The effects of melatonin on the healing of burn wounds in pinealectomized rats

E. Çiğdem Karadağ Sarı, M.D.,1 Nedim Savacı, M.D.2

1Department of Plastic, Reconstructive and Aesthetic Surgery, Acıbadem University Faculty of Medicine, İstanbul-Turkey
2Department of Plastic, Reconstructive and Aesthetic Surgery, Necmettin Erbakan University Meram Faculty of Medicine, Konya-Turkey

ABSTRACT

BACKGROUND: The present study aims to investigate the favorable effects of melatonin on burn wound healing in rats.

METHODS: In this study, forty Wistar-albino-type male rats were divided into four groups. Group 1 was the control group, Group 2 rats were treated using exogenous melatonin, Group 3 rats were pinealectomized, and Group 4 rats were pinealectomized then treated with exogenous melatonin. In all groups, a deep second-degree burn was created on the backs of the rats with a metal plate heated in boiling water. We monitored the progress of burn healing for seven days. At the end of them, we evaluated hydroxyproline levels, type III collagen, edema, inflammatory infiltration, congestion, vascular proliferation, fibrosis, the thickness of the zone of stasis and the epithelium to assess the progress of healing.

RESULTS: The zone of stasis was less thick in Group 2 than the other groups (p=0.009). Type III collagen dyeing (p=0.031), fibrosis (p=0.011) and edema (p=0.031) were higher in Group 2 than the other groups. Congestion was higher in the control group than Group 4 (p=0.031). Other evaluated parameters showed no significant differences among the groups.

CONCLUSION: In this study, it was noted that once total melatonin levels exceeded a certain threshold, a preventive effect was exerted on burn wound damage progression by reducing the zone of stasis. Melatonin may also prevent the development of hypertrophic scarring. Melatonin may be a potential therapeutic option that can supplement traditional treatment in burn wounds; however, further studies with higher doses of exogenous melatonin administered over longer periods are needed to further evaluate the effects noted in this study.

Keywords: Burn; endogenous melatonin; exogenous melatonin; pinealectomy; wound.
no published studies that examine the effects of endogenous melatonin and exogenous melatonin on burn healing. Therefore, in this study, we investigated the effects of the basal release of melatonin, i.e., endogenous melatonin levels, in our pinealectomy group, and whether explored exogenous melatonin has favorable effects on burn wound healing in the groups administered exogenous melatonin.

**MATERIALS AND METHODS**

With the prediction of the difference in the medium effect size accepted for animal experiments in groups as statistically significant, we determined our sample size as 24 rats for 95% power at 0.05 alpha significance level. However, due to the increased mortality and morbidity risk of the pinealectomy procedure, we increased the sample size to 40 animals. Therefore, 40 Wistar-albino-type male rats with body weights ranging from 350 g to 500 g were used in this study.

Five rats per cage were housed in a temperature-controlled room at 22°C ± 1°C and 50% ± 5% humidity on a 12-hour dark-light cycle with free access to water and food. Rats were randomized into four groups (10 rats per group), and burn wounds were created on the backs of all rats in all groups. Group 1 was the control group that received no treatment. Group 2 rats received exogenous melatonin (ExM). Group 3 rats received pinealectomy (Px) and no treatment, and Group 4 rats received Px+ExM. This study was approved by the local ethics committee of the university (project no: 2012/121518019).

**Burn Wound Creation**

Before conducting this study, we reviewed the literature and verified our chosen method with a pilot study in which we tested our method for burn wound creation on one rat. This study was started only after we confirmed the burn model and the degree of the burn using histopathological examination in this pilot application. In all rats, burns were created under general anesthesia induced with intraperitoneal ketamine hydrochloride (8 mg/100 g) and xylazine hydrochloride (1 mg/100 g). The right side of the back skin was shaved in all rats. A circular metal plate with a 1.5-cm-diameter was immersed in boiling water for five minutes measured at 100°C and then placed on the shaved backs of each rat for 15 seconds (without applying pressure) to produce deep second-degree burns. The reason for using a circular metal plate was to assess the wound contraction easily during healing. Burn wounds were left open for the duration of this study, and no topical or other preparations were applied.

**Pinealectomy**

Pinealectomy was performed on rats in Groups 3 and 4 immediately before burn-wound creation. Anesthetized rats were positioned on their abdomens and stabilized. Their heads were cleaned gently with polyvinylpyrrolidone iodine, and a 2-cm incision was made along the midline of the hairless scalp, right behind the eyes. We exposed the junction of the transverse and sagittal sutures and formed an 8-mm diameter circular bone flap using a reverse conic dental drill (Fig. 1a). We cut the dura bilaterally up to the sagittal sinus. The anterior third of the sagittal sinus was doubly ligated with 8/0 polypropylene sutures (Fig. 1b). By folding the caudal portion of the ligated sagittal sinus posteriorly, the pineal gland was exposed beneath the junction of the transverse and sagittal sinuses (Fig. 1c). The gland was carefully removed, the bone flap was replaced, and the skin was stitched closed. All excised pineal glands were confirmed by histopathological evaluation.

**Melatonin Administration**

Powdered melatonin (16.5 mg; Merck Schuchardt OHG, Hohenbrunn, Germany) was dissolved in 0.5 cm³ 100% ethanol; then diluted with 5 cm³ physiological saline to reduce the ethanol concentration to 10%. Melatonin was prepared freshly for each case in a dark environment and injected intraperitoneally to the rats in Groups 2 and 4 at a dose of 10 mg/kg/day between 16:00 and 17:00 hours for seven days.

**Removal of Burn Wound Tissue**

On a postoperative day seven, burn wounds were excised using full-thickness excision under general anesthesia to collect the specimen. Each specimen was dissected into two equal pieces: one piece was fixed in 10% buffered formalin for histopathological analysis, and the other was washed with cold 0.9% NaCl and placed in Eppendorf tubes maintained at -80°C for biochemical analysis. All rats were sacrificed after this procedure.
Evaluation

Photo Analysis

Burn wounds were digitally photographed (Canon EOS60D SLR, 18 megapixels, Canon USA, Japan) on a daily basis, next to a millimetric ruler in all groups under diethyl ether anesthesia. Surface areas of burn wounds were analyzed using ImageJ software (NIH, Bethesda, Maryland, USA). We calibrated the wound area using the millimetric ruler, and the surface area was calculated in square millimeters (Fig. 2). The burn wound margins for rats in each group were traced with a fine-resolution computer mouse on day one and day seven, and the burn wound surface area was calculated. Healing was evaluated based on the progress observed in the burn wound surface area between days one and seven. We also compared the mean progress of healing achieved during this period in each group. Progress of healing was calculated based on the difference between the day one and day seven measurements of the burn wound area in each rat, and the mean healed areas of the groups were compared.

Biochemical Analysis

Specimens were weighed and homogenized in 2N NaOH/g wet tissue. Hydroxyproline levels of the specimens were blind-measured according to the method described by Reddy and Enweremeka. Results were recorded as µg/g wet tissue.

Histopathological and Immunohistochemical Analysis

Specimens were fixed in 10% buffered formalin and embedded in paraffin media. Micron sections (5 µm) were deparaffinized and processed to rehydration, followed by staining with Hematoxylin-Eosin dye (Surgipath, 01562E, 01602E, Peterborough, UK) for histopathological analysis.

For immunohistochemical staining, specimens were prepared using the avidin-biotin peroxidase method (HRP, Thermo Scientific, UK). Collagen anti-type III rat monoclonal protein receptor antibody Thermo Scientific, Clone 1E7-D7 was raised for immunohistochemical analysis. Brown-colored staining of more than 10% was considered positive. Sections stained with histopathological and immunohistochemical methods were examined using light microscopy (Olympus BX40 light microscope). Thicknesses of the zone of stasis and the epithelium were measured from five different points using the Image Analyzing System (BAB B 200proP; Fig. 3a, b). We determined the average in microns.

Vascular proliferation, edema, congestion, fibrosis and inflammatory infiltration were evaluated. Each parameter was scored as negative if none present, + (minimal) if present only on one site, ++ (mild) if present on two sites and +++ (significant) if present on three or more sites, to assess the extent of modification.

The degree of immunohistochemical staining was semi-quantitatively evaluated. Type III collagen staining was scored neg-
ative if <10%, + (minimal) if 11 to 40%, ++ (mild) if 41 to 70%, and +++ (significant) if >71%.

Statistical Analysis
Data were examined using the Shapiro-Wilk test. To investigate the differences between the groups, the Kruskal-Wallis test was used for data outside of normal distribution; analysis of variance was used for data within the normal distribution. In the nonparametric test, subgroup analysis was performed using Mann-Whitney U test and interpreted with Bonferroni correction. P<0.05 was accepted as statistically significant. Data were evaluated using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA).

RESULTS
Two rats in Group 3 died within 24 hours after burn induction, hence excluded from the study.

In the photo analysis performed using the ImageJ software program, no significant differences were found among the four groups concerning their average healed areas (Kruskal-Wallis=5.625, p=0.131), and no significant differences were noted in the biochemical analysis of hydroxyproline levels among the groups (p=0.614).

Table 1. Edema scores (Kruskal-Wallis Test)*

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>Median (Min-Max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>10</td>
<td>2.00</td>
<td>2 (2–2)</td>
</tr>
<tr>
<td>Group 2 (ExM)</td>
<td>10</td>
<td>2.30</td>
<td>2 (2–3)</td>
</tr>
<tr>
<td>Group 3 (Px)</td>
<td>8</td>
<td>2.00</td>
<td>2 (2–2)</td>
</tr>
<tr>
<td>Group 4 (ExM+Px)</td>
<td>10</td>
<td>2.00</td>
<td>2 (2–2)</td>
</tr>
</tbody>
</table>

*Kruskal-Wallis Score = 8.880, p=0.031. Difference = Group 1 and Group 3 and Group 4 vs. Group 2. ExM: Exogenous melatonin; Px: Pinealectomy.

The histopathological and immunohistochemical analysis found significantly higher edema scoring in the ExM group vs. the other groups (p=0.031; Table 1; Fig. 4). Congestion scoring was significantly higher in the control group when compared with the ExM+Px group (p=0.031; Table 2; Fig. 5).

Fibrosis scoring was significantly higher in the ExM group than the other groups (p=0.011; Table 3; Fig. 6). No significant differences were noted among the groups concerning vascular proliferation and inflammatory infiltration.

The average thickness of the zone of stasis in the ExM group was significantly less than the other groups (p=0.009; Table...
However, there were no significant differences among the groups concerning epithelium thickness.

The degree of type III collagen staining in the ExM group was significantly higher than the Px group (p=0.031; Table 5).

**DISCUSSION**

Burn injuries are a major source of physical and physiological trauma worldwide. If second-degree deep burn wounds are not treated promptly and appropriately, they may develop into full-thickness burns due to progressive tissue damage, leading to hypertrophic scars and contractures that require surgical excision and graft repair.[3] Tissue damage in the zone of stasis may be prevented with an appropriate treatment applied within two weeks from the initial injury.[8,9] Inflammation, ischemia and free oxygen radicals increase the progression of damage in burn wounds by affecting the viability of the zone of stasis.[10]

In our literature review, we identified several experimental studies that report their investigations on antioxidants’ ability to prevent the progressive effects of free oxygen radicals in burn wounds. One study [11] reported that necrosis in the zone of stasis significantly reduced in groups treated with N-acetylcysteine than controls. Zor et al., [12] based on nuclear imaging and autoradiography, reported that glutathione helped salvage the zone of stasis. Singer et al. [13] found that curcumin reduced the progression of burn injury in a rat comb burn model. Matsuda et al.[14] suggest that high-dose vitamin C would reduce edema in burn wounds in the porcine burn model, and superoxide dismutase has a salvaging effect on the zone of stasis.[15]

![Figure 6. Fibrotic areas in Group 2 (F) are more significant (ExM) than Group 3 (f) (Px) (H&Ex20).](image-url)
Melatonin’s free oxygen radical scavenging ability is more effective than other antioxidants.\[6\] There are few studies that examine the effects of melatonin on burn healing. Most melatonin studies have focused on non-burn wound healing. Drobnik and Dabrowski\[14\] reported that pinealectomy enhanced collagen deposition in the skin, and melatonin application reduced the pinealectomy-induced elevation of collagen levels. Bulbuller et al.\[17\] on the other hand, indicated that exogenous melatonin reduced collagen synthesis and epithelium proliferation and had negative effects on wound healing in both normal and pinealectomized rats. Contrary to Bulbuller et al.’s findings, we found the level of type III collagen staining to be significantly higher in the exogenous melatonin group than the pinealectomy group. Our finding suggests that melatonin, by enhancing the level of type III collagen in the burn wound, can decrease the likelihood of a hypertrophic scar and help burn wound healing since type III collagen deficiency increases scar formation.\[18\] In our study, fibrosis levels in the exogenous melatonin group were also significantly higher than the pinealectomy groups—a finding that showed the positive effect of melatonin on burn wound healing.

Kayapınar et al.\[10\] assessed melatonin’s efficacy in saving the zone of stasis in rats. Kayapınar et al. divided their rats into two groups, a control group and a melatonin-treated group. Their study did not, however, assess the effects of pinealectomy on burn wound healing, nor did they analyze as many parameters as our study (e.g., collagen levels, epithelial thickness, and hydroxyproline levels). They found most of the inter-spaced areas to be alive in the melatonin-treated group. We found the thickness of the zone of stasis significantly less in our ExM group (the group with the highest total melatonin levels). Given the correlation between the zone of stasis and the depth of the wound; hence, the progress of the burn injury, this outcome proves that total melatonin levels have a profound impact on reducing the progression of burn injuries. Further supporting this, the ExM+Px group rats that could not produce endogenous melatonin presented with thicker zones of stasis and were administered exogenous melatonin. These findings demonstrate that total melatonin production, once exceeding a certain threshold, will exert a preventative effect on burn wound damage progression by reducing the zone of stasis.

Contrary to our findings, Kayapınar et al.\[10\] report higher edema levels in their controls than their melatonin-treated group. In our study, edema levels were higher in the ExM-treated group than controls, suggesting edema may be a side effect of higher melatonin levels. Although we administered exogenous melatonin in the ExM+Px group (Group 4), we found no differences in edema scoring than the control group (Group 1) and the Px group (Group 3). This finding may be due to the relatively low melatonin levels in these groups than the ExM group (Group 2).

The main goal during the recovery period is to minimize the burn wound area by contraction and extracellular matrix synthesis. Contraction of the wound surface area is a clinical indicator of healing. We found no significant differences among the groups concerning the average healed areas after seven days from the injury. We also noted similarities among the groups for epithelial thickness. Our study was designed to evaluate the early effects of melatonin on the healing of burn wounds in pinealectomized rats. A longer study is required to assess its macroscopic effects in the long-term.

Additionally, because hydroxyproline levels of all collagens in the dermis did not show any significant differences among the groups, hydroxyproline was deemed to be less meaningful than the level of type III.

Our study had several limitations. The first limitation was the short duration of seven days. Longer studies are needed to assess the effects of melatonin on wound healing macroscopically. While our study noted the early effects of melatonin burn wound healing, this study design did not allow for a longer-term evaluation of the effects of melatonin on burn wound healing.

While being a significant factor in the progression of burn wound injury, total melatonin levels above a certain threshold will reduce the zone of stasis and exert a preventative effect on burn wound damage progression. Additionally, melatonin may prevent the development of hypertrophic scarring by increasing fibrosis and type III collagen in burn wounds. The results of this study suggest that melatonin can be used as an additional, supplemental treatment in burn wound healing. Because our study aimed to evaluate the early effects of exogenous melatonin, further studies with higher doses administered over longer periods are warranted to assess its macroscopic effects on burn wound healing. A study of melatonin levels in serum or blood in each group would help determine an appropriate dosage of melatonin to affect burn wound healing. Despite promising experimental findings, future studies are needed to address three key areas: optimal dosages, administration timing and route of administration of exogenous melatonin for optimal outcomes in burn wound healing.

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**Ethics Committee Approval:** Ethics Committee Approval: This study was approved by Necmettin Erbakan University Experimental Medicine Research and Application Center Animal Experiments Ethics Committee (Date: 30.07.2012, Decision No: 2012/121518019).

**Peer-review:** Internally peer-reviewed.

**Authorship Contributions:** Concept: E.Ç.K.S., N.S.; Design: E.Ç.K.S.; Supervision: N.S.; Resource: E.Ç.K.S.; Materi-
Pinealektomiye izin verilen cıvanyı yanık yara iyileşmesine etkisi

Dr. E. Çiğdem Karadağ Sarı, 1, Dr. Nedim Savaç 2

1 Acıbadem Üniversitesi Tıp Fakültesi, Plastik, Rekonstrüktif ve Estetik Cerrahi Anabilim Dalı, İstanbul
2 Necmettin Erbakan Üniversitesi, Meram Tıp Fakültesi, Plastik, Rekonstrüktif ve Estetik Cerrahi Anabilim Dalı, Konya

AMAC: Bu çalışmamızın amacı, cıvancıyanların yanık yara iyileşmesine etkisi araştırmaktır.

GEREÇ VE YÖNTEM: Bu çalışmamız 40 adet Wistar-albino cinsi cıvancıyan derisi gruba ayrılmıştır: Grup 1; kontrol grubu, Grup 2; eksöjen melatonin verilen grup, Grup 3; pinealektomi uygulanan grup ve Grup 4; pinealektomi yapılmıştır. Cıvancıyanlar 3 gün evreli, total 8 gün süreli deri yanıkları oluşturuldu. Kontrol ve eksöjen melatonin verilen grupta yanık yaraların iyileşmesi değerlendirildi.

BULGULAR: Staz kalınlığı, tip 3 collagen, fibrozis, fibrozis düzeyi ve epitel kalınlığı değerlendirildi. Eksöjen melatonin verilen grubun staz kalınlığı ve fibrozis düzeyi diğer gruplara kıyasla azaldıktaydı. Eksöjen melatonin verilen gruptaki yanık yaraların iyileşmesi diğer gruplara kıyasla daha iyiydi.

TARTIŞMA: Bu çalışmamız, cıvancıyan yaralarının iyileşmesi için eksöjen melatoninun etkisini göstermektedir. Bu sonucunARDS ve yanık yaraların iyileşmesinde kullanılması konusunda destek olabileceği düşünülmektedir.

Anahtar sözcükler: Eksöjen melatonin; yanık yara; pinealektomi; cıvancıyan; yanık yara iyileşmesi