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Original Article

The Effect of N-acetylcysteine on Biomarkers for Radiation-Induced Oxidative Damage in a Rat Model

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Our study aimed to investigate the potential radioprotective effects of N-acetylcysteine (NAC) by comparing its biochemical effects with those of WR-2721, as a representative of clinically used radioprotectors, in preventing oxidative damage caused by gamma irradiation (single dose, 6 Gy) in normal rat tissue. 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 NAC) and R + WR-2721 (received irradiation and 200 mg/kg WR-2721) rats. Liver tissues and blood samples were harvested and utilized for reduced glutathione (GSH), malondialdehyde (MDA) and myeloperoxidase (MPO) detection. Serum and tissue GSH levels of R rats decreased compared to those of other groups ($p < 0.01$). Tissue MDA levels of R + NAC and R + WR-2721 rats decreased compared to R rats ($p < 0.01$; $p < 0.05$, respectively). Tissue MPO activities of R + NAC and R + WR-2721 rats were higher than those of R rats ($p < 0.001$). Serum MPO levels of R + WR-2721 rats were lower than those of C rats and R rats ($p < 0.01$, $p < 0.001$, respectively). In conclusion, the study suggests that the radioprotective effect against radiation-induced oxidative damage of NAC may be similar to that of WR-2721.

Key words: irradiation-injury, N-acetylcysteine, WR-2721

Oxidative stress occurs when there is excessive free radical production and/or low antioxidant defense, and results in chemical alterations of biomolecules causing structural and functional modifications. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism [1]. Overproduction of ROS is a harmful process that can be an important mediator of damage to cell structures, including lipids, membranes, proteins, and DNA. Most cell damage caused by ionizing radiation is also mediated by ROS generated from the interaction between radiation and water molecules in cells [1, 2].

The antioxidant system includes enzymes and antioxidants that prevent the commencement of oxidative damage and/or its propagation [1, 3, 4]. Essential antioxidants are either endogenous or exogenous. They are typically categorized as free radical scavengers and protective antioxidants.

Since ROS and RNS are highly reactive, the approach most often employed in the study of oxidative stress is assessing the presence and quantity of the oxidative biomarkers of lipids (*e.g.* malondialdehyde), nitrosive biomarkers (*e.g.* nitric oxide), antioxidant biomarkers (*e.g.* glutathione, catalase), and inflammatory biomarkers (*e.g.* neutrophils/myeloperoxi-

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dase) [1, 4, 5].

Reduced glutathione (GSH) is a multifunctional intracellular non-enzymatic antioxidant. GSH is highly abundant in the cytosol, nuclei, and mitochondria and is the major soluble antioxidant in these cell compartments. It is considered to be the major thiol-disulphide redox buffer of the cell [1, 6, 7].

Polyunsaturated fatty acids, when exposed to ROS, can also be oxidized to hydroperoxides that decompose to hydrocarbons and aldehydes such as malondialdehyde (MDA) in the presence of metals [8]. This lipid peroxidation can cause severe impairment of membrane function through increasing membrane permeability and membrane protein oxidation [9, 10]. The causative factors of the events facilitating an inflammatory cascade such as neutrophil activation and the subsequent release of myeloperoxidase (MPO) were also studied [11].

The use of antioxidants has aroused increasing interest since it has been observed that the protection of normal tissues may provide an increase in tumor control by allowing for an increase in the radiation dose [12].

Thiol supplementation to maintain tissue redox balance has been studied by various researchers. Studies on various thiol radioprotectants such as WR-2721 (amifostine) have demonstrated successful prevention of radiation-induced damage to the intestinal epithelial and stem cells [13]. Cysteine and glutathione delivery compounds have been used to protect normal and stem cells from radiation and anti-tumor agents [12, 13]. N-acetylcysteine (NAC), a mucolytic agent and the drug of choice in paracetamol intoxication, indirectly replenishes GSH through deacetylation to cysteine and prevents oxidative damage through the scavenging of ROS [14-16].

The aim of our study was to investigate the potential radioprotective effects of NAC and to compare its effect with that of WR-2721 (amifostine), representing a clinically used radioprotector, in the prevention of oxidative damage caused by gamma-irradiation (single dose, 6 Gy) in normal rat tissue after whole body irradiation. To evaluate the grade of damage or protection, we measured the levels of some biochemical parameters (GSH, MDA, and MPO) of radiation-induced oxidative stress in serum and in the liver homogenate as a representative of normal tissues.

Materials and Methods

Eight-week-old Wistar-Albino female (Gaziantep University, Faculty of Medicine, Experimental Medicine Research Unit; 170±20 g bw) rats were used. The rats were randomly selected and housed in polycarbonate cages with free access to tap water and rat chow with a dark/light cycle of 12: 12h. A 1-week acclimatization period was used. The temperature was 22±2℃ and the 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 at Gaziantep University Faculty of Medicine.

Experimental design. After the stabilization period, the rats were divided randomly into 4 equal-size groups (10 rats per group), namely the 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 the irradiation, R rats received 2.2 ml of saline (i.p.) while the R+NAC and R+WR-2721 rats received 1,000 mg/kg (i.p.) NAC (containing 300 mg of N-acetylcysteine, Asist ampul, Hüsnü Arsan İlaç, İstanbul) and 200 mg/kg (i.p.) WR-2721 (containing 500 mg of amifostine, Ethyol flacon, Er-Kim İlaç, İstanbul), respectively. Saline, NAC and WR-2721 injections in the study groups were performed 15 min before irradiation. The drug doses were chosen according to previous studies since they were observed to have beneficial effects on preventing radiation-induced oxidative damage following irradiation after treatment with NAC or WR-2721 using a similar protocol [12, 17, 18]. A cobalt-60 teletherapy unit (Shandong Xinhua SCC-8000F Zibo, 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 (Eczacıbası, Turkey) anesthesia (35 mg/kg, intramuscularly) 72 h after irradiation. At termination, each animal's liver tissue, which was used as the representative tissue in our study, was

harvested. In addition, blood samples were taken by cardiac puncture and then centrifuged, and the serum was separated. Serum and liver tissues of each animal were stored at −80℃ until biochemical analysis.

Biochemical analyses.

1. Measurement of reduced glutathione

The GSH concentrations of the serum and tissues were measured using the method described by Beutler *et al.* [19]. Fifty-mg specimens of rat liver tissues were homogenized in 5% of NaCl. Three ml of the precipitating solution (1.67g of metaphosphoric acid, 0.2g of EDTA and 30g of NaCl in 100ml of distilled water) was mixed with 2ml of homogenate. The mixtures were centrifuged at 3,000× rpm for 5min. Two ml of filtrate was taken and added to another tube, and then 8ml of the phosphate solution (0.3M disodium phosphate) and 1ml of Ellman's Reagent (DTNB) were added. A blank was prepared with 8ml of phosphate solution, 2ml of diluted precipitating solution (3 parts to 2 parts distilled water), and 1ml of DTNB reagent. A standard solution of the glutathione was prepared (40mg/100ml). The optical density was measured at 412nm in the spectrophotometer. Serum GSH values were expressed as micromol/l while the tissue values were expressed as mmol/mgr tp.

2. Measurement of malondialdehyde levels

Fifty-mg liver tissue specimens were homogenized in 0.15mol/l of KCI for MDA determination. After the homogenate had been centrifuged at 3,000× rpm, the MDA levels in 50 μl of tissue homogenate and serum were determined by thiobarbituric acid (TBA) reaction according to Yagi K. [20]. The principle of the method is based on measuring the absorbance of the pink color produced by the interaction of TBA with MDA at 530nm. Serum and tissue MDA values were expressed as nmol/ml.

3. Measurement of myeloperoxidase activity

Three-hundred-mg rat liver tissue samples were homogenized in 0.02M EDTA (pH4.7) in a Teflon Potter homogenizer. Homogenates and serum were centrifuged at 20,000× g for 15min at +4C. After a pellet was re-homogenized in 1.5ml of 0.5% hexadecyl-tirimethyl-ammonium bromide (HETAB) prepared in 0.05M KPO4 (pH6) buffer, it was re-centrifuged at 20,000× g for 15min at +4C. The determination of the supernatant's MPO activity is based on the fact that it reduces o-dianizidine. Reduced o-dianizidine was measured at 410nm by spectrophotometer [21]. Serum MPO values were expressed as U/ml while the tissue values were expressed as U/gr tp.

Statistical analyses. The descriptive values of data were represented as means ± standard error of the mean (S.E.M.). The one-way analysis of variance (ANOVA) test was used for the determination of significant differences in the levels of GSH, MDA and MPO between groups followed by the Bonferroni post-hoc test. A value of $p < 0.05$ was considered significant, and in all calculations, the SPSS (v 11.5; Lead Technologies, Inc., Chicago, IL, USA) program was used.

Results

GSH levels in serum and liver tissues of rats. Serum and tissue GSH values are shown in Table 1. In R rats, the serum and tissue GSH levels were significantly decreased when compared to those of all other groups of rats after irradiation ($p < 0.01$). The serum and tissue GSH levels of R+NAC and R+WR-2721 rats were higher than those of the R rats and were close to the level of the C rats, indicating that NAC or WR-2721 treatment may be useful for scavenging oxygen-derived free radicals, but these differences were not significant ($p > 0.05$).

MDA levels in serum and liver tissues of rats. Serum and tissue MDA values are shown in Table 1. With regard to the serum MDA levels, there were no statistically significant differences among any of the groups of rats ($p > 0.05$). We found a significant difference between the R and C rats with respect to the tissue MDA levels ($p < 0.001$) while R+NAC and R+WR-2721 rats were not significantly different from C rats ($p > 0.05$). The tissue MDA levels of R+NAC and R+WR-2721 rats were significantly decreased when compared to those of the R rats ($p < 0.01$; $p < 0.05$, respectively).

MPO activity in serum and liver tissues of rats. Serum and tissue MPO values are shown in Table 1. The tissue MPO activity of the R group was significantly reduced when compared to that of the C group ($p < 0.001$). After applications of NAC and WR-2721, there was a marked increase in MPO activity in both R+NAC and R+WR-2721 groups when compared to the R group ($p < 0.001$). The tissue MPO activities of the C and R+NAC rats did not differ

Table 1 The reduced glutathione (GSH), malondialdehyde (MDA) and myeloperoxidase (MPO) levels of the rat groups

	GSH		MDA		MPO	
Groups	Serum (micromol/l)	Liver (mmol/mg tp)	Serum (nmol/ml)	Liver (nmol/ml)	Serum (U/ml)	Liver (U/gr tp)
C	2.26 ± 0.40	0.143 ± 0.021	24.45 ± 1.94	14.87 ± 1.66	0.954 ± 0.056	1.124 ± 0.053
R	$1.00 \pm 0.11^{a\dagger}$	$0.061 \pm 0.006^{a\dagger}$	27.28 ± 2.55	$23.99 \pm 0.61^{a*}$	0.975 ± 0.131	$0.352 \pm 0.029^{a*}$
R+NAC	1.53 ± 0.22	0.115 ± 0.019	26.28 ± 1.50	$15.59 \pm 1.03^{b\dagger}$	0.662 ± 0.064	$0.995 \pm 0.076^{b*}$
R+WR-2721	1.36 ± 0.11	0.097 ± 0.013	25.70 ± 2.05	$17.85 \pm 1.74^{b\ddagger}$	$0.427 \pm 0.072^{a\dagger,b*}$	$0.876 \pm 0.048^{a\ddagger,b*}$

Each group consists of 10 rats. C: Control rats, R: rats that received irradiation (single dose, 6 Gy), R+NAC: rats that received irradiation (single dose, 6 Gy) and were treated with NAC, R+WR-2721: rats that received irradiation (single dose, 6 Gy) and were treated with WR-2721. All values are given as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using the ANOVA test followed by the Bonferroni post hoc test. [a]Compared to C rats, [b]Compared to R rats; $*p<0.001$, $\dagger p<0.01$, $\ddagger p<0.05$. None of the serum and tissue values of the R+NAC and R+WR-2721 rats were different significantly ($p>0.05$). The cases where statistically significant differences were found are given in the Table.

significantly ($p>0.05$) while those of R+WR-2721 rats were significantly reduced when compared to those of the C rats ($p<0.05$). On the other hand, the serum MPO levels of R+WR-2721 rats were found to be decreased significantly when compared to those of the C rats ($p<0.01$), while those of the R and R+NAC rats were not different significantly ($p>0.05$). However, the serum MPO levels of R+WR-2721 rats were significantly decreased in comparison to those of the R rats after irradiation ($p<0.001$).

Discussion

Ionizing radiation interacts with living cells and produces different cytotoxic effects. Many of the actions of ionizing radiation are mediated through the production of ROS [22]. Cumulative results involving animals exposed to either non-lethal or lethal doses of X-radiation show that the biological effects of the ionizing agent are dependent on the radiation dose and post-irradiation time [23].

We particularly investigated whether GSH supplementation or direct radical scavenging would provide the primary protective effect against radiation-induced alteration in the liver homogenate as a representative of normal tissues and serum. In our study, we also measured the activity of MPO, as a major neutrophil protein, in rats exposed to 6 Gy radiation and investigated the protective effect of NAC on the radiation-induced alteration in polimorfonuclear leukocyte function in comparison with that of WR-2721.

The results of the present study demonstrated that whole-body irradiation in rats causes tissue damage in the liver as demonstrated by increased lipid peroxidation (MDA) and decreased GSH levels. The administration of NAC stimulated GSH and inhibited MDA in the liver, which is a similar effect to that observed for WR-2721. In irradiated animals, a marked radiation-induced decline in the MPO activity of liver tissue was found in comparison to the controls. The applications of each of NAC and WR-2721 significantly improved the radiation-induced decrease. The limitations of our study include the absence of sampling of different tissues, single-dose irradiation, single-dose drug treatment and the absence of research in the effects after different lengths of time to observe the possible alteration in the oxidative and anti-oxidative biomarkers.

Among the large number of tested compounds, the most-examined ones were cysteamine derivatives, *i.e.*, aminothiol radioprotectors (for example cysteine, cystamine, WR-2721, and glutathione) [12, 24, 25]. Many studies have shown the radioprotective activity of WR-2721, which has been seen on the jejunum, colon, lung, and bone marrow in preclinical and clinical studies [25-27]. There are many aspects of WR-2721 that limit its use clinically, such as its toxicity and its requirement to be present at the time of irradiation in order to be effective [28]. To discover new drug molecules is an extremely slow and very expensive process, with a high rate of failure. The strategy of identifying new uses for present drugs is more reasonable and profitable.

The intracellular content of glutathione is responsive to environmental factors and is a function of the

balance between use and synthesis. Thus, oxidative stress *in vivo* mainly translates into a deficiency of GSH and/or its precursor, cysteine [6, 7, 24, 29]. GSH deficiency may not contribute to the specific symptoms of the disease and is recognizable mainly by secondary symptoms such as oxidative damage, reduced immune function and an overall decrease in health [29]. Thus, NAC, being a cysteine prodrug, scavenges free oxygen radicals and supplies depleted body glutathione stores. NAC is a well-tolerated drug with a wide toxic-therapeutic window. It has been shown to be beneficial when GSH deficiency occurs, for example under endotoxic and septic conditions [29–35]. These previous studies suggest that NAC is considerably promising. Besides having proven antioxidant, anti-inflammatory and cytoprotective effects, NAC also ensures endothelial protection and enhances microvascular blood flow [36].

The results of the present study, being parallel with these previous reports, suggests that NAC is a very potent agent for replenishing the tissue GSH, which has a major role in the antioxidant defense mechanisms against irradiation injury [17, 37–40]. Recently, Mansour *et al.* reported that pretreatment with NAC resulted in a significant increase in the levels of the antioxidant enzymes, which was in accordance with our results, and DNA damage [18]. They suggested that pre-treatment with NAC offers protection against gamma-radiation-induced cellular damage. Neal *et al.* supported this observation. The results of their study indicate that both isomers of NAC have some, although limited, radioprotective effect on the lung, spleen, liver, and red blood cells of mice [37]. In our rat model, the results obtained were slightly different from their results, and in contrast to the significant decrease in group R, the tissue and serum GSH levels in the group with NAC supplementation with irradiation were maintained close to those of the control group. In addition, MDA levels decreased in our model and approached the control values in the liver, but not in the serum. Also, the effect of NAC was similar to the effect observed for WR-2721, which was in accordance with the results of other previous and recent experimental studies involving animal models and clinical trials, which provided encouraging results [13, 15, 25–29, 37, 39].

Lipid peroxidation can cause severe impairment of membrane function by increasing membrane permeability and membrane protein oxidation [1, 8, 9, 41]. It is suggested that NAC may protect cell membranes against lipid peroxidation and protein oxidation and helps maintain the integrity of cellular organelles [18, 37, 39]. Our study indicated that the administration of NAC stimulates GSH and inhibits MDA to an extent similar to that observed for WR-2721. In a recent study on aged mice, there were significant decreases in lipid peroxide and protein carbonyl levels in the synaptic mitochondria of NAC-supplemented mice [42].

MPO is an essential enzyme for normal neutrophil function. MPO is a heme enzyme that uses the superoxide and hydrogen peroxide generated by the neutrophil oxidative burst to produce hypochlorous acid and other reactive oxidants, and when neutrophils are stimulated by various stimulants, MPO increases like other cellular tissue-damaging substances [1, 24]. Since Klebanoff showed that the myeloperoxidase system is strongly bactericidal, MPO has been considered to be an important component of the neutrophil's antimicrobial defence mechanism [43].

The major dose-limiting sequelae following irradiation are neutropenia and thrombocytopenia. In previous studies, it was reported that irradiation may result in leucopenia and bacteriemi and depressed phagocytosis [23, 44]. In our study, in the liver tissue, the activity of MPO was decreased after irradiation. Konkabaeva *et al.* showed that there are dose-dependent changes; with increasing radiation dosage, the activity of neutrophilic MPO decreases [45]. In another study, on day 1 after 7 Gy irradiation, MPO subcutaneously administered at 200 μg/kg/d or 50 μg/kg/d, twice daily, was effective on multilineage recovery in nonhuman primates after high-dose, radiation-induced myelosuppression [46].

In our study, we investigated the effect of NAC on radiation-mediated leucocyte function. It was shown that the administration of NAC increased MPO activity in the liver tissue to levels approaching that of the control group. With the application of WR-2721, however, there was a significant increase in the MPO activity in comparison to the R group, although this level was lower than that of the C group. In serum MPO activity, no intergroup differences were observed except for the difference in the R+WR-2721 group. In the earlier study with rats and rabbits exposed to either non-lethal or lethal doses of x-radia-

tion, it was shown that radiation depresses at least 2 phases of phagocytosis, namely, the formation of leucocytes and their ability to migrate. The maximum effect on both phenomena occurred on the 3rd to 5th post-irradiation days. The authors suggested that the decrease in leucocyte migration could not be attributed either to leucopenia or to plasma factors [23].

Studies have shown that NAC inhibits both the apoptotic process induced by reactive oxygen species and the imbalances of the redox potential. This activity of NAC depends on the nucleophilic and antioxidant properties of its thiol [47]. In a study on cystic fibrosis, it was reported that oral NAC was able to do both: GSH in blood neutrophils was significantly augmented, and airway neutrophil count and elastase activity were significantly decreased [34]. However, it remains to be determined whether and how NAC influences basic cellular processes such as bacterial clearance, neutrophil-endothelial cell interplay and apoptosis [36]. Since the participation of tissue neutrophils in the radiation-induced oxidative injury of different tissues may occur at different times, time-dependent and different drug dose-dependent research may be required to observe the possible alteration in the oxidative and anti-oxidative parameters such as MPO [5, 23].

In conclusion, considering the present results, the prophylactic use of NAC seems to reduce the damage to the liver during radiotherapy. The results of this study indicated that the administration of NAC stimulates the anti-oxidant enzyme activities, inhibits the lipid peroxidation, and enhances the bactericidal activity of neutrophil by increasing induced MPO activity under radiation-induced oxidative stress. Although the findings of this study are limited to biochemical parameters, they give clues about the probability that the radioprotective effect of NAC, as shown in other studies, may be similar to that of WR-2721. In spite of WR-2721, NAC, as a drug that is safe and easy to use in clinical application, has not yet been used clinically for this purpose. Further experimental studies are needed to prove this result and to rule out the potential protection of tumor cells.

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