Histopathological Evaluation of the Effects of Melatonin as a Protectant Against Oropharyngeal Mucositis Induced by Radiation Therapy in a Pinealectomy Model in Rats

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ABSTRACT

This study presents the histopathological evaluation of the effects of melatonin as a protectant against oropharyngeal mucositis induced by radiation therapy in a pinealectomy model in rats. Thirty-two Wistar rats were divided into four groups. The rats in Group 1 underwent pinealectomy, received melatonin and underwent radiation therapy. The rats in Group 2 underwent pinealectomy, received no melatonin and underwent radiation therapy. The rats in Group 3 underwent sham pinealectomy, received melatonin and underwent radiation therapy. The rats in Group 4 underwent sham pinealectomy, received no melatonin and underwent radiation therapy. The rats underwent euthanasia at six days following radiation therapy. The oropharyngeal tissues were dissected and histopathological evaluation was performed regarding the effects of melatonin as a protectant against oropharyngeal mucositis induced by radiation therapy. Mean item scores for vascular calibration were 2.3 points for Group 1, 2.3 points for Group 2, 1.8 points for Group 3 and 2.1 points for Group 4 (p= 0.44). Mean item scores for vascular permeability were 2.4 points for Group 1, 2.4 points for Group 2, 2.0 points for Group 3 and 2.1 points for Group 4 (p= 0.61). Mean item scores for leukocyte emigration were 2.0 points for Group 1, 2.0 points for Group 2, 1.3 points for Group 3 and 1.6 points for Group 4 (p= 0.02). Mean overall scores for oropharyngeal mucositis were 6.6 points for Group 1, 6.6 points for Group 2, 5.1 points for Group 3 and 5.8 points for Group 4 (p= 0.15). This study elicits the potential effects of melatonin as a protectant against oropharyngeal mucositis induced by radiation therapy.

Key Words: Radiation injury, Mucous membrane, Melatonin, Pinealectomy

ÖZET

Sıçanlarda Bir Pinealektomi Modelinde Radyoterapiye Bağlı Orofaringeal Mukozit Üzerine Melatoninin Koruyucu Etkisinin Histopatolojik Olarak Değerlendirilmesi

Bu çalışmada melatoninin radyoterapi ile uyarılan orofarengeal mukozite karşı bir koruyucu olarak etkilerinin ratlarda bir pinealektomi modelinde histopatolojik değerlendirmesi sunulmaktadır. Otuz iki Wistar türü sıçan dört gruba bölünmüştür. Grup 1'deki sıçanlara pinealektomi uygulanmış, melatonin verilmiş ve radyoterapi uygulanmıştır. Grup 2'deki sıçanlara pinealektomi uygulanmış, melatonin verilmemiş ve radyoterapi uygulanmıştır. Grup 3'teki sıçanlara sahte pinealektomi uygulanmış, melatonin verilmiş ve radyoterapi uygulanmıştır. Grup 4'teki sıçanlara sahte pinealektomi uygulanmış, melatonin verilmemiş ve radyoterapi uygulanmıştır. Radyoterapi uygulamasından 6 gün sonar sıçanlara ötenazi uygulanmıştır. Orofarengeal dokular kesilerek ayrılmış ve melatoninin radyoterapi ile uyarılan orofarengeal mukozite karşı bir koruyucu olarak etkileri açısından histopatolojik değerlendirme yapılmıştır. Ortalama vasküler kalibrasyon skoru Grup 1 için 2.3, Grup 2 için 2.3, Grup 3 için 1.8 ve Grup 4 için 2.1 idi (p= 0.44). Ortalama vasküler permeabilite skoru Grup 1 için 2.4, Grup 2 için 2.4, Grup 3 için 2.0 ve Grup 4 için 2.1 idi (p= 0.61). Ortalama lökosit emigrasyon skoru Grup 1 için 2.0, Grup 2 için 2.0, Grup 3 için 1.3 ve Grup 4 için 1.6 idi (p= 0.02). Orofarengeal mukozit genel skoru Grup 1 için 6.6, Grup 2 için 6.6, Grup 3 için 5.1 ve Grup 4 için 5.8 idi (p= 0.15). Bu çalışma melatoninin radyoterapi ile uyarılan orofarengeal mukozite karşı bir koruyucu olarak potansiyel etkilerini ortaya koymaktadır.

Anahtar Kelimeler: Radyasyon hasarı, Müköz membran, Melatonin, Pinealektomi

INTRODUCTION

Aggressive radiation therapy approaches aiming at improved local control and survival for patients with malignancies of the head and neck come at the expense of increased oropharyngeal mucositis.[1] Oropharyngeal mucositis is thought to be a complex process that involves damaging effects of radiation therapy on the dividing cells of the oropharyngeal epithelium modulated by immunological, inflammatory and bacteriological parameters.[2] Since cellular injury associated with radiation therapy is predominantly brought about as the result of the unstable reactive oxygen species[3], the scavengers of the unstable reactive oxygen species need to be elaborated for protection against their damaging effects.[4] Melatonin, an endogenous peptide that acts as a scavenger of the unstable reactive oxygen species[5], might function as a protectant against the damaging effects of radiation therapy.[6] This study presents the histopathological evaluation of the effects of melatonin as a protectant against oropharyngeal mucositis induced by radiation therapy in a pinealectomy model in rats.

MATERIALS AND METHODS

The study was undertaken at the "Center for Laboratory Animals and Investigational Studies of Gazi 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 Gazi University". Thirty-two female Wistar rats (ages between 12 and 16 weeks and weights between 200 and 250 grams) were divided into four groups, each consisting of eight rats. 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.

The rats in Group 1 underwent craniotomy with pinealectomy, received melatonin and underwent radiation therapy. The rats in Group 2 underwent craniotomy with pinealectomy, received no melatonin and underwent radiation therapy. The rats in Group 3 underwent craniotomy with sham pinealectomy, received melatonin and underwent radiation therapy. The rats in Group 4 underwent craniotomy with sham pinealectomy, received no melatonin and underwent radiation therapy.

Pinealectomy

Prior to craniotomy, the rats received anesthesia using ketamine (Ketalar®) at a dose of 80 mg/kg and xylazine (Rompun®) at a dose of 5 mg/kg administered using an intraperitoneal injection. For the rats in Group 1 and Group 2, pinealectomy was performed following craniotomy as described by

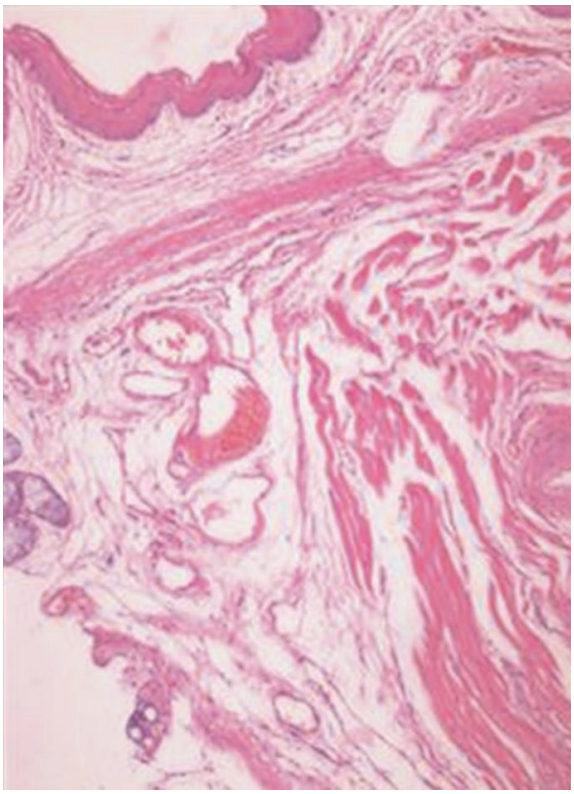

Figure 1. Altered vascular calibration (HE x 100).

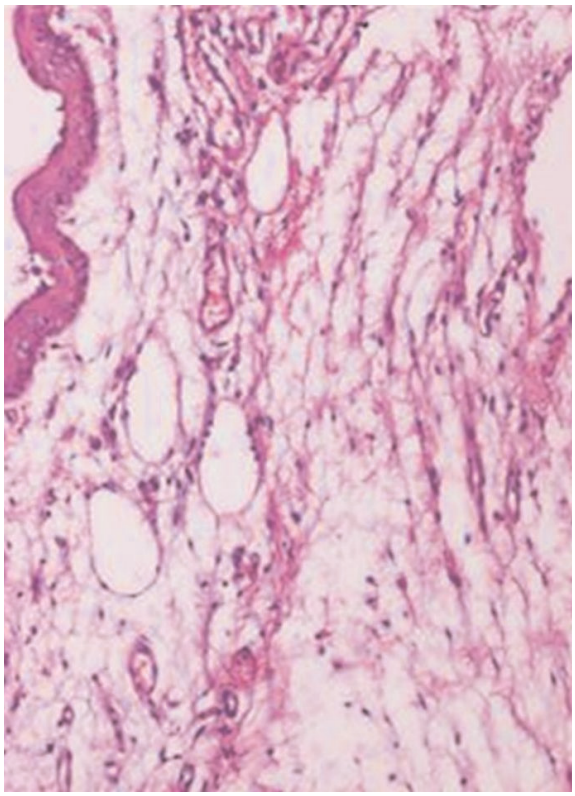

Figure 2. Altered vascular permeability (HE x100).

Kuszak and Rodin.[7] For the rats in Group 3 and Group 4, no pinealectomy was performed following craniotomy.

Melatonin

For the rats in Group 1 and Group 3, melatonin (Melatonin Crystalline, Sigma-Aldrich Corporation, St. Louis, United States of America) was prepared at a concentration of 1 % with dissolution in ethanol and dilution in 0.9 % sodium chloride and administered at a dose of 10 mg/kg using an intraperitoneal injection 10 minutes prior to radiation therapy. For the rats in Group 2 and Group 4, 0.9 % sodium chloride was prepared at the same volume with melatonin and administered using an intraperitoneal injection 10 minutes prior to radiation therapy.

Radiation Therapy

Prior to radiation therapy, the rats received anesthesia using ketamine at a dose of 80 mg/kg administered using an intraperitoneal injection and were immobilized in the supine position on a rough surface by way of taping from the head. Radiation therapy was delivered on a Cobalt-60 unit using a single fraction of 12 Gy defined at the midplane depth through parallel opposed anterior and posterior portals covering the oropharyngeal tissues.

Euthanasia

The rats underwent euthanasia at six days following radiation therapy. Prior to euthanasia, the rats received anesthesia using ketamine at a dose of 80 mg/kg and xylazine at a dose of 5 mg/kg administered using an intraperitoneal injection. Euthanasia was performed by way of transcardiac perfusion using 0.9% sodium chloride. The oropharyngeal tissues were dissected, placed in formaldehyde, embedded in paraffin, sliced into 5 μm thick sections and stained using hematoxylin and eosin.

Histopathological Evaluation

Blinded histopathological evaluation of the dissected oropharyngeal tissues was performed under the

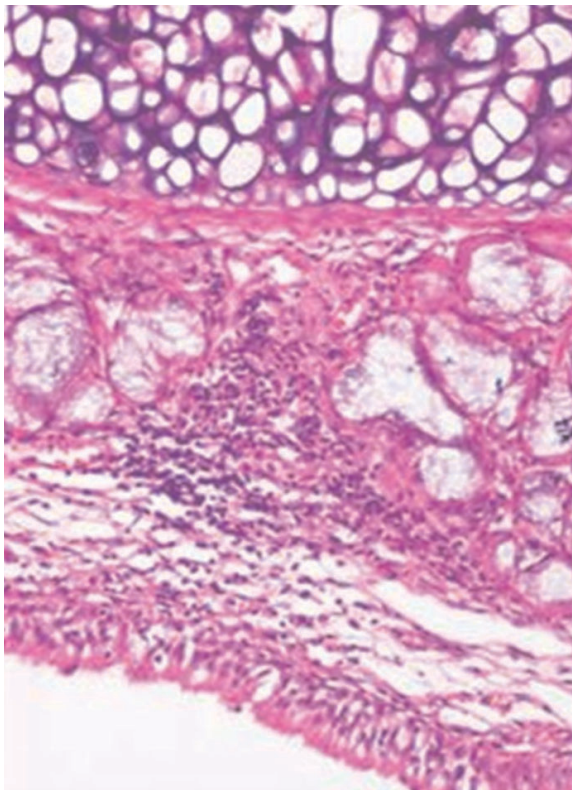

Figure 3. Leukocyte emigration (HE x 100).

light microscope regarding the effects of melatonin as a protectant against oropharyngeal mucositis induced by radiation therapy. Items descriptive for oropharyngeal mucositis including vascular calibration, vascular permeability and leukocyte emigration were individually scored as mildly increased (1 point), moderately increased (2 points) or significantly increased (3 points). Item scores were summated to determine the overall scores.

Statistical Analysis

Mean item scores and mean overall scores for all groups were compared using the one-way analysis of variance test. Statistical analysis was performed using the "10.0" version of the "SPSS for Windows" software package. Statistical significance was defined as the P value being ≤ 0.05.

RESULTS

Mean item scores for vascular calibration were 2.3 ± 0.7 points for Group 1, 2.3 ± 0.5 points for Group 2, 1.8 ± 0.7 points for Group 3 and 2.1 ± 0.8 points for Group 4 (p = 0.44) (Figure 1). Mean item scores for vascular permeability were 2.4 ± 0.5 points for Group 1, 2.4 ± 0.5 points for Group 2, 2.0 ± 0.8 points for Group 3 and 2.1 ± 0.8 points for Group 4 (p = 0.61) (Figure 2). Mean item scores for leukocyte emigration were 2.0 ± 0.5 points for Group 1, 2.0 ± 0.5 points for Group 2, 1.3 ± 0.5 points for Group 3 and 1.6 ± 0.5 points for Group 4 (p = 0.02) (Figure 3). Mean overall scores for oropharyngeal mucositis were 6.6 ± 0.9 points for Group 1, 6.6 ± 1.2 points for Group 2, 5.1 ± 1.8 points for Group 3 and 5.8 ± 1.8 points for Group 4 (p = 0.15) (Table 1).

Table 1. Item scores for vascular calibration, vascular permeability and leukocyte emigration and overall scores for oropharyngeal mucositis by groups (mean ± standard deviation).

	Group 1	Group 2	Group 3	Group 4	p
Mean item score					
Vascular calibration	2.3 ± 0.7	2.3 ± 0.5	1.8 ± 0.7	2.1 ± 0.8	0.44
Vascular permeability	2.4 ± 0.5	2.4 ± 0.5	2.0 ± 0.8	2.1 ± 0.8	0.61
Leukocyte emigration	2.0 ± 0.5	2.0 ± 0.5	1.3 ± 0.5	1.6 ± 0.5	0.02
Mean overall score					
Oropharyngeal mucositis	6.6 ± 0.9	6.6 ± 1.2	5.1 ± 1.8	5.8 ± 1.8	0.15

DISCUSSION

Oropharyngeal mucositis is regarded as the most debilitating morbidity associated with aggressive radiation therapy approaches for patients with malignancies of the head and neck, causing substantial pain, interfering with the ability to swallow and worsening the quality of life.[8] The supportive measures against oropharyngeal mucositis following its onset, such as alleviating pain, providing adequate nutrition, eliminating secondary infections, are of limited effectiveness in attempt to prevent significant interruptions or premature termination of radiation therapy with resultant negative impact on local control and survival; therefore, protective measures against oropharyngeal mucositis prior to its onset are highly desirable.[9] The concept of protecting the oropharyngeal epithelium against the damaging effects of radiation therapy is based on the premise of differential cytoprotection that might be achieved by shifting the balance between cell killing and cell regeneration in favor of the latter.[10] Several protective measures might be taken against oropharyngeal mucositis induced by radiation therapy, including the use of direct cytoprotectants such as sucralfate, prostaglandins, glutamine and amifostine, the use of indirect cytoprotectants such as growth factors and anti-inflammatory agents and the use of antimicrobials such as chlorhexidine, antibiotics and antifungal agents.[11]

Cellular injury associated with radiation therapy is predominantly brought about as the result of the unstable reactive oxygen species, including the superoxide radical, the hydroxyl radical and hydrogen peroxide, that are generated by the hydrolysis of the cellular water content and that are in constant reaction with the cellular DNA content.[3] The antioxidant defense systems including the antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase as well as the scavengers of the unstable reactive oxygen species need to be elaborated for protection against the damaging effects of these species on the cellular DNA content.[4] There is a continued interest as well as an unfulfilled need for the identification of a safe and effective protectant that bears the potential to act as the scavenger of the unstable reactive oxygen species to modulate the biological response to radiation therapy.[12] Melatonin, an endogenous peptide of indole structure that is released from the pineal gland, has been reported to be involved in the antioxidant defense systems through its action as a potent scavenger of the unstable reactive oxygen species and, hence, might appear to be the desired protectant against the damaging effects of radiation therapy in this context.[5]

In a study that examined the protective role of melatonin against injury induced by radiation in various endocrine organs, pretreatment with melatonin has been shown to reverse the histopathological as well as the enzymological changes in the rat thyroid gland.[13] In another study, melatonin has been shown to reduce the injury induced by radiation in the thyroid follicular cells in rats while preserving their nuclear volume.[14] Other in vitro as well as in vivo studies also provide further evidence that apoptosis induced by radiation in neurons[15], retinal cells[16], thymocytes[17] and bone marrow cells[18] might be prevented by melatonin.

Experimental evidence indicates that the endogenous melatonin released from the pineal gland is relevant as a physiological antioxidant, whereas the contribution of the secondary sources to the endogenous melatonin release is not significant.[19] Furthermore, the rhythm of the antioxidant defense systems has been demonstrated to be abolished in experimental pinealectomy models.[20] Exogenous melatonin has been administered in pharmacological doses in a variety of experimental settings, establishing a widespread agreement on its safety.[21] Melatonin displays high lipid solubility as well as high water solubility, facilitating its transportation across the cellular membranes to be readily distributed among a wide range of tissues. Therefore, the plasma level of melatonin displays a quick turnover following its exogenous administration, corresponding to a distribution half-life of two minutes and a metabolic half-life of 20 minutes.[20] Numerous physiological actions of N1-acetyl-N2-formyl-5-ethoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK) indicate that certain actions of melatonin might be mediated, if not amplified, via its metabolites. Due to the generation of such structurally unrelated derivatives during melatonin metabolism, multiple physiological functions of melatonin are predictable. The oxidative status of the organism modifies melatonin metabolism, hence the higher the oxidative state, the more

AFMK and AMK is produced.[22] Considering the design of this study, the effects of the absence of the endogenous melatonin were simulated through the absolute elimination of the endogenous melatonin using a pinealectomy model whereas the effects of the presence of exogenous melatonin in pharmacological doses were simulated through the exogenous administration of melatonin using an intraperitoneal injection just prior to radiation therapy.

When the histopathological evaluation of the damaging effects of radiation therapy on the oropharyngeal epithelium is concerned, items descriptive for mucositis include altered vascular calibration that represents the degree of vascular dilatation, altered vascular permeability that represents the extent of interstitial fluid accumulation and leukocyte emigration that represents the density of leukocyte accumulation, as compared to the unaffected oropharyngeal epithelium.[23] In this study, the histopathological evidence of oropharyngeal mucositis was observed at the highest intensity for the rats that underwent pinealectomy and received no melatonin in comparison to the lowest intensity for the rats that underwent sham pinealectomy and received melatonin, as judged by the mean item scores for vascular calibration, vascular permeability and leukocyte emigration as well as the mean overall scores for oropharyngeal mucositis. Hence, the absence of the endogenous melatonin seemed to aggravate the damaging effects of radiation therapy on the oropharyngeal epithelium while the presence of exogenous melatonin seemed to provide additional, yet modest, protection against these damaging effects. To conclude, this study elicits the potential effects of melatonin as a protectant against oropharyngeal mucositis induced by radiation therapy. Given its desired characteristics as a protectant such as increased safety and effectiveness besides effortless handling in the logistics of radiation therapy, the use of melatonin in experimental settings as a scavenger of the unstable reactive oxygen species needs to be continued.

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