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Day and Night Effect of Single Dose of Ionising Radiation and Melatonin Treatment of Lung Tissue of Rats

[Sıçan Akciger Dokusunda Tek Doz İyonize Radyasyonun ve Melatonin Tedavisinin Gündüz ve Gece Etkileri]

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ABSTRACT

Objectives: We investigated the day-night differences in oxidative-injury and the inflammatory response in rat lung following total body irradiation by measuring thiobarbituric acid reactive substances levels and myeloperoxidase activity.

Materials and Methods: 63 male rats were randomly divided into 6 groups and were exposed to a dose of 8 Gy to the total body region either in the morning or evening together with vehicle or melatonin administration, immediately before, immediately after and 24 h preceeding irradiation (10, 20 and 10 mg/kg, ip, respectively). Rats in the control group didn't receive any treatments. 48 h after irradiation, all animals were sacrificed.

Results: Irradiation increased thiobarbituric acid reactive substances levels. Melatonin administration decreased both thiobarbituric acid reactive substances levels and myeloperoxidase activity when the time of irradiation is not taken into consideration. Depending on the time of irradiation, thiobarbituric acid reactive substances levels increased after total body irradiation only in morning group; treatment with melatonin significantly decreased thiobarbituric acid reactive substances levels. Total body irradiation did not increase myeloperoxidase levels but melatonin treatment in both time-points caused a significant decrease.

Conclusion: The effect of melatonin can be attributed to its antioxidant and free radical scavenger properties. Although further studies are required to define underlying mechanism(s) of time dependent variations in thiobarbituric acid reactive substances levels in rats to which total body irradiation and total body irradiation plus melatonin were applied, this study might help to optimize the treatment time with irradiation.

Key Words: Ionizing radiation, melatonin, circadian rhythm, lung

ÖZET

Amaç: Bu çalışmada, sabah ve akşam olmak üzere, farklı zamanlarda uygulanan tüm vücut radyoterapisi ve melatonin+radyoterapi uygulanması sonrası sıçan akciğer dokusundaki oksidatif stres ve enflamasyona cevabı, tiyobarbitürik asit reaktif ürünlerinin seviyesinin ve miyeloperoksidaz aktivitesinin ölçülerek araştırılması amaçlandı.

Gereç ve Yöntemler: 63 erkek sıçan 6 gruba gelişigüzel dağıtıldı ve tek doz 8 Gy ön-arka tüm vücut ışınlaması yapıldı. Melatonin veya çözücü (etanol) ışınlamadan hemen önce, sonra ve 24 saat sonra sıçanlara uygulandı (melatonin dozu sırasıyla; 10 mg/kg, 20 mg/kg ve 10 mg/kg, ip). Kontrol grubundaki hayvanlara herhangi bir işlem uygulanmamıştır. Işınlamadan 48 saat sonra, tüm hayvanlar ketamin kullanılarak feda edildi.

Bulgular: Işınlama tiyobarbitürik asit reaktif ürünlerinin düzeyini arttırmıştır. Işınlamanın uygulama zamanına bağlı olarak, total vücut ışınlaması sonrası tiyobarbitürik asit reaktif ürünlerinin seviyeleri sadece sabah gruplarında artmıştır, melatonin verilmesinden sonra hem tiyobarbitürik asit reaktif ürünlerinin seviyesi hem de miyeloperoksidaz aktivitesi düşmüştür. Miyeloperoksidaz aktivitesi total vücut ışınlama sonra değişmemiştir, fakat melatonin uygulaması hem sabah hem de akşam gruplarında anlamlı bir düşüşe neden olmuştur.

Sonuçlar: Çalışmamızda melatoninin antioksidan ve serbest radikal süpürücü etkisinin olduğu bir kez daha gösterilmiştir. Ratlarda tüm vücut ışınlaması ve melatonin ile birlikte tüm vücut ışınlaması uygulanan sabah gruplarında gözlenen tiyobarbitürik asit reaktif ürünlerinin düzeylerindeki zaman bağımlı farklılığın altında yatan mekanizmanın açıklanması için yeni çalışmalara gereksinim olmasına karşın bu bulgular ışınlama için optimum zamanın belirlenmesine yardımcı olabilir.

Anahtar Kelimeler: İyonize radyasyon, melatonin, sirkadiyen ritim, akciğer

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Introduction

Ionising radiation remains one of the most effective and essential tools in the therapy of cancer (1) but its immediate and delayed side effects on the normal tissues limit the effectiveness of the therapy (2).

The deleterious effects of ionizing radiation in biological systems are mainly mediated through the generation of reactive oxygen species (ROS) in cells as a result of water radiolysis (3). ROS interact with cellular molecules, including lipids, proteins and DNA (4). Radiation induced lipid peroxidation is a free radical process (5,6) which involves oxidative conversion of polyunsaturated fatty acids to several products including malondialdehyde (MDA) and lipid peroxides (7).

Besides their direct damaging effects on tissues, free radicals seem to trigger the accumulation of leukocytes in target and surrounding tissues and cause further injury through activated neutrophils. It has been shown that activated neutrophils secrete enzymes such as myeloperoxidase (MPO) (8-10).

Several body functions follow distinct day/night rhythms and are controlled by the circadian (approximately 24 hours cycle) system (11). 24-hour fluctuations of both oxidants and antioxidants have been reported (12). Rhythms in free radical formation by leucocytes are demonstrated in mice particularly during inflammation (13,14). Our previous report demonstrated a significant time dependent variation in hepatic superoxide dismutase (SOD) activity in mice (15). Belanger et al. (16) showed that temporal variation in microsomal lipid peroxidation and in the concentration of reduced glutathione exhibited a characteristic circadian rhythm. These rhythms in SOD and glutathione are important in determining the resistance to some toxic substances.

Melatonin is an indole that is synthesized and secreted from the pineal gland during the night and it has been shown to be an effective antioxidant and free radical scavenger (17). Melatonin, because of its small size and high lipophilicity, crosses biological membranes easily, thus reaching all compartments of the cell (18,19). Previously, it has been shown that melatonin has also a capacity to inhibit the proliferation of various tumor cell lines (20,21). Additionally, Akagi et al. have demonstrated that antitumor effect of melatonin was significantly different according to dosing time. Moreover they have shown that the specific binding of melatonin to tumor cells showed rhythmicity (22). Moreover, chronobiological studies showed that there was a circadian rhythmicity in radiation response after whole body irradiation in mice and rats with highest toxicity in light-dark 12h: 12h synchronized animals during their daily activity span (23).

Since response to the radiation and spesific binding of melatonin to its receptor can show circadian rhythmicity, the timing of irradiation and melatonin treatment can expect to change the toxic effects and/or effectiviness of the irradiation. For this reason, in the present study, using rat model, we assessed the day-night differences in lung tissue applying total body irradiation (TBI) by measuring thiobarbituric acid reactive substances (TBARS) levels and myeloperoxidase (MPO) activities. Moreover, we investigated the possible acute radioprotective effect of melatonin in attenuating the deleterious effects of irradiation applied two different time of day on lung tissue.

Methods

Experimental design

The experiments were performed on male Wistar rats, body weights 220-280 g. They were fed a standart rat chow diet, had access to water ad libitum, and were housed in controlled environmental conditions (light, temperature, feeding time, etc.). The animals were synchronized to a light-dark cycle (lights on from 08:00 h to 20:00 h) beginning at least two weeks before the commencement of experiments. The experiments were performed during February-March to avoid the influence of seasonal rhythms on the findings. A total of 63 adult animals were randomly divided into 6 groups. The experimental groups were shown in Table 1.

All animal procedures were carried out according to the rules of Local Ethic Committee. The rats in irradiated groups were exposed to a dose of 8 Gy irradiation to total body following ketamine anesteshia using a ^{60}Co source at a focus 80 cm distance from the skin in two different time of day. Morning irradiation was performed 1 hour after lights on (1 HALO) (at 09:00 a.m.) and evening irradiation was performed at 13 hours after lights on (13 HALO) (at 21:00 p.m.). Melatonin or vehicle (ethanol 20 %) was administered immediately before, after and 24 h preceeding irradiation to the rats (melatonin dose: 10 mg/kg, 20 mg/kg and 10 mg/kg, ip, respectively). Control rats did not receive any treatment. 48 h after irradiation, all animals were sacrificed using ketamine. Lung tissues were removed immediately and divided into three pieces and were taken the upper lobes for this study. Tissues were stored at -80 ^{o}C until the measurement of TBARS levels and MPO activity. Changes in TBARS levels, a marker of lipid peroxidation and MPO activity, an index of neutrophil infiltration, were determined in lung tissue.

Determination of TBARS levels

The level of TBARS was determined in lung tissue homogenized in the ratio of 1/10 (w/v) in 1.15 % cold KCl solution, by the aid of thiobarbituric acid method, and the results were obtained in nmol/g tissue weight (24).

Determination of MPO activity

Lung tissue MPO activity was determined by the method of Koike et al. (25). Lung tissue was homogenized in 20 mmol/L potassium phosphate buffer (pH 7.4) and

Table 1. Experimental groups: A: Morning (1 HALO), B: Evening (13 HALO). Each group consists of 8-12 animals

	Control	Ethanol (% 20)	TBI (8 Gy)	Melatonin
1A (n=8)	+	-	-	-
1B (n=11)	+	-	-	-
2A (n=11)	-	+	+	-
2B (n=12)	-	+	+	-
3A (n=12)	-	-	+	+
3B (n=9)	-	-	+	+

the homogenate then was centrifuged for 5 minutes at 40.000 × g at 4 °C. The supernatant was discarded, and the pellet was resuspended in 50 mmol/L potassium phosphate buffer (pH 6.0) containing 5 % hexadecyltrimethyl ammonium bromide and frozen at −70 °C. Before assay, batched samples were thawed, sonicated for 90 seconds, incubated for 2 hours in a water bath (60 °C), and then centrifuged at 10.000 × g for 5 minutes. The supernatants were used for MPO assay. MPO activity was assessed by measuring the H_2O_2-dependent oxidation of o-dianisidin. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0/min at 410 nm and 37 °C (26).

Statistical analysis

Differences between groups were statistically analysed by two-way analysis of variance (ANOVA). Post-hoc tests were performed using a multiple comparison procedure (Tukey Test). Statistical significance was accepted as $p< 0.05$.

RESULTS

TBARS levels

When the time of irradiation is not taken into consideration, TBARS levels in lung were found to be significantly higher in rats exposed to TBI (Group 2), as compared to that of the control group (Group 1). Treatment with melatonin (Group 3) significantly decreased the irradiation-induced elevations in TBARS levels in TBI rats (Figure 1, two-way ANOVA $p<0.05$)

MPO activity

When the time of irradiation is not taken into account, MPO activity in the lung tissue did not markedly changed after TBI (Group 2) when compared to control group (Group 1), while melatonin administration (Group 3) decreased MPO activity in rats exposed to irradiation (Figure 3, two-way ANOVA, $p<0.05$).

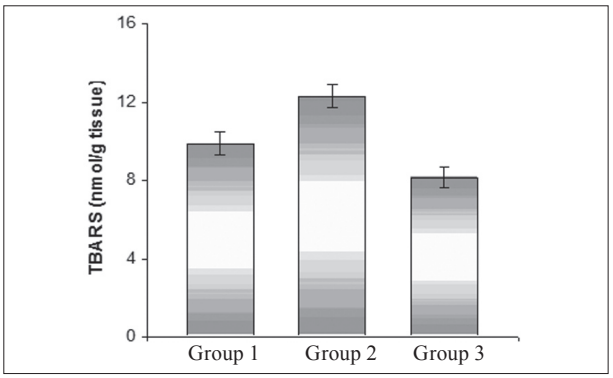

Figure 1. Thiobarbituric acid reactive substances (TBARS) levels in rat lung after total body irradiation (TBI) with vehicle or melatonin (MLT) treatment. All bars represent the pooled data of rats irradiated in the morning (A) and evening (B). Group 1: control , Group 2: TBI, Group 3: TBI + MLT. * $p<0.05$ compared to group 1 , ** $p<0.05$ compared to group 2.

Influence of the time of exposure to TBI on TBARS levels

Depending on the time of irradiation, there was no statistically significant difference between morning (Group 1A) and evening irradiation (Group 1B) in control groups (Figure 2). On the other hand, TBARS levels were found to be increased after TBI in the only morning group (Group 2A) when compared with Group 1A and treatment with melatonin significantly decreased TBARS levels (Group 2A vs Group 3A, two-way ANOVA, $p<0.05$). However, TBI in the evening (Group 2B) did not cause any significant changes in TBARS levels when compared with Group 1B and treatment with melatonin reduced TBARS levels but the difference was not significant (Group 3B vs. Group 2B). An important result is that there was a statistically significant difference between morning and evening irradiation groups in melatonin treatment group (Group 3A vs Group 3B, two-way ANOVA, $p<0.05$). Melatonine treatment was found to be more decreased in TBARS levels in morning irradiation group when compared with evening irradiation group.

Influence of the time of exposure to TBI on MPO activities

Depending on the time of irradiation, there was no statistically significant difference between morning (Group 1A) and evening irradiation (Group 1B) in control groups (Figure 4). Similarly, it was not observed difference between morning and evening irradiation in either TBI group or TBI+MLT group.

MPO activity elevated after TBI (Group 2A) in the morning when compared with Group 1A but the difference was not significant. Melatonin treatment in the morning caused a significant decrease in rats exposed to TBI (Group 2A vs Group 3A; two-way ANOVA, $p<0.05$). Although MPO activity did not change after TBI (group 2B) in the evening when compared with Group 1B, melatonin treatment in the evening caused a significant decrease in rats exposed to TBI (group 2B vs group 3B; $p<0.05$).

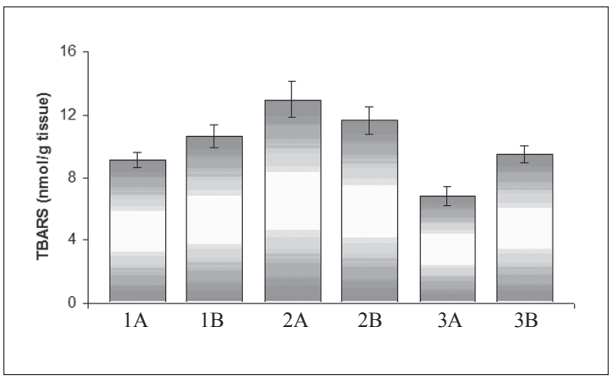

Figure 2. Thiobarbituric acid reactive substances (TBARS) levels in rat lung after total body irradiation (TBI) with vehicle or melatonin (MLT) treatment in the morning (A) or in the evening (B). Group 1: Control, Group 2: TBI, Group 3: TBI + MLT, *p<0.05 compared to group 1A , ** p<0.05 compared to group 2A, *** p<0.05 compared to group 3A.

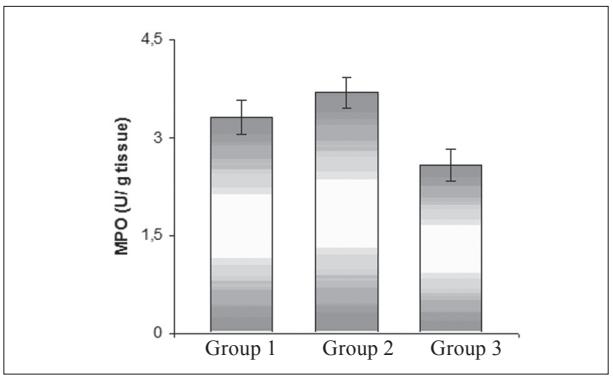

Figure 3. Myeloperoxidase (MPO) activity in rat lung after total body irradiation (TBI) with vehicle or melatonin (MLT) treatment. All bars represent the pooled data of rats irradiated in the morning (A) and evening (B). Group 1: Control, Group 2: TBI, Group 3: TBI + MLT. * p<0.05 compared to group 2.

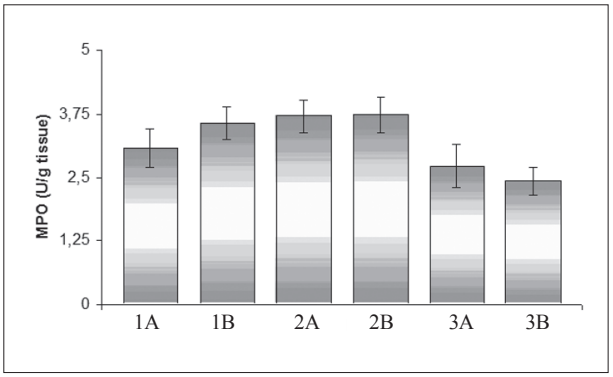

Figure 4. Myeloperoxidase (MPO) activity in rat lung after total body irradiation (TBI) with vehicle or melatonin treatment in the morning (A) or in the evening (B). Group 1: Control, Group 2: TBI, Group 3: TBI + MLT. *p<0.05 compared to group 2A ,**p<0.05 compared to group 2B.

Discussion

In the present study, deleterious effects of ionizing radiation and possible acute radioprotective effect of melatonin on lung tissue were evaluated depending on the time of irradiation. Evaluation was done by measuring TBARS levels and MPO activities.

It was reported that irradiation increased the formation of TBARS, the main product of lipid peroxidation, in the different tissues in some studies (27-28). In the present study, we observed a markedly increased TBARS levels in lung tissue when exposed to single dose (8 Gy) TBI. Our results are in agreement with the published literature. The present results also demonstrated that melatonin treatment which is applied immediately before, after and 24 h preceeding irradiation, protected lung tissue against to oxidative stress, evidenced by decreased TBARS production in the lung compared to the irradiated non-melatonin treated rats (Figure 1). These results are in agreement with those of Sener et al. (29).

TBI causes inflammation by activated inflammatory cells. The activation of these cells leads to the synthesis and release of certain cytokines, inflammatory mediators, and ROS (2,30). The tissue-associated MPO activity, known as an index of neutrophil infiltration, has been shown to be increased in the lung tissue after TBI. MPO plays a fundamental role in oxidant production when neutrophils are stimulated by various stimulants, such as irradiation and causes tissue damage (29,31,32).

Freeman et al. (33) have shown that TBI has a great effect on the circulating levels of white blood cells and granulocytes which recruited to sites of injury has been severely depleted. Also Sener et al. (29) reported that lung MPO activity was markedly reduced at the 72 h following irradiation. In the present study, 48 h after irradiation, MPO activity in the lung was not significantly changed. On the other hand, melatonin treatment further depressed lung neutrophil accumulation markedly (Fig-

ure 3). It appears that lung oxidative injury is not associated with tissue neutrophils in our study protocol.

It is well known that many physiological events show rhythmic variations. These are called as biological rhythm. These rhythms have been determined in various bodily functions such as body temperature, many hematologic and endocrine parameters, enzyme activities and etc (34). Because these biological rhythms, the organism is in different conditions in different times of day. Moreover, biological rhythms may influence the succeptibility of organism to chemical agents (35) or harmful stimulus such as irradiation (23). This phenomena is named as chronesthesy which describes rhythmic differences in the susceptibility or sensitivity of biological target such as receptors, membrane permeability, cells, tissues, organs, etc. to an agent (36). When to treat is the most significant point of any research in chronopharmacology and chronotoxicity. This means the finding of the best circadian time of drug administration not just as to their effectiviness but also as to their toxicity. Chronobiologic studies suggest that arranging the delivery time of drugs may prevent some of the adverse effects of some drugs (37). Significant chronotoxicities are known, particularly among antitumor agents (38).

In the present study, we applied TBI in the morning (1 HALO; early resting period for rats due to nocturnal animals) and in the evening (13 HALO; early activity period); and we determined that TBARS levels were increased in the lung tissues of the rats which were irradiated in the morning compared to the control group and melatonin significantly decreased the elevated TBARS levels in the morning. Besides, TBI applied in the evening did not increase the TBARS levels versus to control. There was not a statistically significant difference between morning and evening TBI groups. On the other hand, we found statistically significant difference between morning and evening irradiation groups in melatonin treatment group (Group 3A vs Group 3B). At this point, we concluded that when irradiation is given in the morning, TBARS levels as an indicator of lipid peroxidation increase significantly and melatonin treatment is more effective in order to prevent the oxidant stress caused by irradiation applied in the morning. So, it seems that rat lung tissue is more prone to oxidative stress in the morning and exogenous melatonin is more effective when given in the morning. However, in the present study, we examined on lung tissues at only two time point (1 HALO vs 13 HALO) and evaluated oxidative stress by two parameter which are TBARS levels and MPO activity at which only 48 h later TBI. These are the limitations of our study. On the other hand, we could not obtain similar results from the point of view MPO activity. In the present study, MPO activity was not changed either in the morning or in the evening after TBI versus to control but melatonin reduced MPO activity, irrespective of the time of irradiation. MPO activity shows dependence neutrophil infiltration whereas TBARS levels are a direct indicator of lipid peroxidation. The reason of different results between MPO activity and TBARS level is not clear but speculative at present, though current evidences suggest that circadian changes in formation ROS and/or TBARS levels can play an important role. Rhythmic changes in oxidative damage of protein and lipid molecules and antioxidative enzyme activities are also reported (12). Kolanjiappan et al. have shown circadian pattern of lipid peroxidation by-products (TBARS) and antioxidants status in 7,12-dimethylb enz(a)anthracene induced rat mammary carcinogenesis in blood at a regular interval of 4 h throughout the 24 h period (39). Similarly, circadian rhythmicity of plasma TBARS levels and antioxidants in oral human squamous cell carcinoma have been described at 6 h intervals (40-41). It has been reported that TBARS levels were higher in rats under continous darkness when compared with altering light-dark cycle (42). Therefore it can be concluded that rats are more prone to oxidative stress in the darkness; and this results do not seem compatible with our results but the protocols of these studies are different. TBARS levels have been investigated in the various central nervous system regions, depending on the entrainment period: 24 h, 48 h, 72 h, 7 days.

Circadian changes related with the number of polymorphonuclear cells and MPO activity in lipopolysaccaharide-induced liver tissues in rats have been defined in literature and that liver tissues have been obtained after 12 h later lipopolysaccaharide injection (43).

Plasma levels of melatonin exhibit a circadian variation with the highest concentrations occurring at night (44) because light suppresses melatonin secretion. Rats are nocturnal animals and their plasma melatonin levels were found to be higher during the dark period and lower during the light period (45). Although we were unable to measure blood melatonin levels, as a speculative, lower melatonin levels and/or diurnal rhythmicity of TBARS and also temporal differences in succeptibility of lung tissue to irradiation might be explain why rat lung tissue is more prone to oxidative stress in the early morning and exogenous melatonin is more effective at that times.

It has been demonstrated that a dose of 14.4 Gy TBI irradiation causes a significant decrease in serum and pineal levels of melatonin 30 min to 6 h postexposure in rats but in the rats irradiated up to total doses 4.8 and 9.6 Gy melatonin levels are not significantly different from the control values (46). In the present study, endogenous melatonin synthesis is not expected to be affected since the dose was 8 Gy.

On the other hand, in the present study, there was no statistically significant difference between morning and evening irradiation in control groups in either TBARS levels or MPO activity This suggests that endogenous TBARS levels or MPO activity displays same properties in rat lung tissues at least when assessed 48 h later TBI.

In our study, the effect of melatonin can be attributed

to its well-known antioxidant and free radical scavenger properties. Although further studies are required to define underlying mechanism(s) of time dependent variations TBARS levels in morning groups applied TBI and TBI plus melatonin, this study might help to elucidate the optimum time(s) to enable better treatment with irradiation without undervalue toxic effects.

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