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Experimental Study / *Deneysel Çalışma*

Evaluation of Oxidant Injury Induced by Irradiation in Brain Tissues of Rats of Different Ages

Farklı Yaşlardaki Ratların Beyin Dokularında Radyasyonla İndüklenen Oksidatif Hasarın Değerlendirilmesi

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Objectives: We aimed to evaluate the age-related changes of oxidative injury in the brain tissues of rats produced by radiotherapy that is widely used on cancer treatment.

Study Design: Fifty-five male Wistar albino rats [ages of rats were 1, 4, 12 weeks (n=10) and 1 year (n=5)] were divided into four groups. Irradiation were performed on a Cobalt-60 unit using a single fraction of 8 Gy. The brain tissues were homogenized and divided into two portions. One portion was used for the measurement of the malondialdehyde (MDA). The other portion was used for the measurement of the protein concentration, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) enzyme activities.

Results: The SOD activity decreased significantly in 1-week-old rats ($p<0.05$). The decreases in GSHPx and CAT activities were more obvious in 1-week and 1-year-old rats than that in others. No significant changes were observed in adolescent and adult rats. The MDA levels of all groups increased. The highest MDA levels were seen in 1-year-old rats ($p<0.05$).

Conclusion: Ionizing radiation used in radiotherapy affected antioxidant systems and increased MDA levels. These changes were more in the 1-week and 1-year-old rats than in others. This can be due to incomplete development of many systems in newborn rats and the loss of physiological capacities associated with aging in 1-year-old rats.

Key words: Radiotherapy; aging; oxidant injury; brain; free radicals.

Amaç: Bu çalışmada, kanser tedavisinde yaygın olarak kullanılan radyoterapinin farklı yaşlardaki ratların beyin dokusunda oluşturduğu oksidatif hasarın değerlendirilmesi amaçlandı.

Çalışma Planı: Elli beş Wistar albino tipi erkek rat, 1, 4 ve 12 haftalık (n=10) ve 1 yaş (n=5) olmak üzere dört gruba ayrıldı. Tüm gruptaki ratlara Co-60 cihazı ile 8 Gy tek fraksiyon radyasyon uygulandı. Ratların beyin dokuları homojenize edilerek ikiye ayrıldı. Bir yarısı ile malondialdehid (MDA) ölçümü yapıldı. Diğer yarısından elde edilen süpernatant ile protein miktar tayini yapıldı, süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GSHPx) aktiviteleri ölçüldü.

Bulgular: SOD aktivitesinin 1 haftalık ratlarda anlamlı derecede azaldığı bulundu ($p<0.05$). GSHPx ve CAT aktivitesindeki azalma 1 haftalık ve 1 yaşındaki ratlarda daha belirgindi. Ergen ve erişkin ratlarda önemli değişmeler olmadı ($p<0.05$). MDA düzeyinde tüm yaş gruplarında artma gözlendi. En yüksek MDA düzeyi 1 yaşındaki ratlarda gözlendi ($p<0.05$).

Sonuç: Radyoterapide kullanılan iyonize radyasyon antioksidan sistemleri etkilemekte ve doku MDA düzeylerini artırmaktadır. Bu değişiklikler 1 haftalık ve 1 yaşındaki ratlarda daha fazladır. Bu durumun yeni doğan ratlarda henüz birçok sistemin tam olarak yerleşmemiş oluşundan ve 1 yaşındaki ratlarda ise yaşlanmaya bağlı olarak ortaya çıkan fizyolojik kapasitedeki kayıplardan kaynaklandığını söyleyebiliriz.

Anahtar sözcükler: Radyoterapi; yaşlanma; oksidan hasar; beyin; serbest radikaller.

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Free oxygen radicals (ROS) have been implicated in the pathogenesis of more than hundred of diseases and conditions in humans, including arthritis, hemorrhagic shock, atherosclerosis, ischemia and reperfusion injury, Alzheimer's disease, Parkinson's disease, gastrointestinal dysfunctions, tumor promotion and carcinogenesis, aging and AIDS.[1] The ROS initiate the oxidant degradation of the unsaturated fatty acids in the cellular membranes through lipid peroxidation[2] and the oxidant degradation of distinct enzyme proteins through the alteration of their active sites as well as through the alteration of their three-dimensional structures.[3] On the other hand, the ROS trigger various enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) as part of the antioxidant defence system for protection against the lipid peroxidation-mediated injury. The concentration of free radicals during normal oxygen metabolism is controlled by various endogen and/or exogen antioxidants and a balance exists between oxidant/prooxidant and antioxidant processes.[4] However, age-associated alterations in the antioxidant status could also contribute to increased oxidative stress in aging. Many living systems, including rats as well as humans, have a varying degree of antioxidant capacity at different periods of their lives. It is known that there is a negative correlation between aging and the response against the oxidative stress.[5]

Due to the abundance of the unsaturated fatty acids in the cellular membranes and the presence of a relatively modest antioxidant defence system, the brain is extremely vulnerable to oxidant injury induced by irradiation through lipid peroxidation The brain involves maturation-dependent cellular and molecular features including altered energy metabolism and perturbed cellular water and electrolyte homeostasis as well as unique structural and functional responses involving the cellular signalling pathways. Therefore, these maturation-dependent discrepancies should be taken into account in attempts at understanding oxidant injury in the brain induced by irradiation through lipid peroxidation.[6,7]

This study presents the evaluation of oxidant injury in the brain induced by irradiation as a function of maturation in a rat model.

MATERIALS AND METHODS

Design

The study was undertaken at the "Laboratory for Experimental Studies of İnönü University" in accordance with the guidelines established in the "Guide for the Care and Use of Laboratory Animals" following the approval of the design by the "Animal Ethics Committee of İnönü University". Fifty-five male Wistar rats were divided into four groups. The rats in Group 1a (n=10) were one week of age and weighed 15 to 20 grams. The rats in Group 2a (n=10) were one month old and weighed 40 to 50 grams. The rats in Group 3a (n=10) were three months old and weighed 220 to 250 grams. The rats in Group 4a (n=5) were twelve months old and weighed 300 to 350 grams. The control subgroups (n=5) were formed for each group (namely Group 1b, Group 2b, Group 3b and Group 4b). The rats in each group were kept in separate cages in rooms with controlled light and temperature and were fed with standard chow and water ad libitum.

Irradiation

The rats in the experimental subgroups underwent irradiation, whereas the rats in the control subgroups underwent sham irradiation. Prior to irradiation or sham irradiation, the rats received anesthesia using ketamine (Ketalar, Pfizer İlaçları Limited Şirketi, İstanbul, Turkey) at a dose of 80 mg/kg and xylazine (Rompun, Bayer Türk Kimya Sanayi Limited Şirketi, İstanbul, Turkey) at a dose of 5 mg/kg administered using an i.p. injection. The rats were immobilized in the prone position in a custom-designed acrylic restrainer. For the rats in the experimental subgroups, whole body irradiation was performed on a Cobalt-60 unit using a single fraction of 8 Gy. For the rats in the control subgroups, sham irradiation was performed on a Cobalt-60 unit over the same fraction duration. Following irradiation or sham irradiation, the animals were closely observed until recovery from anesthesia.

Euthanasia

The rats in the experimental subgroups underwent euthanasia at one hour following irradiation, whereas the rats in the control subgroups underwent euthanasia at one hour following sham irradiation. Prior to euthanasia, the rats received anesthesia using propofol (Propofol, Abbott Laboratuvarı Anonim Şirketi, İstanbul, Turkey) at a dose of 50 mg/kg administered using an i.p. injection. Euthanasia was performed by way of transcardiac perfusion using 0.9% sodium chloride. Subsequently, the brain tissue was dissected for blinded biochemical evaluation and frozen on crushed dry ice.

Biochemical evaluation

Prior to the preparation of the tissue homogenate, the brain tissue was perfused with cold phosphate buffered saline (PBS) solution (50 mM, pH 7.4) in attempt to prevent contamination with blood, dried and weighed. The temperature was kept at +4 °C throughout the preparation of the homogenate. The tissue was homogenized in PBS solution (with a weight to volume ratio of 1 to 5) using a homogenizer. The homogenate was divided into two portions.

One portion of the homogenate was immediately used for the measurement of the malondialdehyde (MDA) level, in accordance with the method of Mihara and Uchiyama.[8] Half ml of the homogenate was mixed with 3 ml of 1% phosphoric acid (H_3PO_4). Following the addition of 1 ml of 0.67% thiobarbituric acid (TBA), the mixture was heated in boiling water for 45 minutes. The colored complex was extracted into n-butanol and the absorbance was measured at 532 nm. with tetramethoxypropane as the standard. The MDA level was expressed in nanomoles per milligram of protein (nmol/mg).

The other portion of the homogenate was sonicated four times for 30 seconds at intervals of 20 seconds and centrifuged at 20.000xg for 15 minutes. The supernatant was separated and stored at –40 °C until the measurement of the SOD, CAT and GPX activities. The protein concentration in the supernatant was measured in accordance with the method of Lowry et al.,[9] with bovine serum albumin as the standard. The SOD activity in the supernatant was measured in accordance with the method of McCord and Fridovich.[10] For Solution A, 100 ml of PBS solution (50 mM, pH 7.4) including 0.1 mM of ethylenediaminetetraacetic acid (EDTA) and 2 μM of Cytochrome c was mixed with 10 ml of 0.001 M sodium hydroxide (NaOH) including 5 μM of xanthine. For Solution B, 0.2 U/ml of xanthine oxidase was mixed with 0.1 mM of EDTA. Subsequently, 50 μl of the supernatant and 50 μl of Solution B was added to 2.9 ml of Solution A. The change in the absorbance was monitored at 550 nm. The change in the absorbance of a blank mixture including all of the ingredients other than the supernatant (substituted with 10 μl of ultrapure water) was also monitored. The SOD activity was expressed in the amount causing a 50% inhibition of the reduction of Cytochrome

Table 1. Brain SOD, CAT, GPx enzyme activities and MDA levels

Groups	SOD (U/mg protein)	GPx (U/mg protein)	CAT (U/mg protein)	MDA (nmol/mg protein)
Group Ia (n=10)	$0.384\pm4.130\times10^{-2}$ a	$0.222\pm2.589\times10^{-2}$ a	1158.74 ± 53.05 d	2.651 ± 0.424
Group IIa (n=10)	$0.623\pm3.096\times10^{-2}$ c,d	$0.165\pm5.517\times10^{-3}$ a,b,c	459.52 ± 39.3 b,c	2.659 ± 0.438 a
Group IIIa (n=10)	$0.613\pm3.363\times10^{-2}$ c	$0.188\pm5.349\times10^{-3}$	354.017 ± 37.52 c,d	2.498 ± 0.359
Group IVa (n=5)	$0.502\pm4.693\times10^{-2}$	$0.206\pm2.878\times10^{-2}$	688.8 ± 145.56	3.560 ± 0.823 a
Group Ib (n=5)	1.061 ± 0.560	0.844 ± 0.564	1640.18 ± 575.56	1.783 ± 0.436
Group IIb (n=5)	$0.528\pm3.744\times10^{-2}$	$0.330\pm8.860\times10^{-2}$	406.49 ± 46.62	1.371 ± 0.184
Group IIIb (n=5)	$0.521\pm3.112\times10^{-2}$	$0.176\pm7.153\times10^{-3}$	437.06 ± 58.96	2.35 ± 0.384
Group IVb (n=5)	$0.557\pm3.464\times10^{-2}$	$0.245\pm5.846\times10^{-2}$	450.71 ± 31.20	$0.869\pm4.726\times10^{-2}$

a: $p<0.05$ when compared with own control; b: $p<0.05$ when compared with Group III; c: $p<0.05$ when compared with Group I; d: $p<0.05$ when compared with Group IV

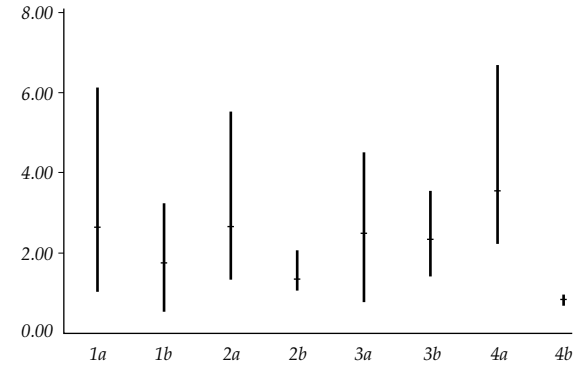

Fig. 1. *MDA levels of all groups.*

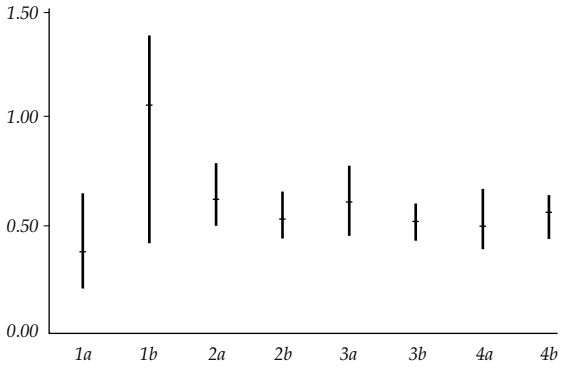

Fig. 2. *SOD levels of all groups.*

c per milligram of protein (U/mg), with bovine copper-zinc SOD (Cu/Zn SOD) as the standard. The CAT activity in the supernatant was measured in accordance with the method of Luck.[11] The decomposition of the substrate H_2O_2 was monitored at 240 nm on a spectrophotometer. The CAT activity was expressed in micromoles of H_2O_2 decomposed per minute per milligram of protein (U/mg). The GPx activity in the supernatant was measured in accordance with the method of Lawrence and Burk.[12] One ml of PBS solution (1 ml, 50 mM, pH 7.4) was incubated at 37 ºC for five minutes together with 5 mM of EDTA, 2 μM of nicotinamide adenine dinucleotide phosphate (NADPH), 20 μM of glutathione, 10 μM of sodium azide (NaN_3) and 23 mU of glutathione reductase. Subsequently, 20 μl of H_2O_2 solution (0.25 mM) and 10 μl of the supernatant were added to the mixture. The change in the absorbance was monitored at 340 nm for three minutes on a spectrophotometer. The change in the absorbance of a blank mixture including all of the ingredients other than the supernatant (substituted with 10 μl of ultrapure water) was also monitored. The GPX activity was expressed in micromoles of NADPH consumed per minute per mg of protein (U/mg), using an appropriate molar absorptivity coefficient (6220 $M^{-1}cm^{-1}$).

Statistical analysis

The mean MDA levels and the mean SOD, CAT and GPx activities were compared using the Kruskal-Wallis Analysis of Variance. Statistical analysis was performed using SPSS for Windows version 10.0 software package. Statistical significance was defined as the P value being less than or equal to 0.05.

RESULTS

Table 1 and the figures show the brain SOD, CAT, GPx enzyme activities and MDA levels. We found increased brain SOD activity in Group Ia ($p<0.05$) compared with its own control.

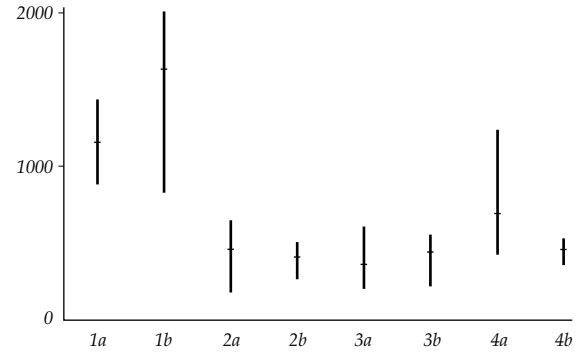

Fig. 3. *CAT levels of all groups.*

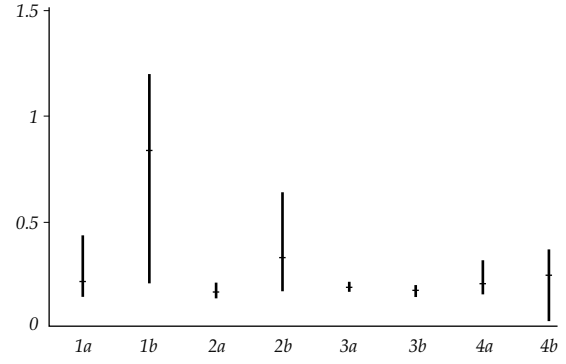

Fig. 4. *GPx levels of all groups.*

However, there was no statistically significant difference in SOD levels between the other groups and their controls ($p>0.05$). Increased GPx levels were determined in Group Ia and IIa compared with their own control groups ($p<0.05$). In addition, GPx activities of Group Ia was higher than Group IIa and IIIa ($p<0.05$). We could not find any difference in CAT levels of all groups compared with controls but CAT activity of Group Ia was higher than the other groups. The highest MDA levels were determined in the oldest group (Group IVa, $p<0.05$) and increased MDA levels were found in Group IIa and IVa compared with their own controls.

DISCUSSION

Radiotherapy technique is one of the major strategies of cancer treatments. However, ionizing radiation is a well-established carcinogen due to the resulting oxidative damage, and the molecule most often reported to be damaged by this physical agent is DNA. About 60-70% of cellular DNA damage produced by ionizing radiation is caused by hydroxyl radicals, produced from the radiolysis of water. Besides nucleic acids, lipids and proteins are also targeted by free radicals induced by ionizing radiation.[13]

Aging refers to the multidimensional process of the accumulation of physical, psychological and social change in an organism over time. Conceivably, a higher metabolic rate would shorten the lifespan by accelerating the accumulation of nuclear errors (e.g., DNA damage) or of cellular damage (e.g., due to free radicals). Inherited differences in the rate of aging make a mouse elderly at three years and a human elderly at 90 years.[14] These genetic differences affect a variety of physiological processes, including the efficiency of DNA repair, activities of antioxidant enzymes and rates of free radical production. Aging is generally characterized by the declining ability to respond to stress and increasing homeostatic imbalance.[15] The free radical theory of aging proposes that age-dependent deterioration in cell function is related to accumulation of molecular oxidative damage, caused by the ROS. It is widely accepted that lipid peroxidation is probably the primary event in the progress of several diseases as well as degenerative processes associated with aging.[16] The products of lipid peroxidation initiate further free radical chain reactions and can cause oxidative damage to proteins and DNA.[17] Accumulation of oxidatively damaged DNA, as a result of lipid peroxidation or direct effect of the ROS, could lead to a decreased mRNA expression and protein production and decline in physiological functions during aging.[18]

The brain shares most of the cellular and molecular aspects of aging, including altered energy metabolism and perturbed cellular ion homeostasis as well as intense oxidant injury to proteins, lipids and DNA.[19] Fatty acids are essential for the structure and the function of the neuronal and the glial cellular membranes, as well as being the crucial components of the myelin sheath. Therefore, their biosynthesis is fundamental for the normal maturation of the brain.[20] Since myelin is most actively synthesized during the perinatal period and reaches a steady state during later in development, it is critical that the correct molecules are present for assembly when the synthesis of myelin is most active.[21] However, due to the molecular complexity of the brain and the post-mitotic nature of the neurons, there also are age-related changes unique to the brain, such as those involving the cellular signalling pathways. Oxidant injury, metabolic compromise and perturbed cellular ion homeostasis interact in a feed-forward manner to promote neuronal dysfunction and degeneration. Such findings are consistent with previous work demonstrating that the brain is particularly vulnerable to oxidative damage during periods of maturation and development and that oxidative episodes occurring during these critical periods seriously impact brain maturation, with consequences ranging from cell death to impaired differentiation of axons and to decreases in synapse formation. This suggests that the effects of oxidative stress in the immature animals might involve additive or synergistic effects between ongoing developmental apoptosis and the stressor stimulus.[22]

In this study, we investigated the possible relationship between irradiation and oxidative

stress parameters in the brain tissues that have been obtained from rats of different age groups. Schindler et al.[23] emphasized that age is an important factor to consider when investigating the radiation response of the brain. In contrast to young adults, older rats show no sustained decrease in number of immature neurons after WBI, but have a greater inflammatory response. The latter may have an enhanced role in the development of radiation-induced cognitive dysfunction in older individuals. Enzymatic response of the brain to oxidative injury declines with age. Niwa et al.[24] reported that the basal activity of the three H202 scavenging enzymes, catalase, glutathione peroxidase, and D-glucose-6-phosphate dehydrogenase was significantly higher in younger adults than in elderly individuals. In addition, in the literature it was expressed many times by different researchers that the basal SOD activity levels decreases with age.[25,26] Sivonova et al.[27] demonstrated a significant decrease in the total antioxidant capacity of plasma for 15-month-old and 26-month-old rats in comparison with the 6-month-old group. However, some researchers have not been able to confirm the decrease in the activity of CAT and GPX with aging.[28]

This study does not aim to make a comparison between the control groups consisting of rats at different age groups. As it is seen, the number of the subjects in the groups is not appropriate for a purpose like this. The main purpose of the study is to comparatively evaluate both the extent of oxidative damage and the depth of antioxidant feedback at the rats which have been exposed to the same radiation doses and at different maturation stages. The study has been designed as appropriate to the purpose and envisaging that one-week-old rats are equivalent to the newborn stage of human; four-week-old rats are equivalent to adolescence stage of human; 12-week-old rats are equivalent to the maturity stage of human and one-year-old rats are equivalent to the senile stage of human. The findings show that malondialdehyde levels are dramatically high in the groups consisting of rats at different edges of the life spectrum. Certain aldehydes such as MDA, the end product of lipid peroxidation reactions, arising from the reactive oxygen radicals degradation of polyunsaturated fatty acids of cellular membranes, can cause cross-linking with proteins and nucleic acids.[29] In newborn group, parallel to high MDA levels, a significant decrease in enzymatic antioxidant defence system is clearly seen. Increasing lipid peroxidation is probably the result of weakening in antioxidant enzyme system. The clear weakening in the enzyme system of this group, in one hand, depends on the decomposition of three dimensional conformations of enzymes stem from the free radicals and on the other hand, expected up-regulation of enzymes has not occurred at genes level as a reaction to the oxidative stress. Similarly, it should be thought that the up-regulation of dehydrogenases which are responsible for purifying the aldehydes, which is product of lipid peroxidation, from the cellular medium has not been inhibited at gene level. The same situation can be stated for the old rats with regard to MDA. On the other hand, an oxidant injury at a low level of intensity, as in irradiation at low doses, might enhance the antioxidant defence system via stimulation, while an oxidant injury at a high level of intensity, as in irradiation at high doses, might exceed the capacity of the antioxidant defence system via oxidant damage of enzymes.[30] However, the changes observed in the antioxidant enzyme levels of this group are quite similar to those of adolescent and mature groups. In general, the most striking changes between the enzymes are seen in GPx. The probable reason of this is that the first defence against the peroxides comes from the GPx and increasing oxidative stress and CAT are involved in this defence.

Based on our findings, we may conclude that radiotherapy application especially in children and elderly people should be supported by exogenous antioxidant agents whose effectiveness is known, regarding the limitations which depend on the radiotherapy.

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