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Protective role of intramuscularly administered vitamin E on the levels of lipid peroxidation and the activities of antioxidant enzymes in the lens of rats made cataractous with gamma-irradiation

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PURPOSE. *To determine the antioxidant role of vitamin E (VE) (10 mg/kg/day) against radiation-induced cataract in lens after total-cranium irradiation of rats with a single dose of 5 Gy.*
METHODS. *Sprague-Dawley rats were divided into three groups. Group 1 did not receive VE or irradiation but received both 0.1 ml physiologic saline intraperitoneally and sham irradiation (control group). Group 2 received to total cranium 5 Gy of gamma irradiation as a single dose (RT group) plus 0.1 ml physiologic saline intraperitoneally. Group 3 received irradiation to total cranium plus 10 mg/kg/day VE (RT+VE group). The rats were irradiated using a cobalt-60 teletherapy unit. Chylack's cataract classification (1) was used in this study. At the end of 10 days, the rats were killed and their eyes were enucleated to measure the antioxidant enzymes (the activity of superoxide dismutase [SOD], glutathione peroxidase [GSH-Px]) and lipid peroxidation level (malondialdehyde [MDA]).*
RESULTS. *While grade 1 cataract development was detectable in seven rats in the RT group, it was detectable only in two rats in the RT+VE group, whereas none of the rats in the control group exhibited any biomicroscopic change in their lenses. MDA level and GSH-Px activity in the rat lens in the RT group was significantly higher than in the control group. SOD activity in the RT group was lower than in the control group. The activity of SOD and GSH-Px enzymes was higher in the RT+VE group, but MDA level was lower in the RT+VE group when compared with the RT group.*
CONCLUSIONS. *Vitamin E has a protective effect on radiation-induced cataract by decreasing oxidative stress. (Eur J Ophthalmol 2004; 14: 478-85)*

KEY WORDS. *Antioxidant enzymes, Gamma irradiation, Lens, Rat, Vitamin E*

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INTRODUCTION

Reactive oxygen species (ROS) are generated by normal cellular metabolism and by exogenous agents. Excess ROS may also damage cellular components. Oxidative stress arises when rates of ROS production outpace rates of removal. Oxidative stress is associated with various degenerative diseases, including cataract, macular degeneration,

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cancer, and arteriosclerosis (2). Exposure of cells to ionizing radiation leads to the formation of ROS that are associated with radiation-induced cytotoxicity. Because of the serious damaging potential of ROS, cells depend on the elaboration of the antioxidant defense system (AODS), both enzymatic and non-enzymatic oxidant defense mechanisms (3). Previous studies suggest that the damaging effect of ionizing radiation on living cells is predominantly due to ROS, including superoxide anion radical (O_2^-), hydroxyl radical ($OH^\cdot$), and hydrogen peroxide (H_2O_2), generated by the decomposition of water (4-6).

Antioxidant defense mechanisms involve both enzymatic and nonenzymatic strategies. Common antioxidants include vitamins A, C, and E, glutathione, and the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GR). They work in synergy with each other and against different types of free radicals (7), and they can offer protection against ionizing radiation-induced oxidants (8). SOD, the first line of defense against oxygen-derived free radicals, catalyzes the dismutation of O_2^- into H_2O_2 (9-11). H_2O_2 can be transformed into H_2O and O_2 by CAT and GSH-Px (10-12). One of the indices of oxidative damage is malondialdehyde (MDA) formation as an end product of lipid peroxidation (13).

Cataract is an opacity of eye lens that interferes with vision. Cataracts are formed in response to a variety of different agents and environmental stresses, and this damage seems in almost all cases to have an oxidative damage component (14). Although cataract of the eye lens is a known late effect of ionizing radiation exposure, most of the experimental work to date has concentrated on single, acute high doses or multiple, fractionated, and chronic exposures. Many articles have dealt with biochemical alterations in metabolism and cellular components, with microscopic and electron microscopic lesions to the epithelial and cortical layers, and with clinical cataract formation. However, the minimum cataractogenic dose for rats has for many years been considered to be about 2 Gy for a single, acute dose of low linear energy transfer (LET) radiation (15). It has been shown that 8 Gy fractionated delivered at the level of the lens can significantly elevate the incidence of cataract development. At higher doses, the percentage of lenses that develop cataract increases to 100% (16).

Vitamin E (VE) not only acts as an effective lipophilic antioxidant and radical scavenger (17-19) but also stabilizes cellular membranes (20, 21). The protective role of VE against radiation-induced oxidative damage was demonstrated *in vitro* (22). Using an injectable form of VE (α-tocopherol), there was a clear improvement in post-irradiation survival compared with the results for dietary administration of VE (23).

METHODS

Rats and experiments

Thirty albino female Sprague-Dawley rats, 8 to 12 weeks old, weighing 185±35 g at the time of radiation, bred at Atatürk University Medical School, Department of Pharmacology Experimental Animal Laboratory, were used for the experiment. All animals received humane care in compliance with the guidelines of criteria of the Atatürk University Research Council. The rats were quarantined for at least 3 days before irradiation, housed 10 to a cage in a windowless laboratory room with automatic temperature (22±1 ºC) and lighting controls (14 h light/10 h dark), and fed standard laboratory chow and water ad libitum.

The rats were divided into three equal groups. Group 1 did not receive VE or irradiation (control group) but received 0.1 ml saline intramuscular (IM) plus sham irradiation. Group 2 received total cranium 5 Gy of gamma irradiation as a single dose (RT group) plus 0.1 ml saline IM. Group 3 received total cranium irradiation plus 10 mg/kg/day VE (RT+VE group). The rats in the RT+VE group received 10 mg/kg/day (0.1 ml for a day) VE (containing 300 mg di-alpha-tocopherol acetate, EVIGEN ampule, Erasılaç, İstanbul, Turkey) daily by IM injection starting from 3 days before irradiation and during 7 days after irradiation (total 10 days). A total of 0.1 ml saline daily by IM injection starting from 3 days before irradiation and during 7 days after irradiation (total 10 days) was injected to both the control group and the RT group. Prior to total cranium radiotherapy, the rats were anesthetized with 80 mg/kg ketamine HCl (Pfizer İlaç, Istanbul, Turkey) and placed on a Plexiglas tray in a prone position. While the rats in the control group received sham irradiation, the rats in the RT and the RT+VE group were irradiated using a cobalt-60 teletherapy unit (Picker-C 9) from a source-to-surface distance of 80 cm, by 5 x 5 cm anterior fields with 5

Gy to the total cranium as a single fraction. A bolus material that has 0.5 cm thickness was placed on the rats' eyes. The dose was calculated for the central axis at a depth of 0.5 cm. The dose rate was 0.59 Gy/min.

Determination of clinical cataract

Chylack's cataract classification was used in this study (1). The lenses were graded by slit-lamp biomicroscopy (Nikon, Zoom-Photo Slit Lamp, FS-3V, Japan) as follows: Grade 0 = normal, clear lenses; grade 1 = lenses showing visible posterior sutures; grade 2 = lenses displaying isolated vacuoles; grade 3 = coalescing vacuoles; grade 4 = peripheral coalescing vacuoles and radial streaks extending into central crystalline opacity. At the beginning of the experiment, the lenses of all rats were classified as grade 0.

Fractionation of lens samples

At the end of 10 days, the rats were anesthetized with ether at first. Then an intracardiac withdrawal of blood was performed. Following the withdrawal of blood, the rats were killed using a high dose of ether, and their eyes were enucleated and the lenses were dissected out immediately. Lenses were homogenized by an OMNI TH International, model TH 220 (Warrenton, VA) homogenizer in isotonic saline (1/20 weight/volume) on ice for 10 seconds in the first speed level. The homogenate was centrifuged at 10,000 g for 60 min at 4 ºC. The supernatant was stored at –80 ºC in aliquots for biochemical measurements. Activities of the antioxidant enzymes SOD and GSH-Px and MDA level were determined from these supernatants spectrophotometrically.

Determination of MDA levels

Measurement of the MDA levels was carried out using the method published by Ohkawa et al (24). In this method, samples less than 0.2 ml of 10% (w/v) tissue homogenate were added to 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid solution (whose pH was adjusted to 3.5 with NaOH), and 1.5 ml of 0.8% aqueous solution thiobarbituric acid (TBA). The mixture was made up to 4.0 ml with distilled water, and heated at 95 ºC for 60 minutes. After cooling, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine mixture (15:1, v/v) were added and the sample was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. Total thiobarbituric acid-reactive substances (TBARS) were expressed as MDA, using a molar extinction coefficient for MDA of 1.56×10^5 cm^{-1} M^{-1}. MDA level was expressed as nmol/mg protein.

Determination of SOD activity

SOD activity was detected according to Sun and coworkers (25). In this method, xanthine-xanthine oxidase complex produces superoxide radicals, which react with nitrobluetetrazolium (NBT) to form the formazan compound. SOD activity is measured at 560 nm by detecting the inhibition of this reaction. By using a blank study in which all reagents except the supernatant sample was present and by determining the absorbance of sample and blank, the activity was calculated and given below. One SOD unit was defined as the enzyme amount causing 50% inhibition in the $NBTH_2$ reduction rate. SOD activity was also expressed as U/mg protein of lens sediment.

Determination of GSH-Px activity

GSH-Px activity was measured according to the Paglia and Valentine method (26). In this method, GSH-Px catalyzes the oxidation of glutathione in the presence of hydrogen peroxide. Oxidized glutathione is converted into the reduced form in the presence of glutathione reductase and NADPH, while NADPH is oxidized to NADP. The reduction in the absorbance of NADPH at 340 nm is measured. By measuring the absorbance change per minute and by using the molar extinction coefficient of NADPH, GSH-Px activity of lens tissue was calculated. GSH-Px activities were expressed as U/mg protein of lens sediment. The protein content was determined by using the Bradford method (27). Biochemical measurements were carried out at room temperature using a spectrophotometer (CECIL CE 3041, Cambridge, UK).

Statistical analyses

The results were given as the median (minimum–maximum). In the study in which we planned to

evaluate the grade of cataract and antioxidant enzymes levels in lens depending on radiotherapy in the rats, statistical analyses were made by using the SPSS packed program (Statistical Package for Social Science; Windows version 10.0) after the necessary data had been collected. Mann Whitney and Fisher exact chi-square tests were used in the comparison of the data means of the data between the control group and the RT group and the RT+VE group. $p<0.05$ was accepted as statistically significant.

RESULTS

Lens grades by slit-lamp biomicroscopy are presented in Table I. At the end of the experiment, the lenses in the control group were classified as grade 0 and those in the RT group as grades 1 and 2 ($p=0.02$). While grade 1 cataract development was detectable in seven rats in the RT group, it was detectable only in two rats in the RT+VE group, whereas none of the rats in the control group exhibited any biomicroscopic change in their lenses. On the other hand, while grade 2 cataract was detected in one rat in the RT group, it was not detected either in the control group or in the RT+VE group. Compared to those in the RT group, significant reduction of cataract formation was observed in the RT+VE group ($p=0.019$).

The level of MDA and the activity of SOD and GSH-Px in the rat lenses are all presented in Table II. In the

TABLE I - LENS GRADES BY SLIT-LAMP MICROSCOPY IN THE CONTROL, RT, AND RT+VE RAT GROUPS

Grade	Control group (n=10)	RTgroup (n=10)	RT+VE group (n=10)	Total
0	10	2	8	20
1	0	7	2	9
2	0	1	0	1
3	0	0	0	0
4	0	0	0	0
Total	10	10	10	30

RT = Radiation therapy; VE = Vitamin E; n = The number of rats in the study; 0 = Normal, clear lenses; 1 = Lenses showing visible posterior sutures; 2 = Lenses displaying isolated vacuoles; 3 = Coalescing vacuoles; 4 = Peripheral coalescing vacuoles and radial streaks extending into central crystalline opacity

TABLE II - THE LEVEL OF MDA AND THE ACTIVITY OF SOD AND GSH-PX IN THE RAT LENS

	MDA, median (minimum–maximum), nmol/mg protein	SOD, median (minimum–maximum), U/mg protein	GSH-Px, median (minimum–maximum), U/mg protein
Control group	17.85[b] (10.76–25.60)	35.19[b] (23.46–62.14)	0.16[b,c] (0.10–0.24)
RT group	25.94[a] (16.89–30.23)	23.85[a,c] (8.08–34.94)	0.27[a] (0.14–0.56)
RT+VE group	17.28[b] (12.59–32.72)	50.76[b] (26.90–67.38)	0.36[a] (0.13–0.60)

[a] $p<0.05$ Compared to control group
[b] $p<0.05$ Compared to RT group
[c] $p<0.05$ Compared to RT+VE group

MDA = Malondialdehyde; SOD = Speroxide dismutase; GSH-Px = Glutathione peroxidase; RT = Radiation therapy; VE = Vitamin E

RT group, the MDA level was higher than that of control group. There was a statistically significant difference between the RT group and the control group ($p=0.003$). In the RT+VE group, the MDA level was lower than that of RT group. There was a significant difference between the RT+VE group and the RT group ($p=0.013$). There was no significant difference between the control group and the RT+VE group. In the control group and the RT+VE group, the SOD activity was higher than that of RT group, and this level was higher in the RT+VE group than in control group. There was a significant difference between the RT group and the control group and the RT+VE group ($p<0.05$). There was also a significant difference between the RT group and the RT+VE group ($p<0.05$). In the control group, the GSH-Px activity was lower than that of the other groups ($p<0.05$). In the RT+VE group, GSH-Px activity was higher than that of RT group, but there was not a significant difference between the two groups.

DISCUSSION

Radiation is a known producer of ROS (28), contributing to radiation injury in cells and formed in cells (9). Ionizing radiation and ultraviolet light (UV) both damage biological tissues, and they produce free radical in aqueous solution, for instance, cell cytoplasm, which in turn can lead to oxidative damage to biological molecules such as nucleic acids, proteins, and lipids, leading to cataract (15, 29-31), and they may also initiate lipid peroxidation (14, 32). One of the mechanisms proposed to explain lens opacification is the oxidation of crystallins, either by radiation or ROS. The other mechanism is formation of opacities with increased calcium release by mitochondrial damage (33-35). This process can be prevented by antioxidant enzymes (SOD, CAT, and GSH-Px) present in the lens epithelium. A decrease in the antioxidant defenses could be responsible for increased lens oxidation and cataract development (35).

Many studies have dealt with the incremental effect of UV radiation (36) in cataract development in addition to antioxidant enzymes level and lipid peroxidation changes in the lens owing to UV radiation (13, 37).

Anwar and Moustafa (37) and Bardak et al (13) reported that lenses of rats exposed to UVA and UVB radiation showed a significant reduction in SOD and GSH-Px activities. Elevated MDA levels served as an index of cellular damage by free radicals. Bhuyan et al (38) evaluated the effect of the free-radical generator menadione on the time of occurrence of cataract in the Emory mouse, a model for human cataract. They found that MDA levels were significantly higher than in controls in the lenses incubated in the presence of menadione, showing that it generated oxidative stress. The observed increases in CAT and GSH-Px activities in the test lenses indicated an early protective response to oxidative insult. Bono et al (39) reported that the GSH-Px activities did not change following X-ray irradiation (2000 rads) in rabbit lens.

In the present study, we found that the MDA level in the rat lens in the RT group was significantly higher than that of control group. This is consistent with the hypothesis that gamma irradiation may generate oxidative stress. We also found that the SOD activity in the RT group was lower than that of control group, and the GSH-Px activity in the RT group was higher than that of control group. These results may also emphasize generating oxidative stress and indicate an early protective response to oxidative damage.

VE is a free radical scavenger, i.e., a sacrificial molecule with which the peroxy radicals preferentially react, rather than with biological molecules, thus preventing damage to cell structures. It also scavenges O_2^-, OH^-, singlet oxygen, lipid peroxyl radicals, and other radical species (40, 41). VE physically stabilizes membrane permeability and fluidity. VE is a potent anti-inflammatory agent. It can prevent apoptosis due to oxidative stress (42). VE exists in four common forms, including α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol. Of these, δ-tocopherol is the most effective scavenger of free radicals and the most predominant tocopherol in human retina and plasma (43). Kumar et al (44) reported that the use of alpha-tocopherol succinate during radiation therapy might have improved the efficacy of radiation therapy by enhancing tumor response and decreasing some of the toxicity toward normal cells. This is one reason why we used di-α-tocopherol acetate as a radioprotector in the present study.

In previous studies, VE prevented increase in oxidative damage to lipids and DNA in liver of osteogenic disorder Shionogi (ODS) rats given total body X-ray irradiation (45), radiation-induced liver damage (46), intestinal injury (8), lethality (47), and cataractogenesis (14, 29).

It has been shown that VE prevented stress-induced changes in visual evoked potentials (48), methylprednisolone-induced cataract (49), VE deficient-related cataract (50), and streptozotocin-induced cataract (51), by improving antioxidant enzymes status and lipid peroxidation.

Umegaki et al (52) showed that total body irradiation with X-rays at 3 Gy decreased both VE level and antioxidants in various tissues, such as bone marrow, liver, and plasma. They suggested that a decrease in antioxidant vitamins was involved in the mechanism of oxidative damage.

In the present study, we found that in the RT group, the MDA level was higher than that of RT+VE group. This result was statistically significant. We also found that the SOD activity was significantly higher in the RT+VE group than in the RT group, and the GSH-Px activity was higher in the RT+VE group than in the RT group, but not significantly. These results led us to believe that VE treatment might accelerate the activity of lens SOD enzyme, and the antioxidant system would be able to clear away gradually free radicals occurring from the environment after abolishing the superoxide dismutase level as a result of VE treatment. We also found that VE treatment decreased lipid peroxidation and that gamma irradiation to total cranium at 5 Gy enhanced cataract formation over 10 days, and VE treatment over 10 days (3 days before and 7 days after irradiation) protected the lenses from radiation-induced cataract.

In conclusion, according to previous studies and our findings, gamma radiation may decrease VE level and antioxidant enzymes activity and lipid peroxidation in the lens. Cataract formation may develop because of these conditions, and VE treatment may decrease these deficits. VE may have a protective effect on radiation-induced cataract by protecting against oxidative stress.

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