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Protective effect of N-acetylcysteine against radiation induced DNA damage and hepatic toxicity in rats

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ABSTRACT

The present study was designed to evaluate the radioprotective effect of *N*- acetylcysteine (NAC) on γ-radiation induced toxicity in hepatic tissue in rat. The cellular changes were estimated using malondialdehyde (MDA, an index of lipid peroxidation), superoxide dismutase (SOD), glutathione peroxidase (GSHPx), reduced glutathione (GSH), and total nitrate/nitrite (NO(x)) as markers of hepatic oxidative stress in rats following γ-irradiation. The DNA damage was determined by agarose gel electrophoresis. To achieve the ultimate goal of this study, 40 adult rats were randomly divided into 4 groups of 10 animals each. Group I was injected intraperitoneally with saline solution for 7 consecutive days and served as control group. Group II was irradiated with a single dose of 6 Gy γ-radiation. Group III was daily injected with NAC (1 g/kg, i.p.) for 7 consecutive days. Group IV received a daily i.p. injection of NAC (1 g/kg, i.p.) for 7 consecutive days and 1 h after the last dose, rats were irradiated with a single dose (6 Gy) γ-radiation. The animals were sacrified after 24 h. DNA damage was observed in tissue after total body irradiation with a single dose of 6 Gy. Malondialdehyde and total nitrate/nitrite were increased significantly whereas the levels of GSH and antioxidant enzymes were significantly decreased in γ-irradiated group. Pretreatment with NAC showed a significant decrease in the levels of MDA, NO(x) and DNA damage. The antioxidant enzymes increased significantly along with the levels of GSH. Moreover, histopathological examination of liver tissues confirmed the biochemical data. Thus, our results show that pretreatment with *N*-acetylcysteine offers protection against γ-radiation induced cellular damage.

1. Introduction

Ionizing radiation (IR) is an important environmental risk factor for various cancers and also a major therapeutic agent for cancer treatment. Exposure of mammalian cells to IR induces several types of damage to DNA, including double- and single-strand breaks, base and sugar damage, as well as DNA–DNA and DNA–protein cross-links [1]. During radiotherapy, ionizing irradiation particles interact with biological systems to induce excessive oxygen free radicals or reactive oxygen species (ROS), which attack various cellular components including DNA, proteins and membrane lipids, thereby leading to significant cellular damage. ROS also negatively impact the antioxidant defense mechanisms, reduce the

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intracellular concentration of glutathione (GSH), and decrease the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) [2]. One of the indices of oxidative damage is the malondialdehyde (MDA) formation as an end product of lipid peroxidation [3]. Lipid radicals are believed to be formed by the reaction of hydroxyl radicals generated by ionizing radiation with polyunsaturated fatty acids, which can subsequently react with oxygen to form lipid peroxyl radical, which can damage the cell and DNA architecture. Further more, lipid peroxidation products such as MDA form adducts with cellular DNA [4]. Thus, scavenging free radicals and inhibiting lipid peroxidation are likely key target activities for developing successful radioprotection strategies [5].

A radioprotector is a chemical compound capable of modifying the normal response of a biological system to radiation-induced toxicity or lethality. They include sulfhydryl compounds, antioxidants, plant extracts, immunomodulators, and other agents [6]. One of the mechanisms of protection, involving free-radical scavenging, is based on the assumption that free radicals resulting from the radiolysis of water are the main cause of radiation damage to cells [7]. Thiol and its derivatives constituted the most effective class of radioprotection compounds [5].

N-Acetylcysteine (NAC), a thiol reducing agent, is a naturally occurring compound found in several vegetables, including garlic, onion [8], peppers and asparagus [9]. It has antioxidant, antiangiogenic, and anticarcinogenic properties [10]. *N*-Acetylcysteine, a GSH precursor, inhibited the elevation of low molecular weight iron and oxidative stress [11]. Numerous studies have demonstrated that NAC is able to inhibit chemically induced oxidative stress and DNA damage [12,13].

The aim of the present study was to investigate the effects of *N*-acetylcysteine against γ-irradiation induced DNA damage, lipid peroxidation and antioxidant status in normal rats.

2. Materials and methods

2.1. Animals

Male adult Wistar albino rats (60–70 days old) weighing 120–170 g were obtained from the Egyptian Organization for Biological Products and Vaccines (VACSERA, Giza, Egypt). Animals were kept under standard conditions and were allowed free access to a standard requirement diet and water ad. Libitum. Animals were kept under a controlled lighting condition (light:dark, 13 h:11 h). The animals' treatment protocol has been approved by the animal care committee of the National Center for Radiation Research and Technology, Cairo, Egypt.

2.2. Chemicals

N-Acetylcysteine, dimethyl sulfoxide, low melting point agarose and ethidium bromide were obtained from Sigma chemicals, Saint Louis, MO, USA. All other chemicals and solvents used were of the highest purity grade available.

2.3. Irradiation

Whole-body γ-irradiation was performed at the National Centre for Radiation Research and Technology (NCRRT), Cairo, Egypt, using an AECL Gamma Cell-40 biological irradiator. Animals were irradiated at an acute single dose level of 6 Gy delivered at a dose rate of 0.012 Gy/s.

2.4. Experimental design

A total of 40 rats were divided into four groups (10 animals per group). In the first group, animals were injected with normal saline (0.5 ml/150 g body weight, intraperitoneally) for 7 consecutive days and served as a control. The animals in the second group were received a single dose of whole-body γ-rays (6 Gy). Whereas animals in the third group were injected with *N*-acetylcystein (1 g/kg body weight, intraperitoneally for 7 consecutive days). The fourth group received a daily injection of *N*-acetylcystein (1 g/kg body weight, intraperitoneally for 7 days), 1 h after the last injection, animals received a single dose of whole-body γ-rays (6 Gy). Twenty-four hours after the last dose of specific treatment, and animals were anaesthetized with ether. Animals were then sacrificed by decapitation after exposure to ether in a dessicator kept in a well-functioning hood. Livers were quickly excised, washed with saline, blotted with a piece of filter paper and homogenized using a Branson sonifier (250, VWR Scientific, Danbury, CT, USA). The homogenates were centrifuged at 800 × *g* for 5 min at 4 °C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 × *g* for 20 min at 4 °C to get the post mitochondrial supernatant which was used to assay superoxide dismutase (SOD) activity.

2.5. Estimation of lipid peroxidation

The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) by the method of Okhawa et al. [14]. Briefly, the reaction mixture consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of 20% (w/v) of post-mitochondrial supernatant (PMS). The mixture was brought up to 4.0 ml with distilled water and heated at 95 °C for 60 min. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of *n*-butanol and pyridine (15:1, v/v) was added and centrifuged. The organic layer was taken out and its absorbance was measured at 532 nm. TBARS were quantified using an extinction coefficient of $1.56 \times 105\ M^{-1}/cm^{-1}$ and expressed as nmol of TBARS per gm tissue. MDA content expressed as nmol/g tissue.

2.6. Estimation of glutathione peroxidase

The activity of GSHPx was determined according to the method of Lawrence and Burk [15]. Absorbance was measured at 340 nm, and the results were expressed as μmol/min/g tissue the changes in the absorbance at 340 nm were recorded at 1-min interval for 5 min. This method is based on measuring the oxidation of reduced nicotinamide adenine

dinucleotide phosphate (NADPH) using hydrogen peroxide as the substrate. A reaction mixture of 1 ml contained 50 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/ml oxidized glutathione reductase and 1 mM GSH was prepared. The homogenate was centrifuged at 105,000 for 15 min at 4 °C. 0.1 ml of the supernatant was added to 0.8 ml of the reaction mixture and the solution was incubated for 5 min at 25 °C. 0.1 ml of 0.25 mM hydrogen peroxide solution was added to initiate the reaction. Absorbance was measured at 340 nm for 5 min, and an extinction coefficient of 6.22×10^{-3} was used for calculation. The results were expressed as μmol/min/g tissue. The changes in the absorbance at 340 nm were recorded at 1-min interval for 5 min

2.7. Estimation of superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity in liver homogenate was determined according to the method of Minami and Yoshikawa [16]. This method is based on the generation of superoxide anions by pyrogallol autoxidation, detection of generated superoxide anions by nitro blue tetrazolium (NBT) formazan color development and measurement of the amount of generated superoxide anions scavenged by SOD (the inhibitory level of formazan color development).The homogenate was centrifuged at 105,000 for 15 min at 4 °C. To 0.25 ml of supernatant, 0.5 ml of Tris cacodylic buffer, 0.1 ml of 16% Triton X-100 and 0.25 ml NBT were added. The reaction was started by the addition of 0.01 ml diluted pyrogallol. Incubation was maintained for 5 min at 37 °C. The reaction was stopped by the addition of 0.3 ml of 2 M formic acid. The formazan color developed was determined spectrophotometrically (Spectronic 501, Shimadzu, Japan). Enzymatic activity was expressed as μg/g of tissue.

2.8. Estimation of reduced glutathione

Reduced glutathione (GSH) in the livers was assayed by the method of Jollow et al. [17]. Briefly, 1.0 ml of the homogenate (20%) was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4 °C for at least 1 h and then subjected to centrifugation at 1200 × *g* for 15 min at 4 °C. The assay mixture contained 0.1 ml filtered aliquot and 2.7 ml phosphate buffer (0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on a spectrophotometer. The GSH level was expressed as μmol/g of tissue.

2.9. Assessment of tissue total nitrate/nitrite (NO(x)) concentration

Total nitrate/nitrite (NO(x)) was measured as stable end product, nitrite, according to the method of Miranda et al. [18]. The assay is based on the reduction of nitrate by vanadium trichloride combined with detection by the acidic Griess reaction. The diazotization of sulfanilic acid with nitrite at acidic pH and subsequent coupling with *N*-(10-naphthyl)-ethylenediamine produced an intensely colored product that is measured spectrophotometrically at 540 nm. A nitrate standard solution (100 μl) was serially diluted in duplicate in a 96-well, flat bottomed microplate (ELISA). After loading the plate with samples (100 μl), addition of vanadium(III) chloride (VCl3) (1 0 0 μl) to each well was rapidly followed by the addition of the Griess reagents, sulfanilamide (50 μl) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (50 μl). The absorbance at 540 nm was measured using a plate reader following incubation (usually 30 min). The levels of NO(x) were expressed as nmol/g wet tissue in homogenate.

2.10. Analysis of DNA fragmentation: agarose gel electrophoresis

According to the method of Katoh et al. [19], the liver tissue was homogenized and lysed in a cold lysis buffer (10 mM Tris–HCl, 5 mM disodium EDTA, and 0.5% Triton X-100, pH 8.0) for 10 min at 4 °C. The DNA was sequentially extracted twice using half volumes of phenol/chloroform and incubated at 55 °C for 10 min. After centrifugation at 3000 rpm for 20 min, the upper layer was incubated with 2 μl proteinase K (20 mg/ml) at 37 °C for 60 min followed by incubation with 2 μl ribonuclease (20 mg/ml) at 37 °C for 60 min. The DNA was precipitated by adding 0.1 vol of 10 M ammonium acetate and 2.5 vol of 100% ethanol and maintained at −20 °C overnight. DNA was collected by centrifugation at 15,000 × *g* for 20 min, air-dried, and resuspended in TE buffer (10 mM Tris–HCl, 5 mM EDTA, pH 7.4). Agarose gel electrophoresis was carried out for the analysis of DNA fragmentation [20]. The resulting DNA preparations were electrophoresed through a 1.4% agarose gel containing ethidium bromide using TBE buffer (Tris-boric acid–EDTA buffer, pH 8.3) at 40 V for 5 h. Equal quantities of DNA (based on optical density measurements at 260 nm) were loaded in each lane, and a molecular DNA marker was used as a molecular mass standard. DNA fragmentation was visualized and photographed under ultraviolet illumination.

2.11. Histopathological study

The samples were fixed in 10% neutral buffered formalin, dehydrated through alcohols, cleared in xylene and then embedded in paraffin wax. Sections (5 μm thick) were stained with haematoxylin and eosin [21].

2.12. Statistical analysis

Differences between obtained values (mean ± S.E.M. $n = 10$) were carried out by one way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test. A P value of 0.05 or less was taken as a criterion for a statistically significant difference.

3. Results

Administration of NAC for 7 consecutive days resulted in non-significant change (24.03 ± 0.9) in MDA level compared to control group (21.34 ± 0.6). γ-Irradiation (6 Gy) induced a significant increase in the level of MDA (40.26 ± 1.6) compared to control group (21.34 ± 0.6) ($P < 0.001$). Pre-treatment with NAC ameliorated the effect of radiation exposure. MDA level

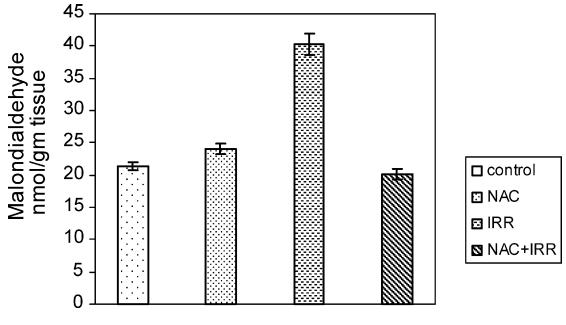

Fig. 1 – Effect of N-acetylcysteine, irradiation and their combination on the level of malondialdehyde in hepatic rat tissue.

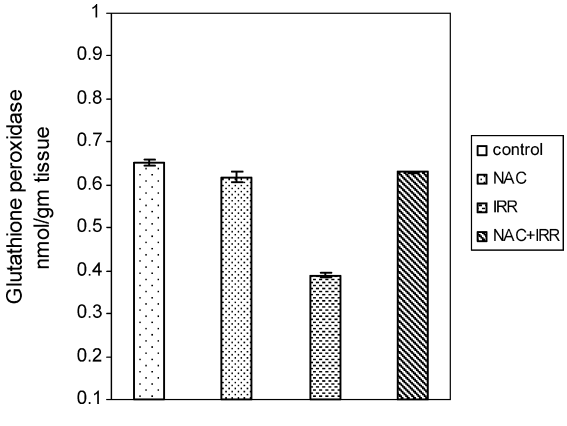

Fig. 2 – Effect of N-acetylcysteine, irradiation and their combination on the activity of glutathione peroxidase in hepatic rat tissue.

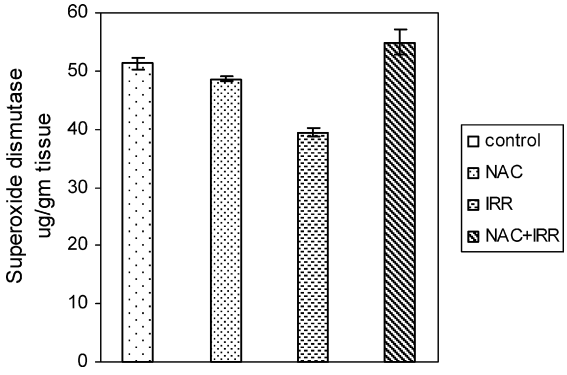

Fig. 3 – Effect of N-acetylcysteine, irradiation and their combination on the activity of superoxide dismutase in hepatic rat tissue.

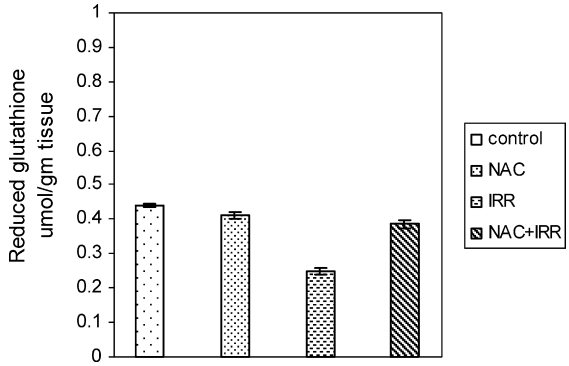

Fig. 4 – Effect of N-acetylcysteine, irradiation and their combination on the level of reduced glutathione in hepatic rat tissue.

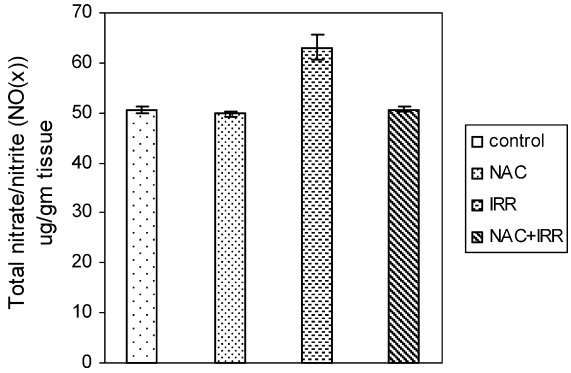

Fig. 5 – Effect of N-acetylcysteine, irradiation and their combination on the concentration of total nitrate/nitrite in hepatic rat tissue.

was significantly decreased compared to irradiated group (0.001) Fig. 1.

Fig. 2 shows that, γ-irradiation induced significant decrease in the activity of GSHPx (0.39 ± 0.004) compared to control group (0.651 ± 0.007) ($P < 0.001$). Treatment with NAC alone induced no change (0.618 ± 0.01) in GSHPx activity compared to control group. Pre-treatment with NAC for 7 consecutive days prior to irradiation resulted in a significant increase (0.63 ± 0.002) in GSHPx activity compared to irradiated group.

Fig. 3 shows that, Administration of NAC for 7 consecutive days resulted in no change (48.6 ± 0.4) in SOD activity compared to control group (51.33 ± 0.9). γ-Irradiation (6 Gy) induced significant decrease (39.45 ± 0.7) in SOD activity compared to control group ($P < 0.001$). Pre-administration of NAC to radiation induced significant amelioration of the change in SOD activity compared to irradiated group ($P < 0.001$).

γ-Irradiation induced significant decrease (0.248 ± 0.008) in GSH level compared to control group (0.439 ± 0.004) ($P < 0.001$). Treatment with NAC resulted in non significant decrease (0.412 ± 0.007) in GSH level compared to the control group. Pre-administration of NAC to radiation induced significant increase (0.387 ± 0.118) in GSH content compared to irradiated group ($P < 0.001$). GSH level was still significantly lower than the control value ($P < 0.01$) (Fig. 4).

In Fig. 5, γ-irradiation (6 Gy) induced a significant increase in NO(x) concentration (63.12 ± 2.48) compared to control group (50.62 ± 0.62) ($P < 0.001$) Administration of NAC induced no change (49.75 ± 0.45) compared to control group. Administration of NAC before irradiation induced a complete restoration of NO(x) concentration to the control level.

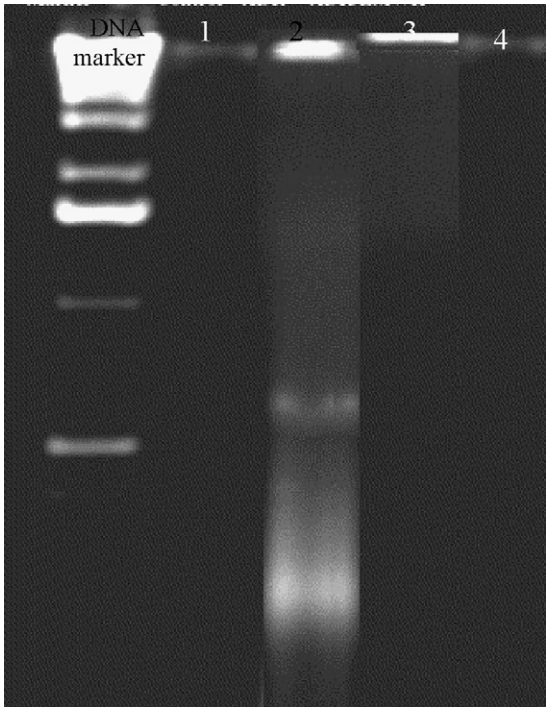

Fig. 6 – Effect of N-acetylcysteine, Irradiation and their combination on DNA fragmentation. Lane 1: control, lane 2: IRR, lane 3: NAC + IRR, lane 4: NAC.

Fig. 6 shows that, γ-irradiation induced significant DNA fragmentation (lane 2). Control sample and NAC sample and lane 4) did not show any band indicating no DNA fragmentation (lane 1). Pretreatment with NAC before radiation showed a small release of DNA (lane 3) compared to lane 2.

Histopathological examination showed that liver of rat treated with 6 Gy γ-radiations (Fig. 7B) displayed fragmentation of the hepatic cells in addition to the presence many of the hepatocytes manifested pyknotic nuclei (↓). Also many of aggregated inflammatory cells were detected (blocked arrow). Liver tissue in rat treated with NAC recorded normal appearance of hepatcytes and hepatic cords (Fig. 7C). Treatment of the experimental animals with NAC combined with 6 Gy of γ-radiation exposure reveled normal appearance of hepatocytes. However many of the inflammatory cells were still detected (↓↓) (7D).

4. Discussion

The goal of radiation treatment is to deliver precisely measured doses of ionizing radiation to a defined tumor volume with the minimum accepted injurious effects of ionizing radiation to surrounding healthy tissue by eliminating tumor cells, giving at reasonable cost to cancer patients [22]. Ionizing radiation is known to induce oxidative stress through generation of ROS in an imbalance in pro-oxidant, antioxidant status in the cells [23].In the present study; we observed increased levels of lipid peroxidation in hepatic tissues in γ-irradiated rats. This may be due to the attack of free radicals on the fatty acid components of membrane lipids

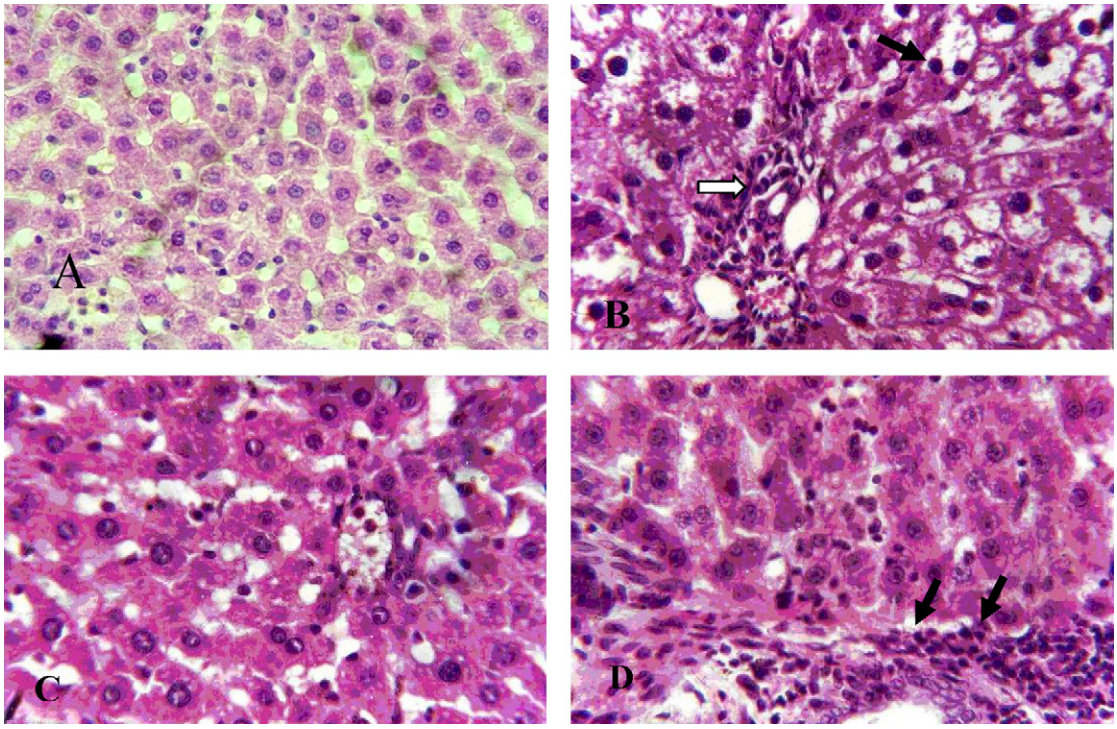

Fig. 7 – (A) Normal control liver tissue section (H&E stain ×400). (B) Section in liver of rat exposed to 6 Gy of γ-radiation (H&E stain ×400). (C) Section in liver of rat treated with NAC (H&E stain ×400). (D) Section in liver of rat treated with NAC and exposed to 6 GY of γ-radiation (H&E stain ×400).

[24]. The decrease in the activities of SOD and GSHPx and the decreased level of GSH in hepatic tissues may be due to their utilization by the enhanced production of ROS [24]. In agreement with our results, Bhatia and Jain [25–27] recorded a significant depletion in the antioxidant system accompanied by enhancement of lipid peroxides after whole body γ-irradiation. Under normal conditions the inherent defense system, including glutathione and the antioxidant enzymes, protects against oxidative damage.

Our results show that whole body γ-irradiation of rats at 6 Gy enhanced the formation of NO(x). Similar results have been reported by Gorbunov et al. [28]. γ-Irradiation may enhance endogenous NO biosynthesis in liver, intestine, lung, kidney, brain, spleen or heart of the animals, presumably by facilitating the entry of Ca^{2+} ions into the membrane as well as the cytosol of NO-producing cells though irradiation-induced membrane lesions. The enhancement of NO production following exposure to a high dose (6 Gy) of γ-rays was attributed to high levels of expression of the inducible nitric oxide synthase [29].

Inducible nitric oxide synthase (iNOS) and NO have been suggested to be involved in acute radiation response in tissues such as the liver, intestine, colon, and brain [30]. Nitric oxide plays an important role in inflammation and carcinogenesis and has now been implicated as an important signaling molecule under normal physiological conditions also. Increased NO results in increased nitration of proteins at tyrosine, which can cause protein dysfunction or alterations in signal transduction pathways [31]. On the other hand, ionizing radiation has been confirmed to potentiate NO production in macrophages. The increase of NO production in irradiated macrophages contributed to tumoricidal activity, with the activation mechanisms differing between high-dose and low-dose irradiation. High-dose irradiation activates macrophages directly, whereas low-dose irradiation acts indirectly through interaction with neighboring cells and the paracrine induction of cytokines [32].

Our results have shown DNA fragmentation in γ-irradiated rats' tissues. ROS are well known to cause DNA damage and induce cytotoxicity. They induce a variety of lesions in DNA, including oxidized bases, abasic sites, DNA strand-breaks and cross-links between DNA and proteins [12].

An increase in the DNA Damage after γ-irradiation has been observed in different studies [33,34]. This may be due to an excessive generation of vasoconstrictors like reactive oxygen species, or due to a reduction of vasodilators such as the nitric oxide, which in turn, can be caused by increased amounts of ROS. On the other hand, Ibuki and Goto [29], suggested that DNA strand breaks caused by hydroxyl radicals formed inside the cells by γ-irradiation, or strand breaks caused by radiation, plays an important role in the enhancement of NO production, but peroxidation of cell membranes has little effect.

NAC treatment prior to radiation was found to decrease the lipid peroxidation, NO(x), DNA fragmentation and significantly increase the antioxidant status when compared with irradiated group. NAC acts as a source of cysteine and stimulates the production of GSH, which protects the body against oxidative stress [35]. Indeed, administration of NAC often causes a time-dependent increase in the cell content of glutathione, the most abundant thiol antioxidant [36] and attenuated the NO effect and abolished the damage of mitochondria [37].

Shao et al. [38] have documented that, NAC a thiol compound reacts directly with NO, can reduce the increased NO generation and reverse the decreased GSH/GSSG ratio, thereby attenuating the cytotoxicity induced by high-dose grape seed proanthocyanidin extract. On the other hand, Yildirim et al. [39] reported that, NAC significantly reduced depletion of glutathione peroxidase, and prevented increases in myeloperoxidase activities, nitric oxide, and malondialdehyde levels in lung tissue produced by bleomycin. Jayalakshmi et al. [40] study indicated that NAC has significant neuroprotective activity during hypoxia in primary hippocampal culture. NAC resulted in a significant cytoprotection, fall in ROS generation, and higher antioxidant levels similar to that of control cells. NAC also inhibited DNA strand breaks induced by hypoxia.

Kao et al. [41] reported that NAC exerts a protective effect on the LPS-induced acute lung injury (ALI) by diminishing the LPS-induced increases in plasma nitrate/nitrite, methyl guanidine (MG), tumor necrosis factor (TNF) alpha and interleukin (IL)-1b. The mechanisms of action may be mediated through the reduction of the production of NO, free radicals and pro-inflammatory cytokines. Majano et al. [42] showed that exposure of hepatocytes to NAC modulated NO synthase expression and NF-kappaB activity, the key responses of the hepatocyte to inflammatory mediators. These data constitute preliminary evidence that NAC might have hepatoprotective actions of potential relevance in chronic inflammatory liver diseases, mediated partially through the modulation of NO production. Recently, Sudheer et al. [43] has been suggested that NAC was effective in protecting against nicotine-induced damage by replenishment of glutathione stores, as well as scavenging of ROS.

Numerous studies have demonstrated that NAC is able to inhibit chemically induced oxidative stress and DNA damage [13]. The present study confirms previous findings that NAC provides protection against diverse oxidative insults [44–46].

Sridharan and Shyamaladevi [47] reported that intragastric pretreatment of NAC prevented the radiation induced damage to an appreciable extent. At relatively high concentrations, NAC scavenges reactive oxygen species produced by ionizing radiation in aqueous solution. On the other hand, Tuttle et al. [48] reported that, the effects of NAC on radiation-induced signal transduction are due to its ability to alter the intracellular reducing environment, and not related to direct scavenging of ROS. NAC affords protection against at least some of the damaging effects of UVB radiation on epidermal DNA, probably by neutralization of UVB induced reactive species [49]. NAC has been shown to have antioxidant properties and to exhibit protective effects against UVA cytotoxicity, DNA damage [50]. On contrary, He and Hader [51] have reported that NAC did not show a substantial effect on lipid peroxidation and DNA strand breaks induced by UVB.

Radioprotection observed with NAC is attributed to the inhibition of lipid peroxidation, increase in the endogenous antioxidative defense enzymes and the reduction in DNA fragmentation.

It has been suggested that, NAC was effective in protecting against radiation-induced damage by replenishment of glutathione stores, as well as scavenging of ROS and inhibition of NO(x). Thus, NAC, by virtue of its free radical scavenging capacity and replenishment of glutathione stores, reduced the cellular and DNA damage caused by γ-radiation.

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