combination was found to be 10 µM arsenic trioxide with 100 µM of docosahexaenoic acid in both pre-treatment and co-treatment studies. Thus the preliminary assays of our study showed that docosahexaenoic acid administration as pre-treatment or co-treatment can aid in reducing arsenic trioxide induced hepatotoxicity. Further studies are required to elucidate the mechanisms behind the protective effects.

Key-Words: Arsenic trioxide, hepatotoxicity, docosahexaenoic acid, cell damage

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INTRODUCTION

Arsenic, the metalloid has received global public health concern since it is a leading cause of toxicity and has the potential to be a human carcinogen [1]. Arsenic trioxide (As_2O_3), the trivalent form of arsenic, is an effective anticancer agent which is used to treat acute promyelocytic leukemia (APL) patients. However, due to its toxicity profile that includes hepatotoxicity and certain other organ toxicity, the clinical use of As_2O_3 is limited [2,3]. Arsenic has shown the potential capability to impede with different body functions and organs including the liver, central nervous system, heart, and kidney. The liver, the major organ dealing with the metabolism of drugs, also serves as the principal target for many toxic chemicals especially arsenic, following acute arsenic toxicity. Research findings from experimental animals around the globe showed that arsenic induce liver injuries along with alterations in the biochemical markers of hepatic functions, as well as morphological changes and apoptosis of liver cells [4,7].

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The regular use of omega-3 polyunsaturated fatty acids (PUFAs) is prescribed due to the favourable effects of these fatty acids on the human system. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) constitutes the two major forms of omega-3 PUFA. The most biologically active form of omega-3 PUFA is docosahexaenoic acid and hence it is considered to be comparatively more important to the body. DHA is more abundant in most human systems and has good curative efficacy against organ abnormalities. Furthermore, the ability of this fatty acid to positively regulate the antioxidant defense system of the body by relieving oxidative stress makes it a more favourable natural product [8,10]. Hence this study was designed to evaluate the protective potential of DHA against As_2O_3 induced cytotoxicity.

The study of the metabolism of yellow methyl tetrazolium salt, 3- (4, 5- dimethylthiazol-2-yl)- 2, 5-diphentyl tetrazoliumbromide (MTT), by mitochondrial dehydrogenases of liver cells into blue formazan crystals is a commonly used biologic assay for cytotoxicity testing. The viability analysis using MTT assay forms a good indicator method of cellular metabolism based on the reduction of MTT by the cells that remain alive after exposure and incubation with a test chemical or device. Mitochondrial dehydrogenase enzymes at the cytochrome b and c sites of live cells catalyze the conversion of the yellow water-soluble form of the salt to an insoluble, intracellular purple formazon metabolite. The solubilization of Formazon by extraction with alcohol or dimethyl sulfoxide (DMSO) can be quantified spectrophotometrically with results related to the proportion of live cells. The cell growth detection by MTT reduction correlated well with indices of cellular protein and viable cell number [11,14]. Lactate dehydrogenase acts as a marker for intact cell. This endogenous enzyme is released into extracellular spaces at higher levels in damaged or dead cells due to cell membrane damage. Compared with several other released enzymes, LDH is more stable in medium for longer periods and this enzyme level gives an idea about the cell viability. Hence, measuring the catalytic activity of this enzyme in the medium in which the cells grow gives an idea about the damage in cells [15,18].

In our study, we employed the above mentioned two methods as preliminary dose standardization assays. The effect of As_2O_3, DHA and their combinations in an *in vitro* model, the Chang liver cells were analyzed by MTT assay and LDH release assay. The assays were performed as Pre-treatment, Co-treatment and post treatment experiments for a period of 24 hours.

MATERIALS AND METHODS

Chemicals: Arsenic trioxide and docosahexaenoic acid were obtained 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 obtained from Hi Media Pvt Ltd (Mumbai, India).

Cell culture: Chang liver cell line was obtained from the cell repository of National Centre for Cell Science (NCCS), Pune, India. The cell line was maintained in Minimum Essential Medium Eagle (MEM), 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 cells were sub-cultured in tissue culture flasks and were allowed to attain confluency at 37ºC in the presence of 5% CO_2 in humidified atmosphere in a CO_2 incubator. The experimental group consists of (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.

Treatment pattern: The cells were subjected to three types of treatment patterns: the Pre-treatment, Co-treatment and Post treatment patterns. In Pre-treatment method, cells were subjected to DHA administration followed by incubation for 24 hours at 37ºC in the presence of 5% CO_2 in humidified atmosphere in a CO_2 incubator before the administration of As_2O_3. The Co-treatment pattern was where the cells were administered with As_2O_3 and DHA simultaneously and then incubated for 24 hours. The Post treatment method comprises As_2O_3 administration followed by DHA.

Estimation of cell viability: The *in vitro* experimental model, Chang liver cells, was allowed to attain confluency of about 80% in well plates. On attaining confluency, different concentrations of As_2O_3, DHA and their combinations were administered as Pre-treatment, Co-treatment and Post treatment patterns. The viability of cells belonging to various experimental groups was determined colorimetrically after 24 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 and it indicates the ability of cells to carry out metabolic activities. The cells were subjected to washing with 1 X PBS twice. Then MTT solution having a concentration of 5 mg of MTT / L of PBS was added to the cells. The cells with the MTT reagent were incubated for 3 hours in a CO_2 incubator thermostated at 37^0C with a supply of 5% CO_2. After the incubation period is over, the cells were washed with 1x PBS and were then solubilised with the organic solvent dimethyl sulfoxide. The MTT enters and passes into the mitochondria of the cells, where it is reduced to an insoluble, coloured formazan product. The released formaza product was measured using an ELISA plate reader (Erba Manheim, Germany) at 540 nm. Since reduction of MTT can occur only in metabolically active cells, the level of activity is a measure of the viability of the cells [13]. MTT assay was done using commercially available kits from Hi Media Pvt Ltd (Mumbai, India).

Estimation of cell damage by lactate dehydrogenase release: The enzyme lactate dehydrogenase (LDH), an important member of the glycolytic pathway of cells, is normally present in the cytoplasm. This enzyme is associated with the catalytic conversion of pyruvate to lactate. Damage to the membranes of cells results in the leakage of this enzyme into the extracellular spaces of cells. In this way LDH acts as an indicator of cell membrane damage in cells and thereby it indicates irreversible cell damage leading to apoptosis. The level of LDH in the culture medium was estimated by the method of [19]. 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 340 nm.

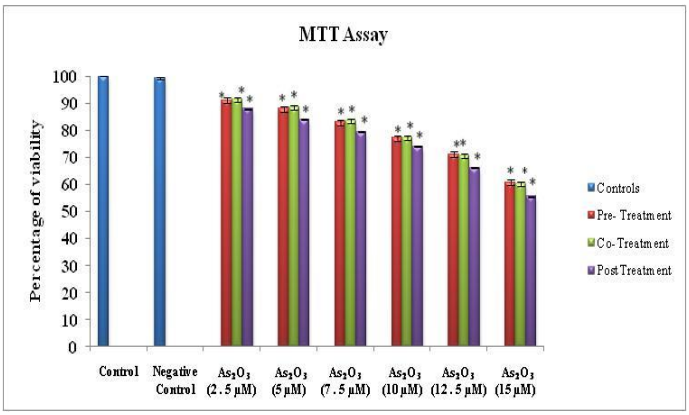
Statistical Analysis: The experimental data were obtained from repeated experiments. Results obtained from the experiments were represented as mean (±Standard deviation). The experimental results were analyzed using the statistical program Origin, version 7 (OriginLab Corporation, Northampton, USA). $p \leq 0.05$ was considered significant.

RESULTS

Docosahexaenoic acid safe guards the viability of cells

$As_2O_3$was found to cause significant ($p \leq 0.05$) dose dependant reduction of cell viability in Chang liver cells after 24 hours of incubation with this chemical. The lowest level of viability (55.52±0.1963) was found in cells subjected to incubation with 15 µM of As_2O_3 (The earlier mentioned viability was found with As_2O_3 at a dose of 12.5 µM). This indicates the toxic potential of this chemical agent in liver cells (Fig 1a). However the administration of the poly unsaturated fatty acid, DHA, was found not to cause any significant reduction in viability of cells. This was the indica-

tion of non-toxic effect of DHA on cells (Fig 1b). Hence the combination of As_2O_3 along with DHA was administered to test the effect of their combination in Chang liver cell viability. The combination treatments were done using Pre-treatment, Co-treatment and Post treatment methods. It was found that Pre-treatment and Co treatment methods have protective effect in Chang liver cells against As_2O_3 induced toxicity. DHA at a dose of 100 µM was found to be more effective and the highest dose of As_2O_3 at which it showed the most efficiency was 10 µM with 97.47±0.269 in Pre-treatment and 95.21±0.635 in Co-treatment methods. However DHA was found not to be effective in post treatment method against As_2O_3 where it failed to produce any significant variation from the As_2O_3 treated groups (Fig. 1C– 1H).



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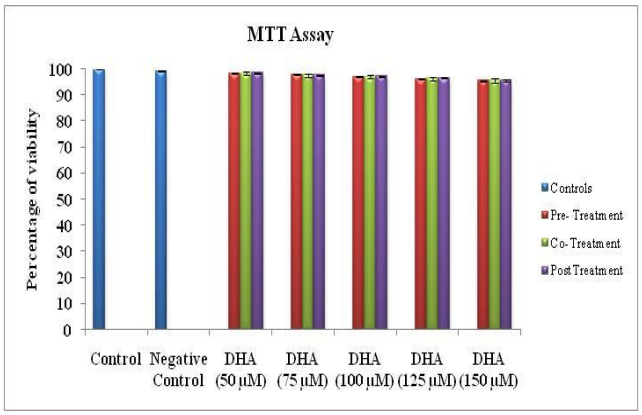

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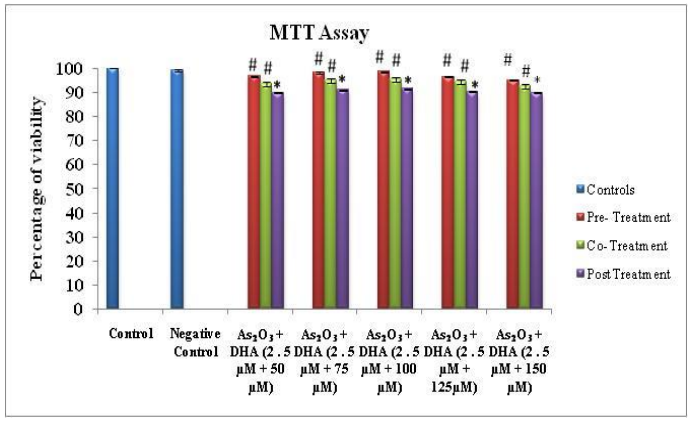

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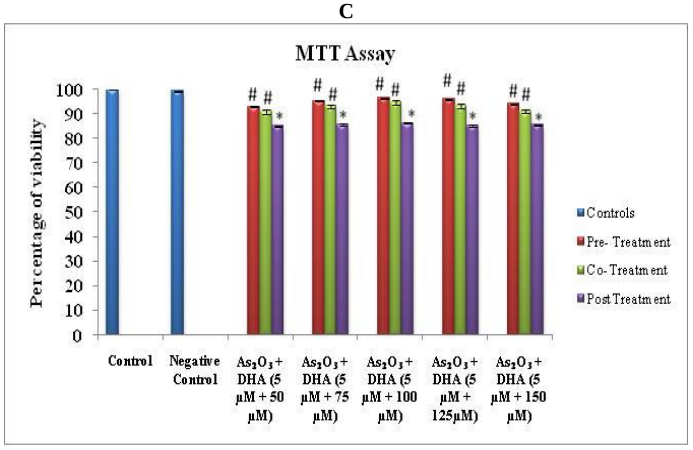

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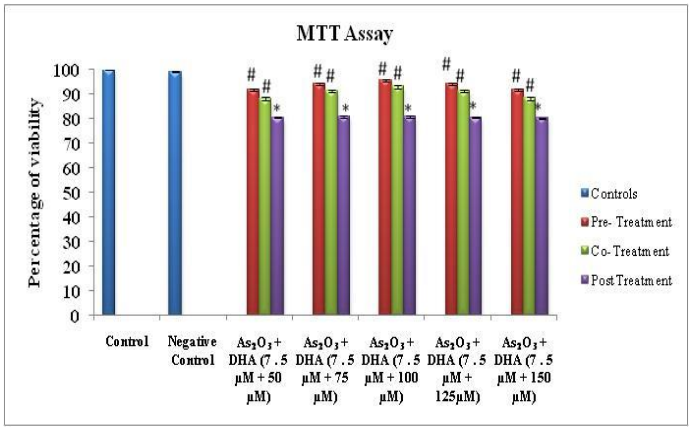

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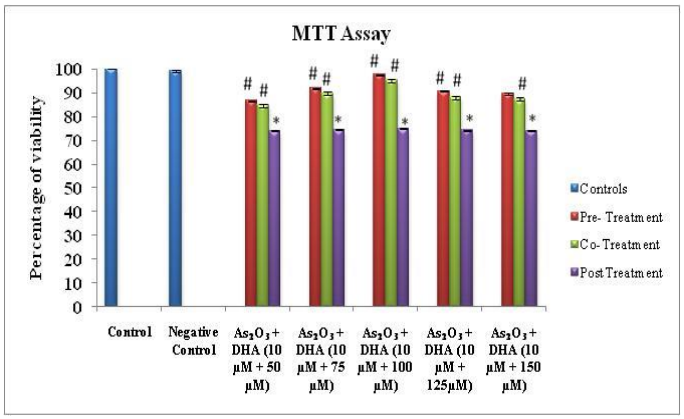

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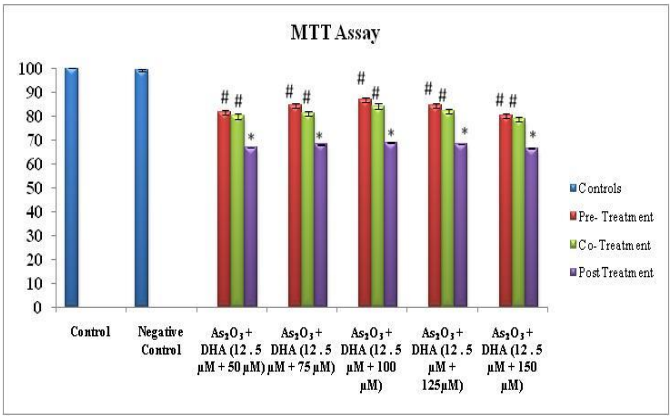

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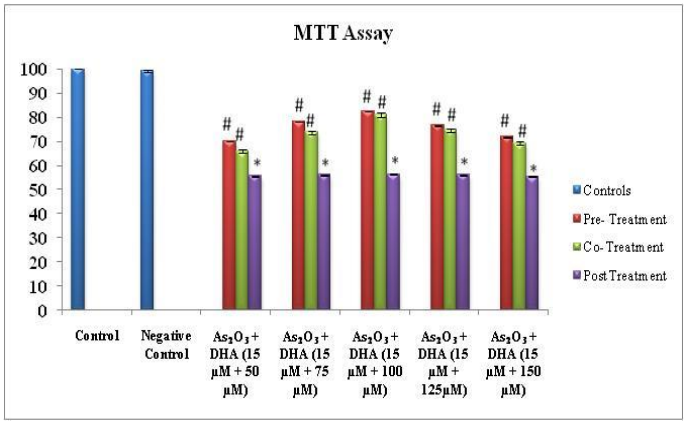
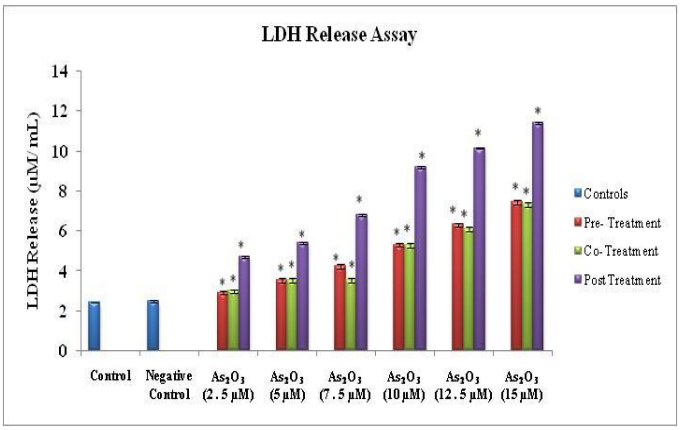


H

Fig. 1: MTT Assay (24 hours) of Chang liver cells (As_2O_3 - Arsenic trioxide; DHA - Docosahexaenoic acid); 0.2% Ethanol is used as negative control. Data represented as mean± SD,* $p \leq 0.05$ versus normal control group

DHA was effective in reducing the lactate dehydrogenase release from Chang liver cells

Chang liver cells after 24 hours of incubation with As_2O_3 showed significant ($p \leq 0.05$) dose dependant enhancement of LDH release. The highest level of LDH release was found in cells subjected to incubation with 15 µM of As_2O_3. This again showed that As_2O_3 is cytotoxic to the liver cells. The enhanced LDH release indicated that the membrane of cells subjected to As_2O_3 administration had undergone irreversible damage, leading to the release of endogenous LDH to the cell culture media. DHA alone treated groups showed no significant variation from the normal control, again indicating that DHA was not toxic to Chang liver cells. The combined administration of DHA along with As_2O_3 as Pre-treatment and Co-treatment methods resulted in bringing down the LDH levels which were significant ($p \leq 0.05$) from the As_2O_3 alone treated groups. This showed the protective potential of DHA indicating that DHA has membrane protecting effect. DHA at a dose of 100 µM was found to be most effective and it showed the maximum efficacy with 10 µM of As_2O_3 in both Pre-treatment and Co- treatment experiments. However the Post treatment method failed to produce any protective effect in Chang liver cells (Fig 2C–2H).



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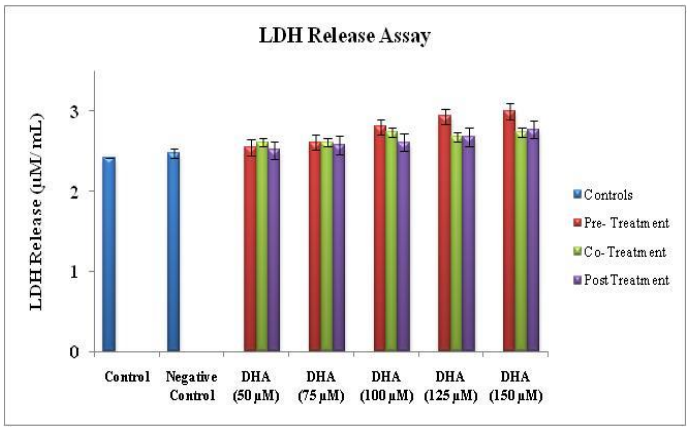

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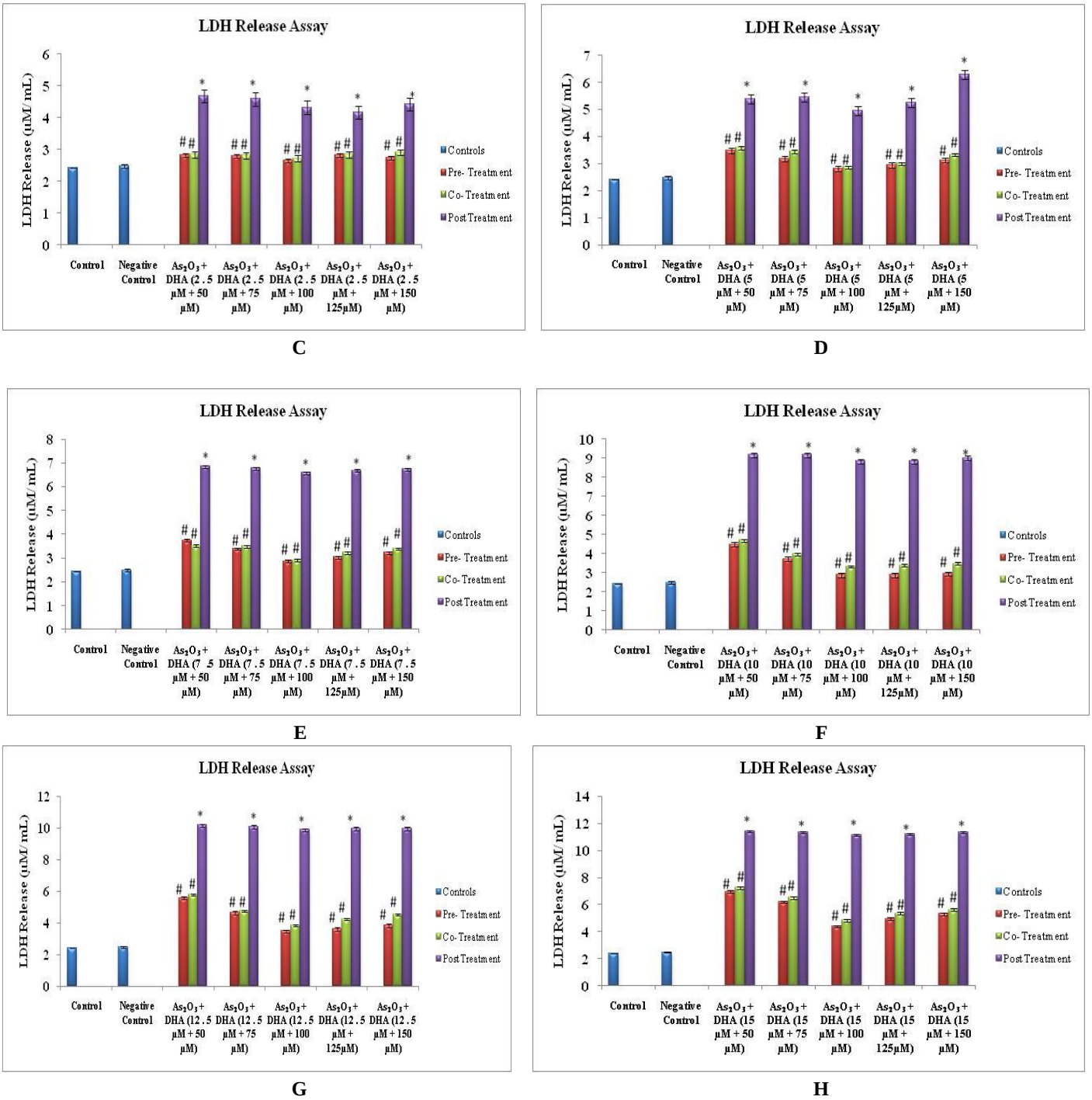

Fig. 2: LDH releasing assay of Chang liver cells; 24 hours (As_2O_3- Arsenic trioxide; DHA-Docosahexaenoic acid); 0.2% ethanol is used as negative control. Data represented as mean±SD,* $p \leq 0.05$ versus normal control

DISCUSSION

The term 'cytotoxicity' is commonly used to depict the cascade of molecular events that impede with macromolecular synthesis, resulting in unequivocal cellular functional and structural damage [14]. Arsenic trioxide, the front line cancer drug against APL, is also known to cause side effects in humans. This negative aspect of arsenic trioxide therapy remains a major hindrance for elucidating its therapeutic potential. The prominent side effect, hepatotoxicity, occurs mainly due to alterations in membrane structure resulting in enhanced permeability of liver cells, mainly due to enhanced oxidative stress [6]. The liver is the major organ responsible for xenobiotic metabolism in the human body by which toxic products get eradicated. Hence like other chemical substances, the major part of arsenic metabolism also takes place in the liver. Arsenic absorbed by the small intestine is subjected to biomethylation in liver forming monomethylarsonic acid [MMA(V)] and dimethylarsinic acid [DMA(V)]. As (III) is normally transported by aquaglyceroporins 7 and 9, which are the main transporters of water. Since arsenic resembles the substrates of these transporting proteins, it becomes easy for the inorganic arsenic compounds to enter the liver cells [3-5,20,21]. Research findings have reported that arsenic has the potential to accumulate in organs including the liver [21,22]. Since liver is the major organ responsible for the regulation of metabolism and detoxification, any damage to this organ may cause adverse effects in the entire human system. Hence this study is focused towards the detection of arsenic trioxide induced toxicity in liver, by using an *in vitro* model, the Chang liver cells. The Chang liver cells forms a widely used model to study liver pathophysiologies [23,24].

The MTT method is widely considered as a sensitive index to assess the cytotoxicity of various chemicals. The results from this viability test reflect not only the cell number but also the cellular metabolic status. This method has several advantages, which include simplicity, rapidity, repeatability and it does not require radioisotopes [14,25]. We found a significant dose dependent reduction in viability of As_2O_3 treated groups of Chang liver cells from the MTT assay. This showed that as the concentration of As_2O_3 increases, the cellular metabolic status along with cell viability has been adversely affected. The concentration of LDH in the medium was found to increase with the elevation in the concentration of As_2O_3. Research findings have suggested that enhanced leakage of LDH may be due to the damage of cell membranes. The damage of membrane architecture may result in the leakage of cellular constituents to the extracellular spaces which may ultimately result in apoptosis [15-18]. Observations from LDH release assay agrees with the results obtained with the MTT assay. So in together, these two assays indicated the cytotoxic effect of As_2O_3.

DHA is the most biologically active, longest and most unsaturated form of omega-3 fatty acids. This long chain fatty acid serves as a vital component of cell membranes, especially in the brain, retina, liver and heart. They also act as the precursor of signaling molecules called docosanoids. Research findings have reported that docosahexaenoic acid has anti-inflammatory, hypolipidemic and antioxidant properties and hence exerts favourable effects on organ function [8-10,26]. Hence our study was aimed at investigating the toxic potential of As_2O_3 and the ameliorative potential of DHA on this toxicity.

The results obtained from our study indicated that DHA administration along with As_2O_3 as Co-treatment and Pre-treatment patterns can reverse the cytotoxic effects of As_2O_3 by safeguarding the cellular viability along with bringing down the LDH release from cells. However, post administration of DHA failed to produce any protective effect. This indicated that regular uptake of DHA may reduce the side effects of arsenic chemotherapy. Research findings have suggested that DHA may possess membrane stabilizing effect. Studies have reported that supply of DHA

results in increased uptake of this fatty acid by the cells. It is hypothesized that the uptake DHA has been incorporated into the phospholipids of cell membranes [27-30]. The incorporated DHA has been suggested to offer protection to the liver cells from injury by safeguarding the normal cellular architecture. The reduced leakage of LDH observed from our study also indicated the membrane protective potential of DHA. The inability of Post treatment method to protect cells indicates that DHA cannot impart any protective effect once the cells get damaged by arsenic.

CONCLUSIONS

The conclusion of our study is that arsenic trioxide causes toxic side effects in Chang liver cells, which was indicated by the reduced levels of cell viability coupled with enhanced leakage of LDH from the cells. These assays also indicated that the metabolic status in cells was affected with cell membrane damage. The combined administration of As_2O_3 with DHA as Co-treatment and Pre-treatment patterns were found to be effective in protecting the liver cells from toxicity. But the Post treatment with DHA cannot offer any protection to the cells. Thus, our preliminary studies showed that DHA can be used as an effective adjuvant in cancer treatment using As_2O_3. More in depth studies at molecular levels are required to explore the mechanisms behind the protective effect of combined administration of As_2O_3 with DHA.

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