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ORIGINAL ARTICLE

N-acetylcysteine ameliorates nitrosative stress on radiation-inducible damage in rat liver

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Summary

Purpose: *The present study was designed to investigate the potential radioprotective effects of N-acetylcysteine (NAC) on radiation-induced nitrosative stress caused by gamma irradiation (single dose, 6 Gy) in rat liver.*

Methods: *The rats (n=40) were divided randomly and equally into 4 groups: Control (C), Radiation (R), R+NAC (received irradiation and 1,000 mg/kg of NAC) and R+WR-2721 (received irradiation and 200 mg/kg of WR-2721). Liver tissue of each animal was harvested and utilized for 3-nitrotyrosine (3-NT) detection using high-performance liquid chromatography-ultraviolet (HPLC-UV) system.*

Results: *In the R rats, 3-NT levels significantly increased when compared to those of the C rats ($p<0.05$). There were no significant differences in the 3-NT levels among R+NAC and R+WR-2721 rats. Histologically examined liver tissue samples showed no obvious differences.*

Conclusion: *The present study suggests that irradiation has a negative effect on the cellular proteins by enhancing 3-NT formation. The prophylactic use of NAC seems to reduce the nitrosative damage during radiotherapy.*

Key words: irradiation injury, *N*-acetylcysteine, nitrosative stress,WR-2721

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism [1]. Overproduction of ROS and RNS is a harmful process that can lead to damage of cell structures, including lipids, membranes, proteins, and DNA [1,2]. 3-NT is one of the most common products of the action of RNS on proteins [3]. The nitration of aromatic amino acids, principally tyrosine and tryptophan, in proteins results in alteration of their functions [4]. 3-NT represents a specific peroxynitrite-mediated protein modification that is different from the modifications mediated by ROS. 3-NT, formed by the reaction of peroxynitrite with either free or protein-bound tyrosine residues, has been proposed as a nitrosative biomarker [5]. Several studies demonstrate that 3-NT increases under oxidative stress and radiation-induced damage [5,6].

To evaluate the potential protective role of NAC in our previous studies we had measured the levels of the oxidative biomarkers (glutathione [GSH], malondialdehyde [MDA], myeloperoxidase [MPO]) of radiation-induced damage and had also evaluated its effect on genocytotoxicity [7,8]. In the present study, the effect of NAC on nitrosative stress was investigated and compared with that of WR-2721 (amifostine), which is a clinically used radioprotector in the prevention of damage caused by gamma irradiation (single dose whole-body irradiation, 6 Gy) in normal rat liver. We measured the levels of 3-NT in the liver homogenate. Besides, the radioprotective effects of these agents on liver were histologically evaluated.

Methods

Eight-week-old Wistar-Albino female rats (Gaziantep University, Faculty of Medicine, Experimen-

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tal Medicine Research Unit; 170 ± 20 g/body weight [bw]) were used. The rats were randomly selected and housed in polycarbonate cage with free access to tap water and rat chow with a dark/light cycle of 12:12 h. A 1-week acclimatization period was used. The temperature was 22 ± 2° C and relative humidity was 50-70%. All procedures in this study were performed in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals and also were approved by the Institutional Animal Care and Use Committee in the Faculty of Medicine at Gaziantep University.

Experimental design

After the stabilization period, the rats were divided randomly into 4 equal-sized groups (10 rats per group), namely, Control (C), irradiation (R), irradiation+NAC (R+NAC) and irradiation+WR-2721 (R+WR-2721) groups. C rats received neither radioprotector nor irradiation, but 2.2 ml of saline was injected intraperitoneally (i.p.). All groups of rats in the study (R, R+NAC and R+WR-2721) received whole-body gamma irradiation as a single dose of 6 Gy (50% lethal total body irradiation dose for rats). Besides irradiation, R rats received 2.2 ml of saline i.p., while the R+NAC and R+WR-2721 rats received 1000 mg/kg i.p. NAC (containing 300 mg of *N*-acetylcysteine, Asist ampul, Husnu Arsan Ilac, Istanbul) and 200 mg/kg i.p. WR-2721 (containing 500 mg of amifostine, Ethyol flacon, Er-Kim Ilac, Istanbul), respectively. Saline, NAC and WR-2721 injections in the study groups were given 15 min before irradiation. A cobalt-60 teletherapy unit (Shandong Xinhua SCC-8000F, China) was used for all irradiations. The dose rate was 1.80 Gy/min at a distance of 80 cm.

The study was terminated by sacrificing the rats under Ketalar anesthesia (Eczacibasi, Turkey, 35 mg/kg, intramuscularly/i.m.) 72 h after irradiation. At termination, liver tissue was harvested from each animal. Liver tissues were stored at –80° C until biochemical analysis.

Biochemical analyses

Measurements of tyrosine nitration, 3-NT and tyrosine were obtained from Sigma Chemical (St. Louis, USA). H_2O_2, sodium acetate, citrate, NaOH, MnO_2, H_3PO_4, KH_2PO_4, and K_2HPO_4 were purchased from Merck Chemicals (Deisenhofen, Germany). All organic solvents were HPLC-UV graded. The tissues were homogenized in ice-cold phosphate-buffered saline (pH 7.4). Equivalent amounts (50 mg) of each sample were hydrolyzed in 6 N HCl at 100° C for 18-24 h, and then samples were analyzed on an Agilent 1100 series HPLC apparatus (Germany). The analytical column was a Spherisorb ODS-2 C18 reverse-phase column (4.6 × 250 mm; HICHROM, Waters Spherisorb, UK) with a pore size of 5 μm. Guard column was a C18 cartridge (HICHROM, Waters Spherisorb, UK). The mobile phase was 50 mmol/l sodium acetate/50 mmol/l citrate/8% (v/v) methanol, pH 3.1. HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml min-1 and UV detector set at 274 nm. 3-NT and tyrosine peaks were determined according to their retention time, and the peaks were confirmed by spiking with added exogenous 3-NT and tyrosine (10 μmol/l) [9]. 3-NT levels were expressed as 3-NT (nmol/l)/total tyrosine (nmol/l).

Histological examination

The liver samples were fixed in 10% formaldehyde and embedded in paraffin for histological assessments. Five-micrometer-thick slices of skin were stained with hematoxylin and eosin for evaluation with light microscopy according to standard procedures. Slides were examined blindly by an experienced pathologist. The liver samples were evaluated for tissue response to radiation-induced injury including hepatocyte degeneration, hepatocyte necrosis, inflammation, regeneration and fibrosis. These features were referred to as the characteristics of hepatic injury, regardless of cause [10].

Statistical analysis

The two-way analysis of variance (ANOVA) test was used to determine significant differences at the level of 3-NT between groups; this was followed by the Bonferroni *post hoc* test. Type I error rate was accepted as 0.05, and in all calculations, the SPSS (v 11.5; Lead Technologies, Inc., USA) program was used.

Results

Tyrosine nitration measurements in control and irradiated groups of rats

3-NT levels of the liver are shown in Figure 1. We found a significant difference between the 3-NT levels of the R and C rats ($p < 0.05$), while R+NAC and R+WR-2721 rats were not significantly different from the C rats ($p > 0.05$). In the R rats, 3-NT levels significantly increased when compared to those of R+NAC and R+WR-2721 rats, indicating that the irradiation might stimulate the peroxynitrite ($ONOO^-$) formation in female liver ($p < 0.05$). The effect of NAC was similar to the effect observed for WR-2721 ($p > 0.05$).

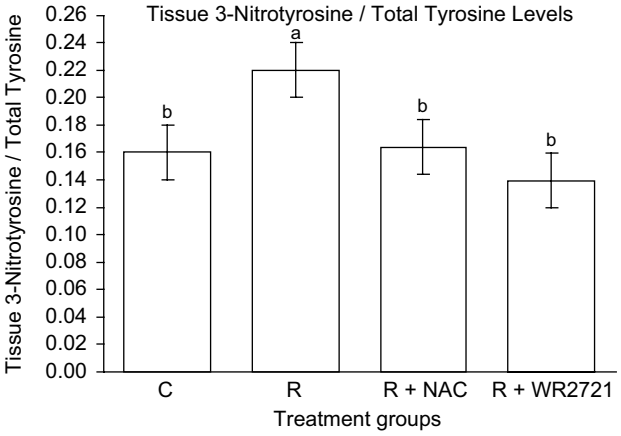

Figure 1. Each group consisted of 10 rats. **C:** control rats; **R:** rats that received irradiation (single dose, 6 Gy); **R+NAC:** rats that received irradiation and treated with NAC, 1000 mg/kg; **R+WR2721:** rats that received irradiation and treated with WR-2721, 200 mg/kg. All irradiated rats were exposed to the same irradiation procedure. Each bar represents the mean± standard error of the mean (SEM). [a]Compared to C rats, [b]Compared to R rats; R rats vs. C rats, p=0.000; R+NAC rats vs. R rats, p=0.000; R+WR 2721 rats vs. R rats, p=0.000.

Histological examination

According to the histopathological evaluation with respect to hepatocyte degeneration, hepatocyte necrosis, inflammation, regeneration and fibrosis, the only finding was hepatocyte degeneration in the NAC group (Figure 2). In terms of these parameters, no intergroup differences were observed. On the other hand, we observed a marked sinusoidal dilatation in the R+WR-2721 and R groups and congestion in R+NAC and R+WR-2721 groups.

Discussion

In many studies, the widely indicated pathogenesis of radiation-induced tissue injury is that radiation stimulates cells to generate various cytokines, leading to tissue damage and subsequent fibrosis [11]. Exacerbations of ROS and nitric oxide synthase (NOS) expression after

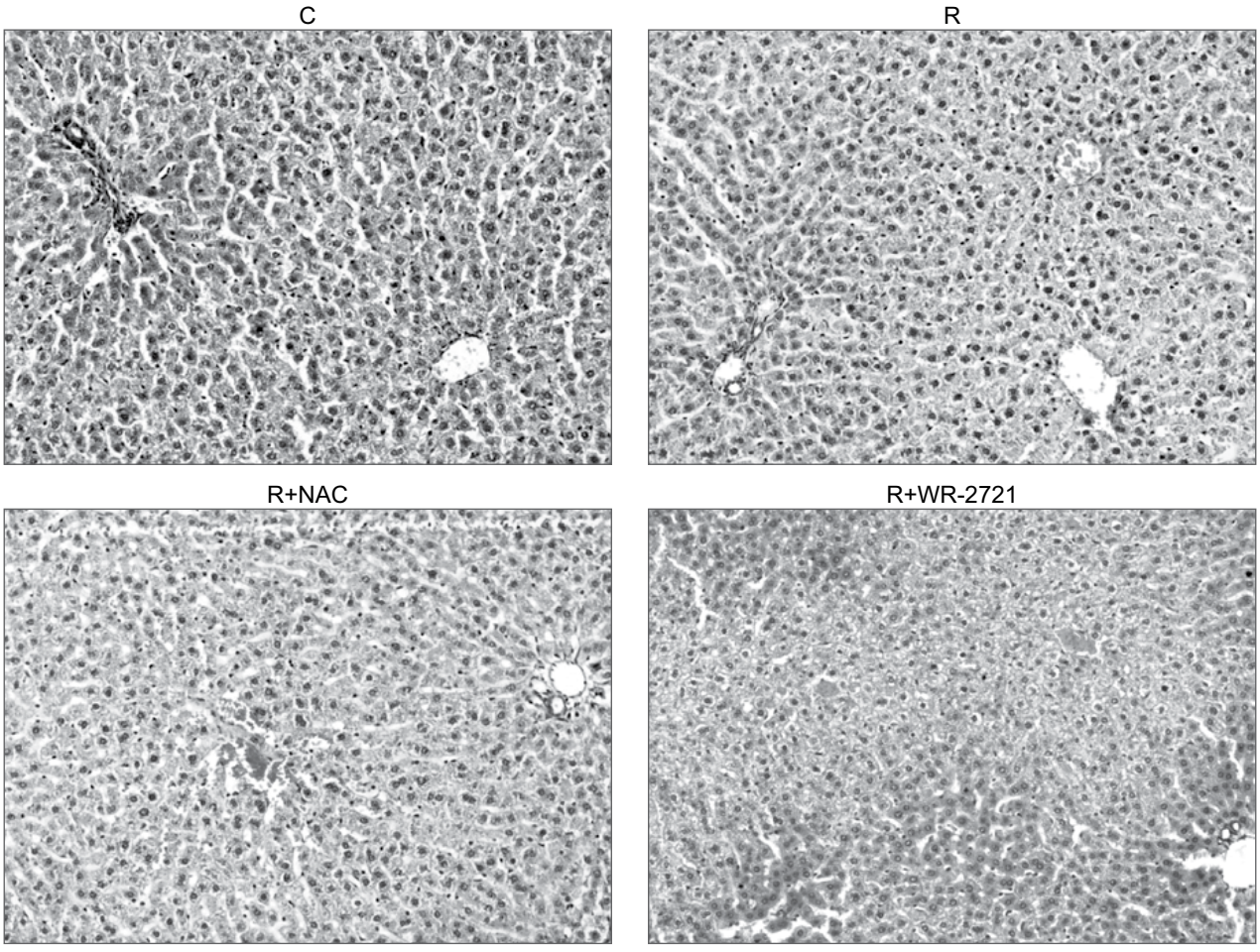

Figure 2. The histological images of all groups in the present study. **C:** Control rats. Liver parenchymal cells and sinusoids with normal appearance. **R:** rats that received irradiation (single dose, 6 Gy). Radiation damage on the liver samples. Only sinusoidal dilatation on the liver parenchymal areas are observed. **R+NAC:** rats that received irradiation (single dose, 6 Gy) and treated with NAC; **R+WR-2721:** rats that received irradiation (single dose, 6 Gy) and treated with WR-2721. Marked congestion in the hepatic lobules are seen by radiation in the R+NAC and R+WR2721 groups (H&E ×20).

irradiation have been reported in various tissues [7,8,12]. Oxidative/nitrosative stresses occur when there is excessive free radical generation and/or low antioxidant defense and result in chemical alterations of biomolecules, causing structural and functional modifications [1]. As ROS and RNS are highly reactive, the most often used approach in the studies of oxidative and nitrosative stress is to determine the extent of related biomarkers (e.g. nitric oxide [NO], GSH, MDA), and inflammatory biomarkers (e.g. neutrophils/MPO) [1,13,14].

NO is a highly diffusible, moderately reactive, and unstable free radical produced *in vivo* by both essential and inducible isoforms of the enzyme NOS [15,16]. NO plays a crucial role in numerous physiological and pathological conditions, such as inflammation and carcinogenesis, and in acute radiation response in tissues [1,15-18]. Endogenous NO biosynthesis may be triggered by radiation *in vivo* in the liver and in other tissues. It seems to be related to radiation-induced membrane lesions by the entry of Ca_2+ ions into the membrane and cytosol of NO-producing cells [19]. $ONOO^-$ is a strong oxidant agent formed by the near-diffusion limited reaction between NO and superoxide (a member of ROS, O_2^-) [17,20]. $ONOO^-$ has the capacity to nitrate free tyrosine and tyrosine residues in proteins [20,21].

$ONOO^-$-mediated tissue damage has been involved in several disease pathologies and radiation-induced damage [22,23]. $ONOO^-$ nitrates tyrosine residues to form 3-NT, a stable product that has been indicated as a nitrosative biomarker of $ONOO^-$-mediated tissue damage in several studies [17,22]. 3-NT increases under oxidative and nitrosative stresses [15]. The results of the present study demonstrate that whole-body irradiation in rats causes tissue damage as proved by increased 3-NT levels, indicating that the irradiation might stimulate the $ONOO^-$ formation in rat liver [15,24].

In our previous studies, we particularly investigated whether GSH supplementation, direct radical scavenging, would be protective against radiation-induced alteration in the different tissues (e.g. liver, bone marrow, skin) and serum [7,8,25]. The intracellular content of GSH is responsive to environmental factors, and it is correlated with the balance between use and synthesis [1]. Thus, oxidative and nitrosative stresses *in vivo* are mainly interpreted as deficiency of GSH and/or its precursor cysteine [26,27].

The use of antioxidants has gained increasing interest due to their potential properties on radioprotection of normal tissues which may provide an better tumor control by allowing for an increase in the radiation dose [28]. To this purpose, the most examined ones were aminothiol radioprotectors (i.e. cysteine, cystamine, WR-2721, and GSH) [28,29]. Aminothiol radioprotectors that cover the SH with a phosphate group reduce radiation-induced cytogenotoxicity. The radioprotective activity of WR-2721 has been shown in many preclinical studies [7,8,28,29]. In our previous studies [7,8,25] we observed the protective effect of WR-2721 against radiation-induced damage, and this result was in accordance with the results of other studies in the literature [29,30]. Although WR-2721 is thought to be a broad-spectrum cytoprotective agent, it may produce dose-limiting toxicity at the maximum effective dose [29,31].

NAC is a well-tolerated drug with a wide toxic-therapeutic window. It has been shown to be beneficial when GSH deficiency occurs, e.g. endotoxic and septic conditions [32,33]. Thus, NAC, as a cysteine prodrug, can regulate redox status in cells, supply depleted body GSH stores and scavenge ROS and RNS by providing sulphydryl groups [34,35]. In addition to its proven antioxidant, anti-inflammatory and cytoprotective properties, NAC also protects endothelial cells and improves microvascular blood flow [36].

The results of our previous studies [7,8,25] related to radiation-induced oxidative stress, have indicated that the administration of NAC can stimulate the anti-oxidant enzyme activities mainly by supplying depleted body GSH stores, inhibiting the lipid peroxidation and enhancing the activity of neutrophils by increasing induced MPO activity during radiotherapy, giving clues about the beneficial effects of NAC against radiation-induced cytogenotoxicity in the bone marrow. Despite the presence of some debatable findings in these studies, they showed that NAC's effect may be comparable to that observed with WR-2721. In the present study, we measured the levels of 3-NT in the liver homogenate as a representative of normal tissues. Furthermore, the radioprotective effects of these agents on liver were histopathologically evaluated. The tissue 3-NT levels of R+NAC and R+WR-2721 rats were lower than those of the R rats and were close to the level of the C rats, indicating that these agents may be useful for scavenging radiation-induced free radicals in nitrosative stress as well; however, these effects were not histopathologically obvious.

Inflammation, regeneration and fibrosis are the changes identified in response to damage in liver tissue, and these changes require time. However, the duration of our experimental study might be not enough for these changes to occur. On the other hand, the sinusoidal dilatation and congestion may be considered as tissue reactions that developed in the early period. Trajkovic et al. demonstrated that tissue damage scores for both degenerative and vascular changes in non-protected animals as well as in protected ones were lower on the 7th day than on the 28th day after irradiation, implying aggravation of radiation-induced damaging processes in

the subsequent postradiation course [37]. On the other hand, Mansour et al. showed that the liver of rats treated with 6 Gy gamma-radiation displayed fragmentation of the hepatic cells in addition to the presence of pyknotic nuclei in many of the hepatocytes and aggregated inflammatory cells after 24 h of irradiation. NAC administration with radiation exposure revealed normal appearance of hepatocytes. However, many of the inflammatory cells were still detected [17].

Yakovlev et al. suggested that tyrosine nitration is not commonly studied in the context of signal transduction, although it is an important post-translational regulatory modification for nuclear factor (NF)-kappaB activation, which is stimulated by ionizing radiation and in the therapeutic dose range, and possibly for other signaling molecules modulated by mild and transient oxidative/nitrosative stresses [38]. Many studies have demonstrated that NAC can inhibit oxidative/nitrosative stresses induced by different stimuli. Majano et al. showed that exposure of hepatocytes to NAC regulated NOS expression and NF-kappaB activity, the key responses of the hepatocyte to inflammatory mediators. They suggested that NAC might have hepatoprotective actions of potential relevance in chronic inflammatory liver diseases, mediated partially through the modulation of NO production [39]. In a chick cardiomyocyte study, Shao et al. have demonstrated that high level of NO contributed to increased apoptotic cell death and was also associated with the depletion of intracellular GSH, probably related to increased consumption by NO with the formation of S-nitrosoglutathione. They showed that NAC, a thiol compound reacting directly with NO, can reduce the increased NO production and reverse the decreased GSH/GSSG ratio, thereby attenuating the induced cytotoxicity induced by high-dose grape seed proanthocyanidin extract [40]. Mansour et al. reported that pretreatment with NAC prevented the radiation-induced damage, decreased the levels of MDA and NO(x), and increased antioxidant enzymes and GSH level [22].

In conclusion, the present study suggests that irradiation may enhance the nitrosative stress in the liver of rats and has a negative effect on cellular proteins by enhancing 3-NT formation. The prophylactic use of NAC seems to reduce the nitrosative damage of the liver during radiotherapy. Although the findings of this study were limited to 3-NT levels, they imply a potential radioprotective effect of NAC in nitrosative stress.

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