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ORIGINAL ARTICLE

Protective effect of an aminothiazole compound against γ-radiation induced oxidative damage

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Abstract
Ionizing radiation causes its biological effects mainly through oxidative damage induced by reactive oxygen species. During radiotherapy of cancer, one of the undesirable side-effects is toxicity to normal cells. Compounds with antioxidant activities are being tried as 'prophylactic radioprotectants' to overcome this problem. We evaluated the protective effect of an aminothiazole compound, in the form of dendrodoine analogue (DA) originally derived from a marine tunicate, against γ-radiation-induced damage to lipid, protein, and DNA besides its cytotoxicity. Oxidative damage was examined by different biochemcial assays. Our studies reveal that DA gave significant protection, in fairly low concentrations, against damage induced by γ-radiation to rat liver mitochondria, plasmid pBR322 DNA, and mouse splenic lymphocytes *in vitro*. It also protected against oxidative damage in whole-body irradiated mice exposed to therapeutic dose of radiation (2 Gy) *in vivo*. Spleen, a major target organ for radiation damage, of the irradiated mice showed significant protection when treated with DA, as examined by histopathology. In conclusion, due to the possible protective effects against normal cells/tissues both *in vitro* and *in vivo*, DA shows potential to be a radioprotector for possible use during radiotherapy.

Keywords: *antioxidant,dendrodoine analogue, mouse radioprotection, splenocytes*

Abbreviations: *ALT, alanine aminotransferase; AST, aspartate aminotransferase; DA, Dendrodoine analogue (4-Amino-5-benzoyl-2-(4-methoxy phenylamino) thiazole); DCFDA, 2′,7′-Dichlorodihydrofluorescein diacetate; DMEM Dulbecco's modified Eagle's medium; DNPH, dinitrophenyl hydrazine; EDTA ethylene diamine tetra acetic acid; FCS, foetal calf serum; GSH, glutathione; LDH, lactate dehydrogenase; LOOH, lipid hydroperoxide; MCB, monochlorobimane; NADPH, nicotinamide adenine dinucleotide phosphate reduced; PBS, phosphate buffered saline; PI, propidium iodide; PMS, phenazine methosulphate; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; TBA, 2-thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid.*

Introduction

In the modern day world, ionizing radiation is used for the benefit of mankind in various ways. It has become a vital component of modern medicine, being used in a large number of diagnostic and therapeutic applications. It is also utilized for the generation of nuclear power, developing new varieties of high-yielding crops, for industrial applications, and enhancing storage-period of food materials [1–3].

In recent years, radiotherapy has emerged as one of the most common therapies for treating human cancers. However, ionizing radiation, employed in radiotherapy of various cancers, is non-selective in its action because it affects both tumour and normal cells. When cells/tissues are exposed to ionizing radiation such as γ-rays, the water molecules undergo dissociation (radiolysis) producing free radicals and related species in the form of reactive oxygen species (ROS). These, in turn, can act on biomolecules such as DNA, lipids and proteins, and cause oxidative damage [1,4,5]. Hence, the damage induced is mostly indirect. DNA is the primary target of radiation damage. In the indirect effect, DNA damage is mainly induced by abstraction of the H atom from the C-4 position of the deoxyribose or by attack of the bases via the hydroxyl radicals [6]. The ROS also react with

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unsaturated lipids generating hydroperoxides, which in turn can induce changes in lipid bilayer thereby altering the membrane permeability and inducing lipid peroxidation. An antioxidant, due to its ability to neutralize ROS or their actions can protect cellular constituents against radiation and, hence, can be a good radioprotector.

A radioprotector has to meet several criteria to be clinically applicable during radiotherapy. It has to be non-cytotoxic. The normal tissues should be protected against radiation injury. To obtain better tumour control, a cautious balance between the total dose of radiotherapy delivered and the threshold limit of the surrounding normal critical tissues is required. Hence, there is a need for finding more compounds with a potential to reduce toxicity to normal tissues without effecting radiation-induced toxicity to cancer cells. Among the molecular radioprotectors, amifostine is the most thoroughly investigated radioprotective drug that has been approved by the FDA, USA for alleviating the side-effects (xerostomia) associated with radiotherapy [9,10,12]. However, there are reports about contra-indications in some cases [13]. Thus, the role of 'prophylactic radioprotective' compounds, which can be administered prior to radiotherapy to reduce the side-effects, is very important in clinical radiotherapy. Several novel approaches are on to locate a potent radioprotector for possible application in radiotherapy and for protecting personnel during unplanned or accidental exposures [1,6,8–11, and references cited therein]. Many natural and synthetic compounds are being examined for their ability to modulate radiation damage in normal and cancer cells/tissues [1,2,9,14, and references cited therein].

Sea is a major natural source for novel bio-active compounds, many of which belong to totally new chemical classes not found in terrestrial sources. Several of these unique compounds have shown pharmacological activities for diseases like cancer, AIDS, diabetes, arthritis, etc. Dendrodoine, [3-(dimethylamino)-1,2,4-thiadiazol-5-yl]-(1H-indol-3-yl)methanone] an alkaloid extracted from *Dendrodoa grossularia* is an aminothiazole possessing a 1,2,4-thiadiazole unit, a rarity among natural products. Though its synthesis has been reported [15], not many biological studies have been carried out on it and its analogue (Figure 1). Aminothiazoles have a wide range of biological activities such as anti-tumour, anti-anoxic and antioxidant properties. Recent reports show that they can enter blood brain barrier and also are potential therapeutic agents for prion disease [16].

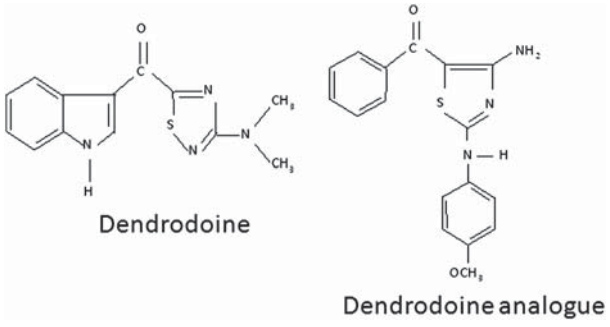

Figure 1. Structure of Dendrodoine and its analogue.

Dendrodoine analogue (DA) [4-amino-5-benzoyl-2-(4-methoxyphenyl)aminothioazole] has been chemically synthesized [17] and is similar to dendrodoine. Our earlier study [18] as well as that of Kalpana et al. [19] reported the antioxidant activity of this compound. DA also has been shown to have other beneficial effects such as protection against ethanol and thermally oxidized sunflower oil toxicity [20] and cytotoxicity towards cancer cells [21]. Several natural/synthetic compounds, especially with antioxidant abilities are known to be radioprotective [1,9,14]. A recent study also showed protective ability of DA in human lymphocytes cultured *in vitro*, exposed to X-rays [22]. The aim of the present study is to examine the protective ability of DA against γ-radiation in normal cells/tissues using *in vitro, in vivo,* and cell culture model systems and possible mechanisms.

Materials and Methods

Chemicals

2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA), dinitrophenyl hydrazine (DNPH), ethylene diamine tetra acetic acid (EDTA), ferric chloride, ferrous sulphate, Folin-Ciocalteu reagent, glutathione, glutathione reductase, guanidine hydrochloride, N-(1-naphthyl)-ethylenediamine, nicotinamide adenine dinucleotide phosphate reduced (NADPH), phenazine methosulphate (PMS), propidium iodide, superoxide dismutase, 1,1,3,3-tetraethoxypropane, 2-thiobarbituric acid (TBA), trichloroacetic acid, Triton X 100, Tris-base and xylenol orange were purchased from Sigma-Aldrich Chemical Co., U.S.A. pBR322 plasmid DNA was purchased from Bangalore Genei Limited. Other chemicals used in our studies were of the highest quality commercially available from reputed suppliers. Dendrodoine analogue (Molecular weight 325.4) was a gift from Dr. V. P. Menon of Annamalai University, Annamalai Nagar, India. It was synthesized by his group as described by Rajasekharan et al. [17] (see Figure 1).

Animals and their maintenance

Three months old female Wistar rats weighing 250–300 g and 8–10 weeks old female Swiss mice weighing 20–25 g, reared in the animal house of Bhabha Atomic Research Centre were used. They were housed in temperature and humidity controlled room (24 ± 2°C) with a 12/12 h light/dark cycle and were maintained on a standard laboratory diet and water *ad libitum*. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre,

Government of India, regarding the conduct of experiments, maintenance, and dissection of small animals were strictly followed. For *in vivo* studies, DA was administered at a dose of 5 mg/kg, intra-peritoneally, daily, for 5 days prior to whole body irradiation. The optimum concentration of DA was selected from an earlier *in vivo* study demonstrating the beneficial effect of DA [20] and from our own studies on related protective effects.

Preparation of homogenate and isolation of mitochondria from liver

In brief, rat/mouse livers were removed from respective animals fasted overnight and homogenized in 0.25 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at $3000 \times g$ for 10 min to remove cell debris and the nuclear fraction. The resultant supernatant was centrifuged at $10\,000 \times g$ for 10 min to sediment mitochondria. This pellet was washed three times with 5 mM potassium phosphate buffer, pH 7.4, to remove sucrose [23]. Protein was estimated by Lowry's method [24], and pellets were suspended in the above buffer at the concentration of 10 mg protein/ml.

Systems Used for Ionizing radiation (^{60}Cobalt-gamma source)

Oxidative damage was induced in mitochondria, cells, or plasmid DNA, by exposure to γ-rays from a ^{60}Co source. All *in vivo* and *in vitro* gamma irradiations were carried out using Gamma irradiator, Blood irradiator, and Junior theratron teletherapy machine. Mitochondria from rat liver treated with or without test compound *in vitro* were exposed to 450 Gy gamma radiation at a dose rate of 60 Gy/min. Plasmid pBR322 DNA or murine splenocytes treated with test compounds *in vitro* were exposed to 2–15 Gy gamma radiation at a dose rate of 3.1 Gy/min. For whole body irradiation, animals were placed in perspex covered boxes and were exposed to 2 Gy at a dose rate of 3.1 Gy/min.

Parameters Used to Assess Membrane Damage during Oxidative Stress

Lipid peroxidation and oxidation of proteins. Lipid hydroperoxides (LOOH) formed from lipid peroxidation were detected by FOX reagent. Hydroperoxides oxidize Fe^{2+} to Fe^{3+} under acidic conditions [25]. The dye, xylenol orange, complexes with an equal molar concentration of Fe^{3+} to produce a blue-purple color complex with an apparent extinction coefficient of $1.5 \times 10^4\ M^{-1}cm^{-1}$ at 560 nm.

TBARS (thiobarbituric acid reactive substances) method involved heating of biological samples with TBA reagent (20% TCA, 0.5% TBA, 2.5 N HCl and 6 mM EDTA) for 20 min in a boiling water bath. After cooling, the solution was centrifuged at 2000 rpm for 10 min, and the precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm against a blank that contained all the reagents except the biological sample. Concentration of TBARS was then calculated with the help of standard graph using 1,1,3,3-tetraethoxypropane, as malondialdehyde equivalents [26].

The method for protein carbonyls is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form a 2,4-dinitrophenylhydrazone, which can be measured spectrophotometrically at 366 nm [27]. The difference in optical density between blank and corresponding experiment samples gives amount of carbonyls formed. Protein sulphydryls were estimated according to the method of Santos et al. [28].

Enzyme assays and estimation of glutathione. The antioxidant enzymes like glutathione peroxidase and glutathione reductase were estimated using kits procured from RANDOX Laboratories, UK (Glutathione reductase using GLUTRED (GR2368); glutathione peroxidase using RANSEL (RD 504/ RS 505)). Catalase was estimated using the protocol by Beers and Sizer [29] where the disappearance of peroxide is followed spectrophotometrically at 240 nm. Cells (2×10^6 cells/well in a six well plate) were either γ-irradiated or treated with various concentrations of DA for 4 h at 37°C. Following treatment, cells were incubated further for 18–20 h and then harvested, pelleted and used for the enzyme assays.

Glutathione was estimated using monochlorobimane (MCB). The cells (2×10^6 cells/ well in a six well plate) were treated with test compound (DA) and then harvested after 2 h. MCB was then added to the cells and incubated for 30 min. Fluorescence reading was then taken at an excitation of 360 nm and emission of 465 nm using a plate reader (Fluostar OPTIMA, BMG Biotek). Serum and plasma were separated from blood after centrifugation and used for assay of marker enzymes such as lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using diagnostic kits from Agappe chemicals Ltd.

Histopathology examinations. The livers of the experimental animals were washed immediately with phosphate buffered saline (PBS) and then fixed in 10% neutral buffered-formalin prior to routine processing in paraffin-embedded blocks. Sections (4 μm thick) were cut and stained using hematoxylin-eosin (H&E) and examined under high power microscope (40X) and photomicrographs were taken.

Isolation of splenic lymphocytes and estimation of cell viability

Splenocytes were obtained by gently squeezing the spleen from Swiss mouse through a nylon mesh in a petriplate containing RPMI 1640 medium supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. The RBCs were lysed by brief hypotonic shock. Lymphocytes were suspended in RPMI 1640 medium. Cell viability was assessed by trypan blue dye exclusion. For measuring cytotoxicity of DA, 2×10^6 cells were seeded in 12 well plate in presence and absence of DA for 24 h. The total number of cells and viability of different groups were estimated by trypan blue dye exclusion test using haemocytometer.

Assessment of DNA damage. To study the effect of DA on single strand breaks in DNA, 300 ng pBR322 plasmid DNA was incubated in 5 mM potassium phosphate buffer (pH 7.4). It was irradiated by γ–rays in the presence and absence of different concentrations of DA [31]. It was then subjected to agarose gel electrophoresis as described by Devasagayam et al. [32]. DNA bands were stained with ethidium bromide and quantified by Advanced American Biotechnology software.

Radiation-induced damage to DNA in the splenic lymphocytes was measured as strand breaks using alkaline single cell gel electrophoresis ('comet assay'). Comets are visualized by fluorescent microscopy, and the amount of DNA in the tail, relative to the head, is proportional to the amount of strand breaks [33]. The cells were incubated with DA for 4 h and irradiated at a dose of 2 Gy. 150 µl cells were mixed with 1 ml of low melting agarose and layered on prelabelled clean frosted glass slide. After solidification, the slides were kept in cold lysis buffer solution and incubated in dark for 1 h at 4°C. The slides were then placed in alkali solution for 5 min and allowed to stabilize followed by electrophoresis (25 V and 350 mA) for 30 min. The slides were then placed in neutralization buffer for 15 min and kept in a moist chamber till analysis. The slides were stained with 50 µl of propidium iodide (PI, 20 µg/ml) and visualized using a Carl Zeiss Fluorescent microscope (Axioskop) with bright field phase-contrast and epi-fluorescence facility. The images (40–50 cells/slide) were captured with high-performance GANZ (model: ZC- Y11PH 4) color video camera. The integral frame grabber used in this system (Cvfbo1p) is a PC based card and it accepts color composite video output of the camera. The quantification of the DNA strand breaks of the stored images was done using the CASP software by which %DNA in tail, tail length, tail moment, and Olive tail moment could be obtained directly.

DNA fragmentation assay

The splenic lymphocytes, which were cultured in RPMI 1640 medium containing 1% FCS for 24 h in the presence or absence of DA, were centrifuged. The cell pellet was incubated with a 100 µl lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1% SDS (sodium dodecyl sulphate), and 80 mg/ml proteinase K) at 55°C for 1 h. After this 10 µl RNAse A (10 mg/ml) was added and further incubated at 55°C for 1 h and then at 65°C for another 2 min. 15 µl of this reaction mixture was mixed with 3 µl 6X DNA loading dye and was resolved in a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide in 1X Tris–borate–EDTA buffer. The bands were visualized and photographed under UV light using Geldoc (Syngene, UK).

DCFDA Assay for ROS Scavenging in Cellular Systems. The fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH/DA) was used to monitor net intracellular generation of ROS induced by γ–radiation. To detect intracellular ROS, cells were pre-incubated with 20 µM DCFDA for 20 min at 37°C before being treated with various concentrations of DA for 4 h followed by γ–radiation (2 Gy). The increase in fluorescence resulting from oxidation of DCFH to DCF was recorded at excitation wavelength of 485 nm and emission wavelength of 535 nm. The mean fluorescence intensity was calculated [34].

Statistical analysis

Data were presented as mean ± SE of four different experiments. Statistical analysis was done by One way ANOVA followed by post-hoc analysis using Scheffe's test. $P < 0.05$ was considered statistically significant.

Results

Most of the natural radioprotectors are believed to exert radioprotective effect by scavenging the radiation-induced ROS. The *in vitro* radioprotective effect of DA was studied using assays corresponding to their ability to inhibit the damage caused to biomolecules. DA gave protection against radiation-induced oxidative damage in all our experiments.

In vitro *and cell culture radioprotective study*

Protection against lipid and protein damage during radiation-induced oxidative stress. The capacity of DA to prevent mitochondrial damage induced by radiation was examined in terms of lipid peroxidation and protein oxidation. Lipid peroxidation was assessed as formation of LOOH and TBARS. Before performing experiment on radioprotection a dose response study was carried out and the optimum dose of 450 Gy was selected for further studies to produce reproducible results (Data not included). Dose of 450 Gy has been used in earlier studies to demonstrate the radioprotective effect of certain

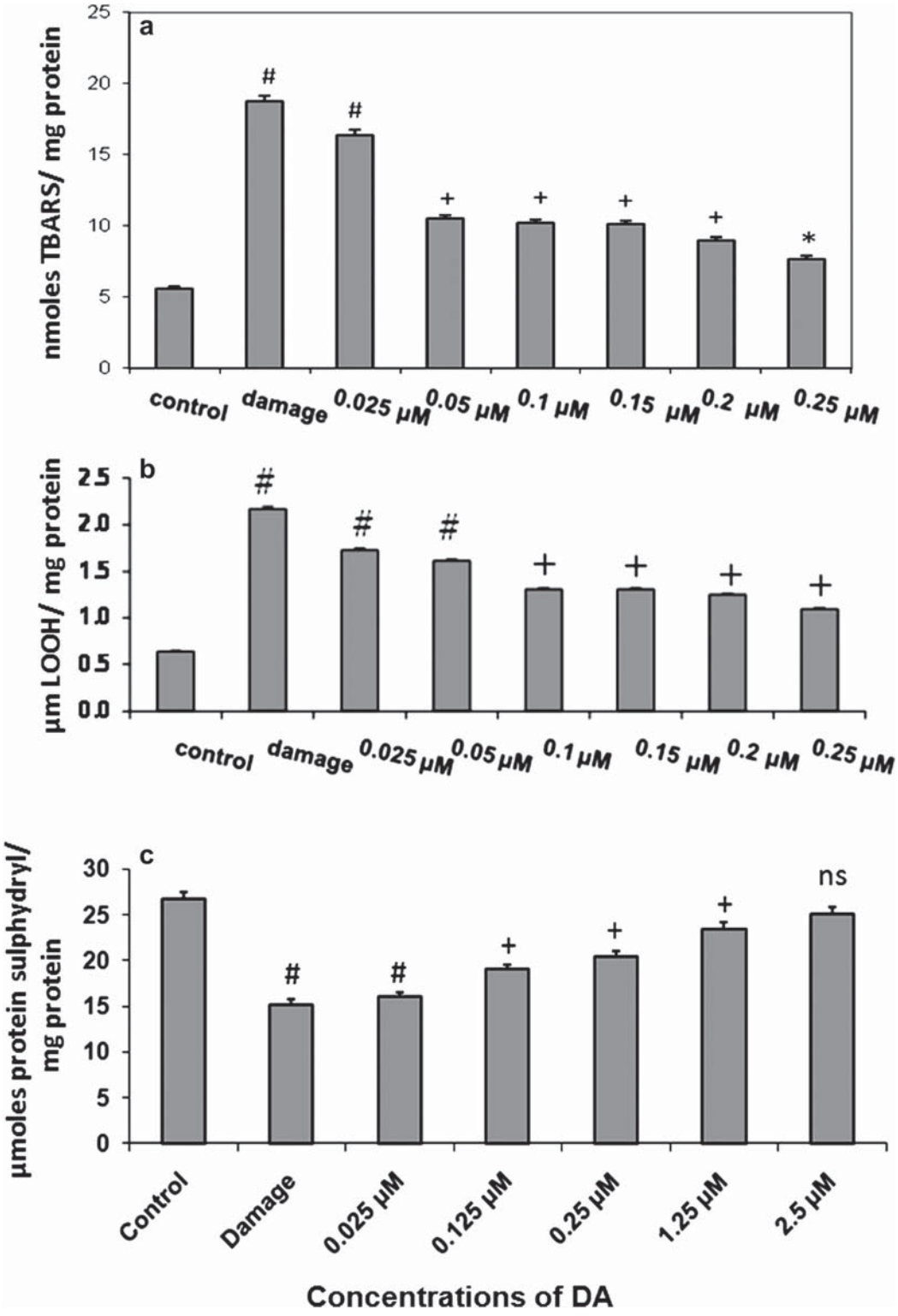

Figure 2. Protection against radiation-induced lipid peroxidation in terms of TBARS (a), LOOH (b) and protein sulphydryl depletion (c) in rat liver mitochondria. The dose of radiation given was 450 Gy. Data represented as mean ± S.E. from three or four individual experiments. $^{*}p < 0.05$; $+\ p < 0.01$, $^{\#}p < 0.001$ and ns – non-significant, as compared to control.

polyphenols from *Phyllanthus amarus* [35] and betel leaf extracts [36]. Lipid peroxidation increases as a function of radiation dose. All experiments were carried out with different concentrations of DA in order to investigate whether an increase in the concentration influences the radioprotective activity. The Figures 2a and b reveal that DA inhibits γ-radiation-induced lipid peroxidation significantly (more than 70% protection) even at a low concentration of 0.1 μM. The percent protection against TBARS formation observed with 25, 50, 100, 150, 200, and 250 nmoles were 18.08, 62.55, 65.29, 65.75, 74.22, and

84.21 respectively. Similar protection against LOOH formation observed with 25, 50, 100, 150, 200, and 250 nmoles were 28.86, 36.37, 56.31, 56.31, 60.07, and 70.20 respectively.

Free radicals can oxidize proteins increasing their hydrophobicity and sensitivity to proteolysis. Free radicals may react with amino acids or sulfur groups, leading to cross-linking and aggregation in proteins [37]. Protection against protein oxidation was assessed as formation of protein carbonyls and depletion of protein sulphydryls. Concentration range of 0.025–2.5 μM of DA was used (Table I). DA inhibits γ-radiation-induced protein oxidation significantly even at a low concentration of 0.25 μM (more than 75% protection). Radiation depletes protein sulphydryls and DA gave significant protection against this depletion (Figure 2c). Concentrations of 0.025, 0.125, 0.25, 1.25, and 2.5 μM DA gave percent protections of 60.15, 76.77, 76.40, 88.07, and 94.04 respectively.

Protection against radiation-induced damage to DNA and ROS scavenging. Agarose gel electrophoresis pattern of pBR322 DNA exposed to different doses of γ-radiation (dose = 4–15 Gy) showed that maximum damage was obtained from 8 Gy onwards (Data not shown). Using a γ-radiation dose of 10 Gy the protective effect of DA was examined. On exposure of plasmid DNA to γ-radiation the intensity of the band corresponding to Form II (open circular) was increased. DA protected against γ-radiation-induced single strand breaks in pBR322 DNA in a concentration-dependent manner. 0.25, 2.5, and 5 μM DA gave 58, 71, and 75% protection, respectively.

Our present study using comet assay, and utilizing different parameters to measure damage such as tail DNA, tail length, tail moment and Olive tail moment, indicated that it also protected the cellular DNA (from murine splenocytes) upon γ-radiation exposure. Tail length is given in micrometers and tail moment is a relative value obtained as a product of % DNA in tail and tail length. Gamma-radiation at a dose of 2 Gy significantly induced the formation of 'comet' due to extensive DNA damage and presence of DA prevented this damage (Figure 3a and b). When time is given for repair, there is a reduction in damage in both radiation-exposed and radiation plus DA treated group. The possible reason for the observed radioprotective effect of DA might be associated with both DNA repair and its antioxidant property, which can be because of radical scavenging.

Table I. Protection against radiation-induced protein carbonyl formation in rat liver mitochondria by different concentrations of DA.

Concentration of DA	nmoles of protein carbonyls/mg protein*	% Protection
Control	2.5 ± 0.005	------
Damage	8.81 ± 0.001#	------
0.025 μM	5.24 ± 0.0012#	56.60
0.05 μM	4.99 ± 0.002#	60.38
0.1 μM	3.80 ± 0.00025#	79.25
0.5 μM	2.38 ± 0.005#	>100

Data represented as mean ± S.E., from four individual experiments. *Rat liver mitochondria (2 mg protein/ml in 5 mM phosphate buffer, pH 7.4). #$p < 0.001$ compared to control.

Ionizing radiations such as γ-rays exert their biological effects through generation of ROS. Most radioprotectors exert radioprotective effect by scavenging the radiation-induced ROS. Our earlier studies showed that DA is a potential antioxidant and a free radical scavenger. To ascertain the possible mechanisms of the radioprotective effect of DA, its ROS scavenging ability was estimated by DCFDA assay. An increase in intracellular oxidant levels (51%) was observed in murine splenic lymphocytes after exposure to 2 Gy of γ-radiation. DA per se decreased the basal ROS levels (49% with 10 μM, 41% with 25 μM, 53% with 50 μM, and 57% with 100 μM). It also scavenged γ-radiation-induced ROS formation (22% above control with 10 μM, as compared to 51% in radiation treated, 20 % with 25 μM, 8 % with 50 μM, and control levels with 100 μM) in a concentration dependent manner when pre-incubated for 1 h prior to radiation exposure.

Effect of DA on the endogenous antioxidant status. To ascertain the other possible mechanisms of the radioprotective effect of DA, its effect on the antioxidant enzymes (catalase, glutathione reductase, and glutathione peroxidase) besides level of glutathione were estimated. Radiation at the dose used (2 Gy) did not significantly alter the levels of antioxidant enzymes or GSH levels (Figure 4). However, the DA alone treated splenic lymphocytes showed an increase in the levels of the enzymatic antioxidant status. Increase in GSH levels with increasing concentrations of DA was also observed. Therefore, increase in the levels of antioxidant enzymes and GSH levels could be another possible mechanism, by which DA exhibits its radioprotective activity when the antioxidant levels can possibly be lowered at higher doses of radiation.

Inhibition of radiation induced apoptosis and cytotoxicity of DA. We wanted to find out whether DA treatment *in vitro* can protect against apoptosis induced by radiation. In the control group, the prevalence of DNA fragmentation was low (Figure 5). The radiation-treated groups showed an increase in the length of DNA fragments indicating damage. Addition of different concentrations of DA restored DNA damage to near normal levels indicating its efficacy. A radioprotector has to meet several criteria to be clinically applicable and has to be non-cytotoxic. Hence, we studied the cytotoxicity of DA in murine splenic lymphocytes using trypan blue dye. There is no cytotoxicity of DA at the tested concentration, up to 100 μM in the normal cells.

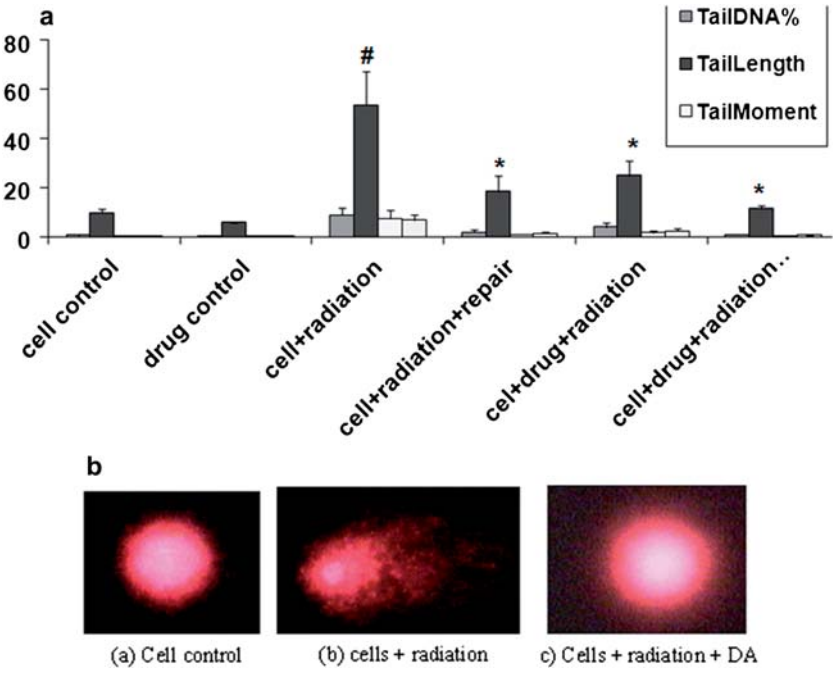

Figure 3. Protection of mouse splenic lymphocyte DNA by DA (1 μM) after 4 Gy of γ-irradiation (a). Mouse Splenocytes were exposed to γ-irradiation in the absence and presence of DA. After radiation exposures, samples were subjected to alkaline comet assay to monitor DNA strand breaks. In the repair group, the samples were subjected to comet assay after 20 min. $^{*}p < 0.05$; $^{\#}p < 0.001$, as compared to control. A representative comet assay photographs for protection of mouse splenic lymphocyte DNA by DA (1 μM) after 2 Gy of γ-irradiation dose (b). Tail length is given in micrometers and tail moment is a relative value obtained as a product of % DNA in tail and tail length.

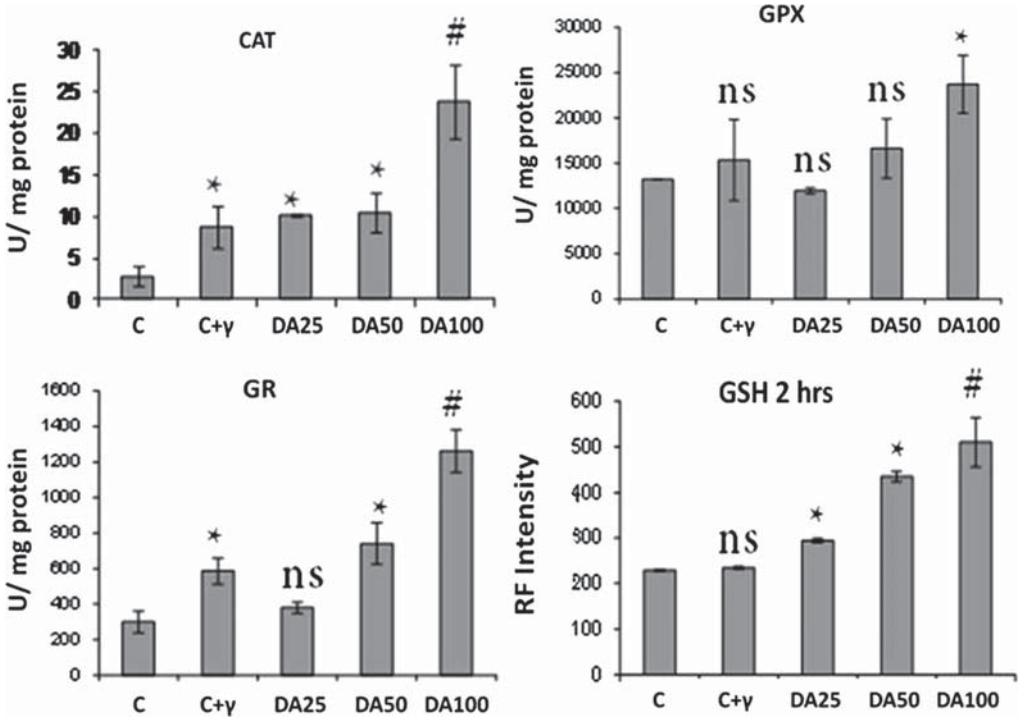

Figure 4. Effect of DA on the antioxidant enzymes in mouse splenic lymphocytes. Cells (2×10^6 cells/well in a six well plate) were either γ-irradiated with 2 Gy or treated with various concentration of DA for 4 h at 37°C. Following treatment cells were incubated further for 18–20 h and then harvested and pelleted and used for the enzyme assay. For estimation of glutathione cells were harvested after 2 h. C – control; C + γ - irradiated; DA25, DA50, DA100 – treated with various concentrations of DA (μg/ml). Data represented as mean ± S.E., from four individual experiments. $^{*}p < 0.05$; $+p < 0.01$; $^{\#}p < 0.001$; ns – non-significant, as compared to control. In the assay for GPx, DA25 and DA50 were not significant with respect to control, while DA100 is significant at $p < 0.05$. CAT: catalase, GR: glutathione reductase, GPx: Glutathione peroxidase.

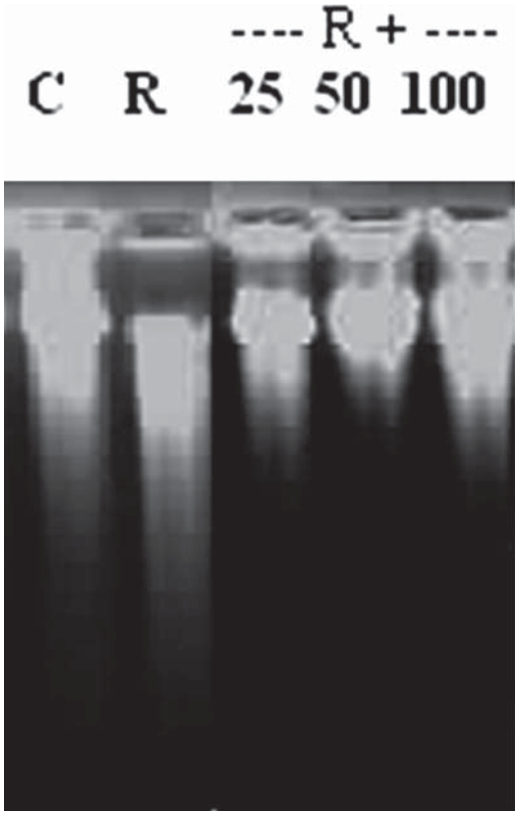

Figure 5. Effect of DA on DNA fragmentation in splenic lymphocytes subjected to radiation (2 Gy). Splenic lymphocytes were treated with DA *in vitro* for 4 h and exposed to 2 Gy gamma-radiation and DNA fragmentation assay was carried out.

In vivo *radioprotective study*

Effect of DA on spleen index, spleen histology, serum marker enzymes and membrane damage. We used a γ-radiation dose of 2 Gy (for whole body irradiation) which is a clinically accepted dose for human patients. A significant reduction of relative spleen weight was seen in radiation-treated group. Pre-administration of DA significantly increased the weight of spleen, suggesting the possibility of DA to give radioprotection *in vivo*. Control animals had a spleen index of 2.65, which decreased to 1.70 with radiation. DA treatment alone resulted in a spleen index of 2.59 while DA treatment along with radiation treatment resulted in a spleen index of 2.05. The radiation group (R) shows smaller spleen size than the control group (C). Pre- administration of DA did not show decrease in the spleen size and weight even after radiation treatment (DA + R) (Figure 6a). The spleen sections were stained with Hematoxylin & Eosin and examined for the histopathological assessment (Figure 6b). Splenic tissue sections of control and DA group revealed red and white pulp in the histology sections. The radiation group revealed enlarged fused white pulp with increased sinusoidal spaces i.e. the matrix is completely destroyed when compared to the control group. Pre-treatment with DA prevented the radiation-induced damage as can be revealed by the normal histology.

Exposure of mice to radiation causes an increase in the activities of LDH, AST, and ALT in serum (Figure 7). Our results showed a significant rise in the levels of these enzymes in the irradiated group. The DA treatment lowered the enzyme activities.

We have observed an increase in the levels of TBARS and LOOH in mouse liver mitochondria in the irradiated group. Elevation of these lipid peroxidation products in irradiated groups could be attributed to the accumulation of irreversible damage to the membrane lipids. Administration of DA prior to radiation exposure to mice significantly decreased the levels of lipid peroxidation and protein oxidation to near normal values (Figure 8).

Discussion

Radiation is an important source of free radicals in living systems. Ionizing radiations such as γ-rays exert their biological effects through generation of ROS and by damaging biological macromolecules such as lipids, proteins, carbohydrates, and DNA. Antioxidants are capable of preventing damage caused by free radicals. Presently, the focus is on developing natural compounds as radioprotectors, especially those with antioxidant activity that would scavenge radiation-induced ROS when present during irradiation. Most of the natural radioprotectors are believed to exert radioprotective effect by scavenging the radiation-induced ROS. Many natural and synthetic chemicals have been investigated in the recent past for their efficacy to protect against radiation-induced damage in biological systems [1,2,9,10,14 and references cited therein]. Though a large number of compounds have been shown to be promising as radioprotectors in laboratory studies, few could pass the transition from bench to bedside. However, the inherent toxicity of some of the synthetic agents at the effective radioprotective concentration warranted further search for safer and more effective radioprotectors. Thus, we also tried to examine the radioprotective ability of DA, which has been shown to have beneficial effects such as antioxidant activity, protection against ethanol and thermal induced toxicity and anti-cancer effects besides protection against X-ray induced cellular damage in human lymphocytes [18–22]. The study by Kalpana et al [22] showed radioprotective effect in human lymphocytes against X-rays while that of ours showed protection against γ-radiation induced damage using different model systems like *in vitro* models, cell cultures, and mouse (*in vivo* system). Hence these two studies are not strictly comparable. The radiation dose range used in our studies varies in different model systems, in the earlier studies as compared to our present study. Their results indicated a radiation dose-dependent increase in both genetic damage and TBARS, accompanied by a significant decrease in the antioxidant status in the irradiated

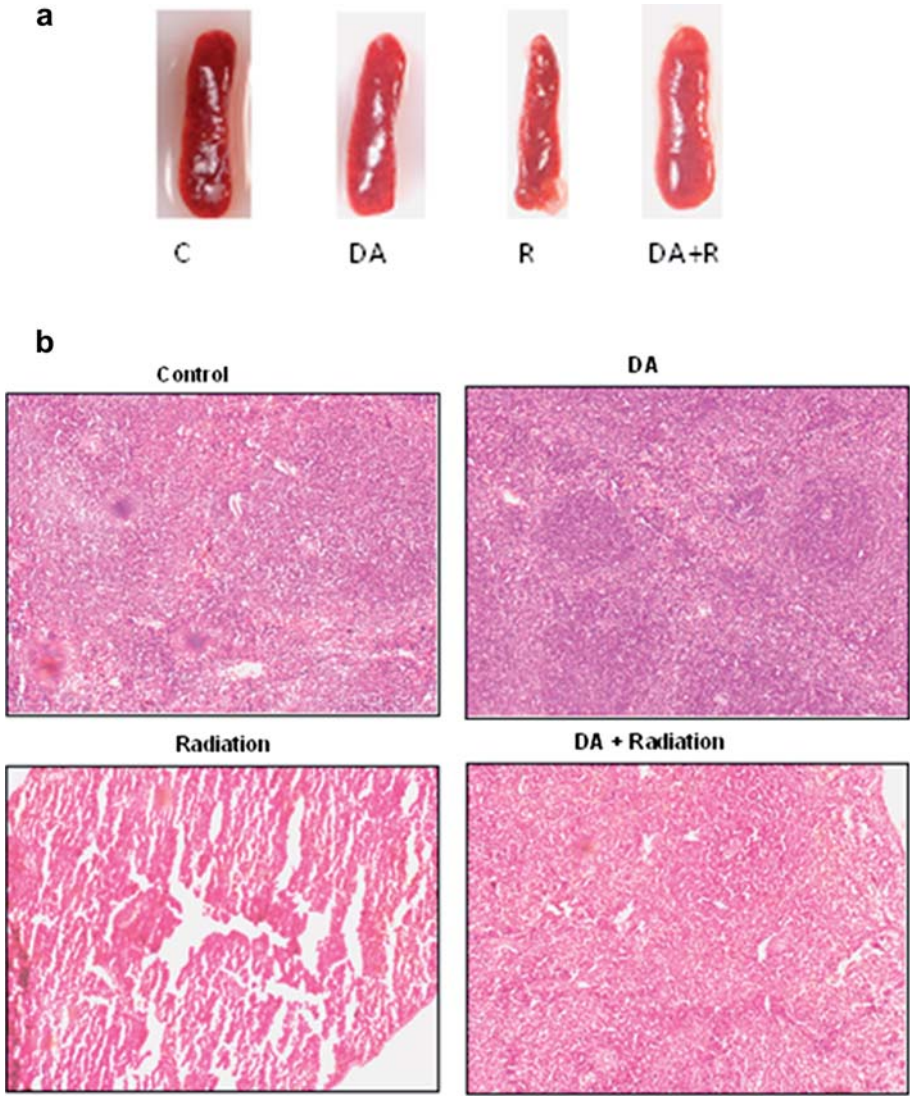

Figure 6. Effect of DA on mouse spleen morphology (a). DA was given at a dose of 5 mg/kg body weight daily to mice, for 5 days, prior to irradiation (2 Gy). C: control, DA: Only dendrodoine analogue, R: only irradiation (2 Gy); DA + R: DA and irradiation. Photomicrographs of spleen demonstrating the protective effect of DA on the spleen of irradiated mice. Spleen Histology (Magnification: 10 ×) (b). Histopathological demonstration of the protective effect of DA on the spleen of irradiated mice. Photomicrographs of spleen sections stained with H&E staining. DA was given at a dose of 5 mg/kg body weight daily, for 5 days, prior to irradiation (2 Gy).

groups compared to DA treated groups which modulated the toxic effects through its antioxidant potential. Their study used only human peripheral lymphocytes cultured *in vitro* as model systems. Whereas in our studies we used *in vitro* systems like rat liver mitochondria, plasmid DNA, cultured mouse splenic lymphocytes, and the whole mouse model. The duration of DA treatment in our studies and theirs differs significantly. Apart from the parameters like lipid peroxidation, antioxidant enzymes and comet assay they have used, we also used protein carbonyls, protein sulphydryls, ROS levels, serum marker enzymes, spleen index, histology etc. for measuring damage. We observed protection by DA in these systems but to different extents. Our study especially using *in vivo* model (mouse) is quite different from that of the earlier study where only a cell culture system was used. Hence ours can be considered as more whole some, especially if extrapolated to conditions similar to that in radiotherapy. The energy levels of X-rays used in their studies and γ-radiation used in our studies may also be different.

The protective effect of DA against γ-radiation induced oxidative damage was studied using assays corresponding to their ability to inhibit the damage caused to biomolecules. DA gave protection against radiation-induced oxidative damage in all our experiments. Significant membrane damage was observed with 450 Gy. Mitochondria are crucial targets for γ-radiation and free radical-mediated damage and a dose of 450 Gy is considered to induce optimum damage required for radioprotection studies [38–40]. This dose of radiation is much higher than those used in radiotherapy (1–6 Gy) or for radioprotection pertinent to mammals (LD_{50} in the range of 5–7 Gy). Mitochondria are fairly resistant to γ-radiation. Hence,

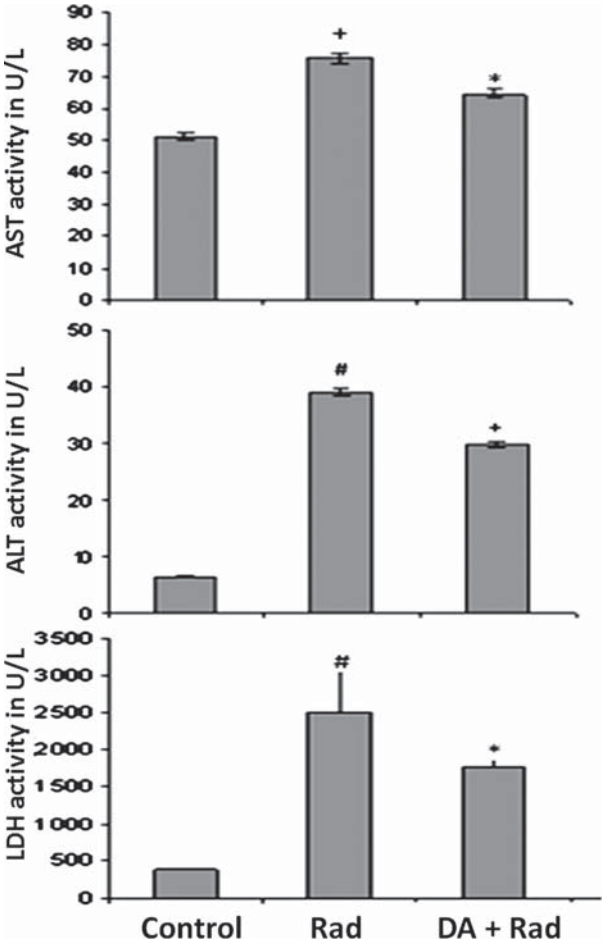

Figure 7. Effect of DA on serum marker enzymes in mice exposed to whole body irradiation. Data represented as mean ± S.E. from four individual experiments. AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase; DA (5 mg/kg BW) was administered for 5 days prior to 2 Gy whole body irradiation. $^{*}p < 0.05$; $+\ p < 0.01$ and $^{\#}p < 0.001$, as compared to control.

a dose of 450 Gy is needed to achieve optimum concentration of free radicals capable of inducing significant damage measurable by simple spectrophotometric assays [40]. DA gave significant protection against radiation induced lipid peroxidation and protein oxidation in rat liver mitochondria.

Ionizing radiation-induced damages to cellular DNA are of biological importance. The types of damages suffered by DNA due to ionizing radiation include strand breaks of single and double-strand types, base damage, elimination of bases, and sugar damage. Protecting cellular DNA from radiation damage might result in prevention of the cancers/mutations induced by radiation. The present study shows that DNA was protected from deleterious effects of γ-radiation by DA. Our study using splenic lymphocytes indicated that it protected the cellular DNA upon γ-radiation exposure. The possible reason for the observed radioprotective effect of DA might be associated with both DNA repair and its antioxidant property, which can be because of radical scavenging.

Administration of DA alone intra-peritoneally did not produce any mortality or toxic side effects up to 50 mg/kg body weight indicating its non-toxic nature. Our related studies have shown that DA is non-toxic up to 50 mg/kg. Exposure of animals to 2 Gy γ-radiation induced radiation sickness. When animals were exposed to 2 Gy of γ-radiation no mortality was seen in all irradiated groups up to eight days of post irradiation.

Apart from enhancing lipid, protein, and DNA damage, radiation treatment is known to inactivate antioxidant enzymes (1,38,39). Our study showed that DA increased the levels of GSH and antioxidant enzymes in splenic lymphocytes. The antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT) can block the initiation of free radical chain reactions [41]. Juan et al. [42] showed that these enzymes may play a central role in protecting cells against ROS induced injury during ionizing radiation exposure. Radiosensitivity of cells depends on the intracellular thiol levels. GSH, a thiol, is also an important cellular antioxidant. Role of GSH in reductive processes are important for the synthesis and degradation of proteins, regulation of enzymes, formation of the deoxyribonucleotide precursors of DNA and protection of the cells against ROS produced even in normal metabolism. GSH is a versatile protector. Several distinct mechanisms of radioprotection by GSH has been recognized, including radical scavenging, restoration of damaged molecules by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the reduced state. Of these mechanisms, the most important one is probably hydrogen donation to DNA radicals and this mechanism requires a high concentration of GSH [43]. It is postulated that ionizing radiation would rapidly oxidize the thiol groups of cells. In accordance with this hypothesis, radiation decreases the cellular concentration of GSH and leads to formation of glutathione disulfide. Administration of various thiols can protect the cells and animals against the effects of radiation [44]. Some investigators measured GSH levels in animals exposed to radiation [45,46] and noted that the GSH levels were significantly lower in the groups exposed to radiation compared with the control groups. In the present study, DA pre-treatment significantly lowered the radiation-induced lipid peroxidation.

When liver cell membrane is damaged, a variety of enzymes is released from the cytosol into the blood, thereby causing increased enzyme levels in the serum. The estimation of enzymes in the serum is a useful marker of damage. The elevated activities of AST, ALT, and LDH in serum are indicative of cellular leakage and loss of functional integrity of cell membranes in liver. Pre-treatment of the mice with DA for 5 days before radiation resulted in a significant

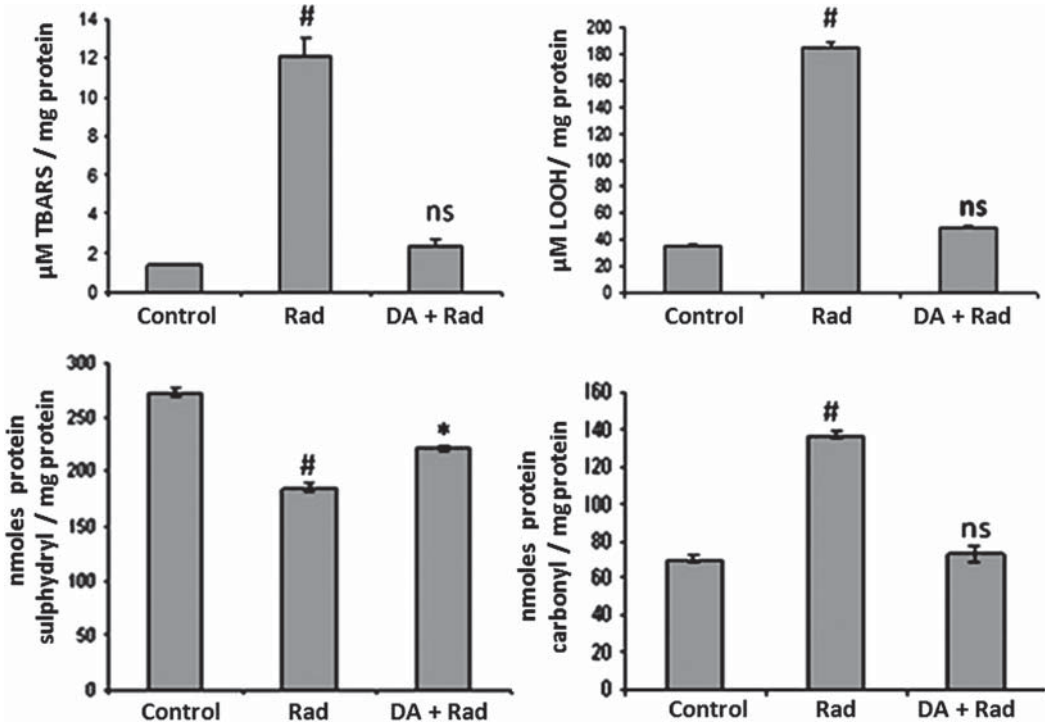

Figure 8. Effect of DA on lipid peroxidation and protein oxidation in hepatic mitochondria of mice exposed to whole body irradiation. TBARS: Thiobarbituric acid reactive substances, LOOH: Lipid hydroperoxide. Mouse liver mitochondria (2 mg protein/ml in 5 mM phosphate buffer pH 7.4). Data represented as mean ± S.E. from four individual experiments. DA was given at a dose of 5 mg/kg body weight daily, for 5 days, prior to irradiation (2 Gy). $^{*}p < 0.05$; $^{\#}p < 0.001$; ns – non-significant, as compared to control.

protection of γ-radiation-induced elevation of these serum marker enzymes. This indicates that DA could repair the radiation-induced damage, which was supported by the histopathological findings.

We wanted to find whether *in vivo* DA administration can protect against DNA damage induced by γ-radiation. DA showed protective effects against DNA damage too. We also wanted to determine whether DA can protect against apoptosis, as indicated by DNA fragmentation. DA protected against apoptosis also.

In conclusion, the present study suggests that DA can prevent damage to lipids, proteins, and DNA from γ-radiation induced oxidative stress in normal cells/tissues. The possible reason for the observed radioprotective effect of DA can be due to its antioxidant property. Our studies, therefore, reveal that DA, an analogue of a compound from a marine tunicate can be a valuable radioprotector with potential applications in health, possibly during radiotherapy.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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