The Effect of Melatonin on Experimental Cold Injury of the Skin

Deride Deneysel Olarak Oluşturulan Soğuk Hasara Melatoninin Etkisi

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Geliş Tarihi/*Received:* 03.06.2008
Kabul Tarihi/*Accepted:* 21.08.2008

The study was supported by Hacettepe University, Faculty of Medicine, Research Centre Office and was presented at the British Association of Clinical Anatomists Winter Scientific Meeting, 2007, London.

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ABSTRACT Objective: The human skin is one of the major organs in continuous interaction with environmental factors. The effects of frostbite on the skin have been extensively studied and many drugs for its treatment have been tested over the years. The effects of melatonin in the treatment of tissue injuries have also been investigated. The present study was performed to examine the effects of melatonin on frostbite of the skin. **Material and Methods:** This study was performed in 2006 in the Anatomy Department of Hacettepe University, Faculty of Medicine. Ears of five rabbits were used in each group and specimens were taken by punch biopsy. Groups were allocated as follows: Control group: rabbit ears with no induced injury or treatment; Trauma group: rabbit ears subjected to cold injury; Treatment group: rabbit ears treated with melatonin after cold injury; and Sham group: rabbit ears treated with 0.9% NaCl after cold injury. Specimens were evaluated by transmission electron microscope and were scored statistically. **Results:** In the trauma group, the cells of the stratum corneum layer of the epidermis had separated. Intra-cytoplasmic edema and vacuoles in these cells were visible. Although there was loss of chromatin and evident edema in the nucleus of some of these cells, there was no clear defect in the distribution of the nuclear chromatin in general. Separation between the cells of the stratum corneum of the melatonin group was absent. The cells were normal, collagen fibers were regular and the fibroblasts were inactive. **Conclusion:** The results obtained in this rabbit model are significant for suggesting the favorable effects of melatonin on cold injury.

Key Words: Skin; melatonin; freezing

ÖZET Amaç: Deri, dış ortam ile en çok temas halinde olan organımızdır. Bu nedenle soğuğa yoğun bir şekilde maruz kalmaktadır. Donmanın deri üzerindeki etkisi uzun yıllar boyunca çalışılmıştır. Bu çalışmaların sonucunda ise birtakım tedavi edici maddeler denenmiştir. Günümüzde travmalara bağlı doku zedelenmelerinde melatoninin tedavi edici etkisi yoğun bir şekilde denenmektedir. Çalışmamız, deride oluşturulan soğuk hasara bağlı oluşan zedelenmede melatoninin etkisini incelemek amacı ile yapılmıştır. **Gereç ve Yöntemler:** Bu çalışmada, 5 tavşanın kulakları kullanılmıştır ve hayvanlar 4 grupta incelenmiştir. 1, Kontrol grubu: Hiç bir uygulama yapmadan, kontrol amacı ile sağlam kulaklardan alınan biyopsi materyallerinin incelenmesi. 2, Travma grubu: Donma derecesine kadar soğutulan kulaklardan alınan biyopsi materyallerinde doku harabiyetinin incelenmesi. 3, Tedavi grubu: Kulakları donma derecesine kadar soğutulan hayvanlara intra-peritoneal melatonin uygulayarak, alınan biyopsi materyallerinde melatoninin etkilerinin incelenmesi. 4, Sham grubu: Melatonin serum fizyolojik ile sulandırıldığından SF'in etkisini görmek için sadece SF kullanılan gruptur. Bu örnekler transmisyon elektron mikroskobu ile incelenmiştir. **Bulgular:** Kesitlerin incelenmesi sonucunda, travma grubundaki örneklerde epidermis stratum corneum tabakasında hücreler arasında ayrılma olduğu, hücreler incelendiğinde ise intrasitoplazmik ödem ve vakuollerin mevcut olduğu izlendi. Bazı hücrelerin nukleuslarında kromatin kaybı ve belirgin ödem olmasına rağmen genel olarak nükleer kromatin dağılımında belirgin bir değişiklik olmadığı gözlendi. Melatonin grubunun epidermis stratum corneum'unda hücreler arasında ayrılma olmadığı, hücrelerin normal, kollajen diziliminin çok düzenli, fibroblastların inaktif olduğu izlendi. **Sonuç:** Melatoninin tedavi edici etkisi istatistiksel olarak grafik şeklinde gösterildi. Elde edilen sonuçlar, deride oluşturulan soğuk hasara bağlı oluşan zedelenmeye karşı melatoninin etkisini ortaya koyması açısından önem taşımaktadır.

Anahtar Kelimeler: Deri; melatonin; soğuk hasar

Turkiye Klinikleri J Med Sci 2008;28(6):828-33

Human skin is a major human organ in continuous interaction with environmental factors. Thus, it is excessively exposed to cold. Cold injuries are evaluated in two groups as general and local. Local injuries occur below the freezing point. The skin is the most important organ protecting us against cold. The reaction of the skin to local cold injury is a vascular response, and a number of agents have been evaluated extensively for its treatment.[1-9] The treatment effects of melatonin on tissue damage in trauma have been studied in the recent years in view of its powerful antioxidant properties and its potency as a protective agent against damage after experimental thermal injuries. Furthermore, it is recognized as an extremely efficient free radical scavenger and it has lipophilic and hydrophilic properties that enable it to protect against oxidative stress throughout the body.[10-20]

Melatonin was also experimentally used in acute cardiac and spinal cold injuries and cerebral edema and it improved recovery in these tissues.[10,12] However, to our knowledge, the ultrastructural effects of local cold injury on the epidermis and dermis layers of the skin and the possible recovery effects of melatonin in this condition have not been reported in the literature.

MATERIAL AND METHODS

This study was performed in 2006 in the Anatomy Department of Hacettepe University, Faculty of Medicine. The ears of five rabbits (weighing 4000-5000 g) were used in each group. Specimens were taken by punch biopsy. Groups were allocated as follows: Control group: rabbit ears were shaved, and no other procedure was performed. Trauma group: ears were shaved and than cooled to the freezing point with liquid nitrogen using a metal probe for 30 seconds. Biopsy materials were taken 24 hours after the cooling process and specimens were evaluated for tissue damage. Melatonin group: ears were shaved and cooled until the freezing point with liquid nitrogen using a metal probe for 30 seconds. Ten minutes after the cooling process 50 mg/kg melatonin (diluted by 0.9% NaCl) was applied intraperitoneally and biopsy materials were taken after 24 hours to investigate the recovery effect of melatonin. Sham group: ears were shaved and cooled to the freezing point with liquid nitrogen using a metal probe for 30 seconds. Ten minutes after the cooling process, intraperitoneal 0.9% NaCl was applied and biopsy materials were taken 24 hours later to evaluate the effects of 0.9% NaCl.

The biopsy specimens were prepared and evaluated by transmission electron microscope. All specimens were fixed for 24 hours with 2.5% glutaraldehyde and then washed with Sorenson's phosphate buffer (SPB). They were then post-fixed in OsO4 for 1 hour. After dehydration in alcohol with increasing grade, specimens were treated with propylene and then embedded in Araldite CY212. Semi-thin sections were stained with methylene-blue and were examined under the light microscope; then 60-90 nm thick ultra-thin sections were stained and contrasted with uranyl acetate and lead citrate. They were examined, scored statistically and photographed under JEOL JEM 1200 EX transmission electron microscope. Scores obtained by evaluation of several sections of dermis and epidermis were compared across study groups using the Kruskal-Wallis test to analyze the presence of overall effect of the experiment. When an overall effect was observed, pairwise comparisons were performed using the Mann-Whitney U test. The type-I error level of 0.05 was used to infer statistical significance.

The present study was performed in accordance with 'Guide for the Care and Use of the Laboratory Animals' principles and was approved by the ethic committee of Hacettepe University, Faculty of Medicine.

RESULTS

The epidermis cells and connective tissue fibers were normal, fibroblasts were inactive, and gland cells and hair follicles were normal in appearance in the control group. In the trauma group, there was separation between the cells of the stratum corneum layer of epidermis, and intra-cytoplasmic edema and vacuoles were visible in these cells. Although there was loss of chromatin and evident edema in the nucleus of some of these cells, there was no clear defect in the distribution of the nuc-

lear chromatin in general (Figure 1). In the melatonin group, no separation between the cells of the stratum corneum was seen. The cells and the basal lamina were more normal in appearance than in the trauma group, although there was a mild edema in the cells (Figure 2).

Irregularity, disarrangement and edema (*) between the collagen fibers were present in the dermis layer of the trauma group (Figure 3). However, collagen fibers of the melatonin group were rather well arranged (Figure 4). The fibroblasts were quite active compared to the melatonin group.

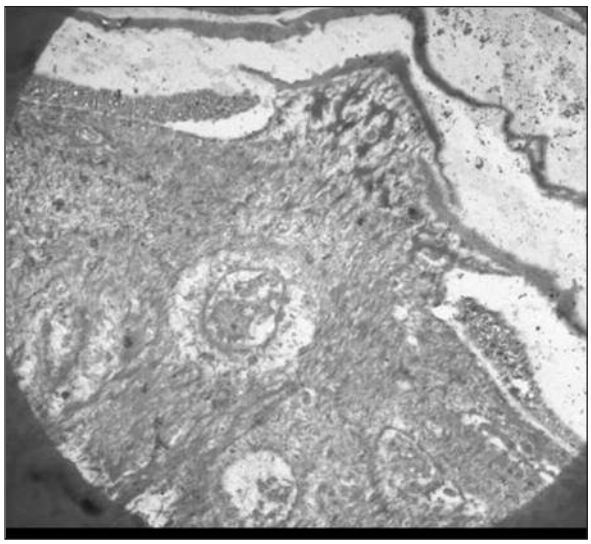

FIGURE 1: Separation between the cells of the stratum corneum layer of the epidermis (Original magnification x 2500, uranyl acetate and lead citrate).

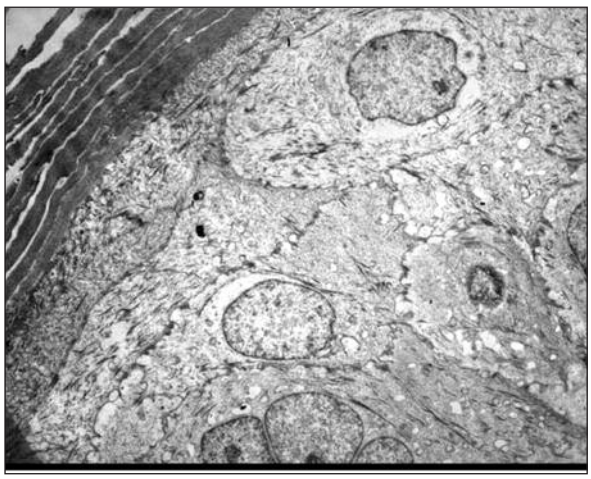

FIGURE 2: Epidermis layer in the melatonin group (x 2500, uranyl acetate and lead citrate).

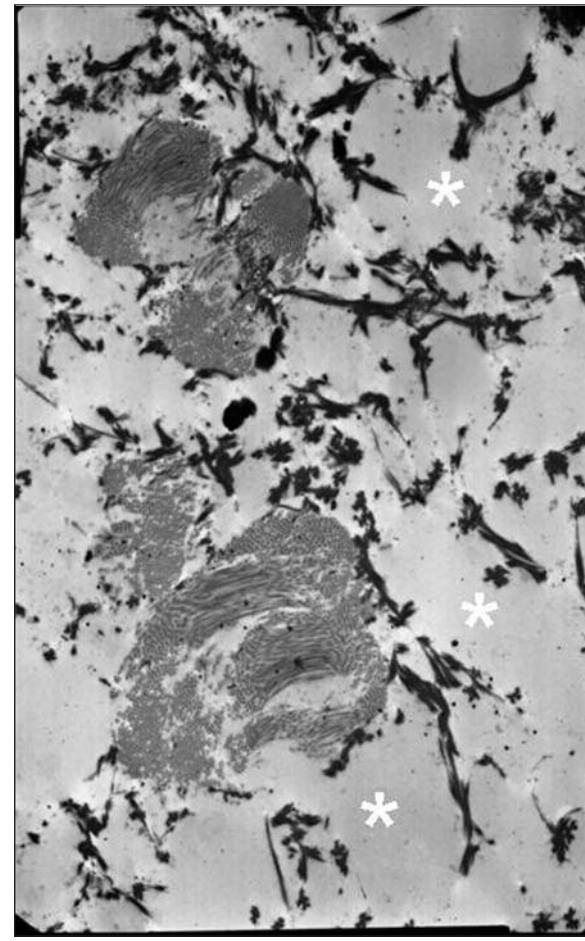

FIGURE 3: Collagen fibers in the trauma group, edema (*) between the collagen fibers (x 2500, uranyl acetate and lead citrate).

Although some of the endothelial cells of the trauma group seemed normal, there was margination (m) and clumping in the nuclei in the majority of the cells. Edema (*) and vacuolization (v) were seen in the cytoplasm of these cells (Figure 5a). Conversely, the endothelial cells of the melatonin group were normal in appearance (Figure 5b). According to evaluation of the peripheral nerve, edema in perineurium (*), axonal degeneration (arrow) and axonal edema (*) were predominant in the trauma group (Figure 6a). Normal axonal cytoplasm and perineurium were seen in the melatonin group (Figure 6b).

The results of the sham group were similar to those of the trauma group.

Evaluating the ultrastructural effect of melatonin on the chart showed that melatonin signifi-

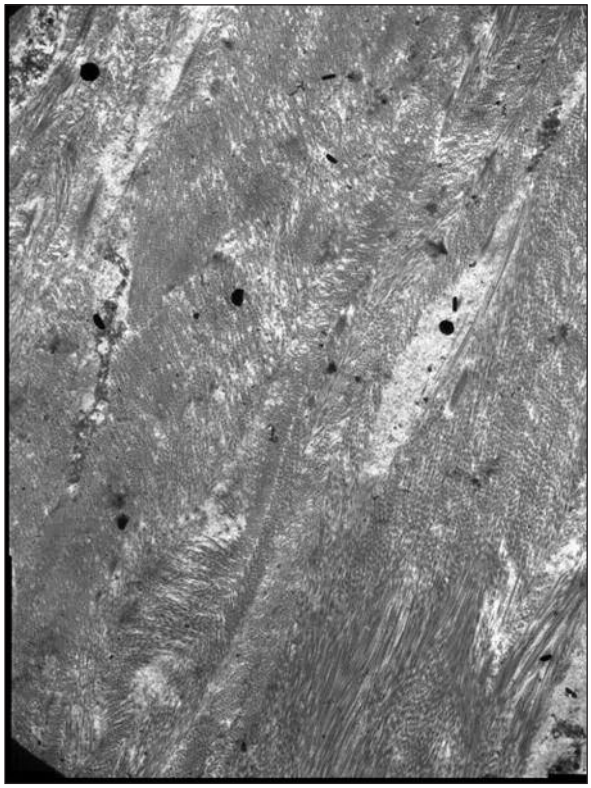

FIGURE 4: Collagen fibers in the melatonin group (x 2500, uranyl acetate and lead citrate).

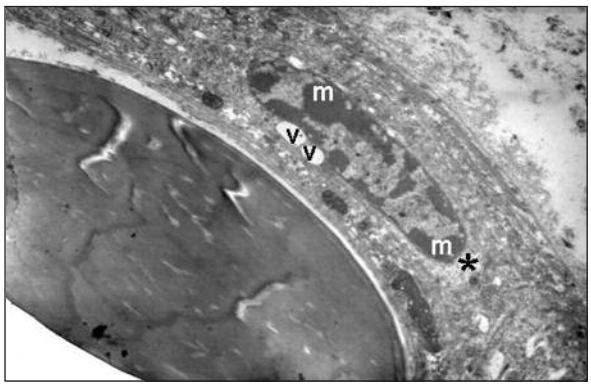

FIGURE 5a: Collagen fibers in the melatonin group x 2500, uranyl acetate and lead citrate). m: morgination, v: vaculization.

cantly reduced the trauma scores ($p< 0.01$). Comparison of the melatonin and sham groups indicated that decreases in the sham group scores, except for intranuclear and interstitial edema in the epidermis, were statistically significant. Values in the melatonin and control groups were parallel regarding fibroblast activity and interstitial edema at the dermis layer ($p> 0.05$) (Figure 7). The results obtained in this animal model suggest a favorable effect of melatonin on cold injury.

DISCUSSION

Melatonin is a very significant compound with well-known antioxidant properties. It is able to directly scavenge a variety of toxic oxygen and nitrogen-based reactants, stimulates antioxidative enzymes, increases the efficiency of the electron transport chain, thereby limiting electron leakage and free radical generation, and promotes ATP synthesis. Via these actions, melatonin preserves the integrity of the mitochondria and helps to maintain cell functions and survival.[16] Regulation of the immune system is among the most significant pleiotropic effects of melatonin.[21]

The reaction of skin to local cold injury as a vascular response and treating agents have been evaluated extensively.[1-9] Marzella et al studied freezing injury in rabbit ears using serial biopsies examined by light and electron microscopy. They mentioned that the morphological evidence of skin injury due to freezing was localized exclusively in the endothelial cells, particularly in the arterioles, so they did not perform detailed morphological analysis. According to Marzella, freezing caused an immediate separation of the endothelial cells. In the

FIGURE 5b: Endothelial cells in the melatonin group (x 2500, uranyl acetate and lead citrate).

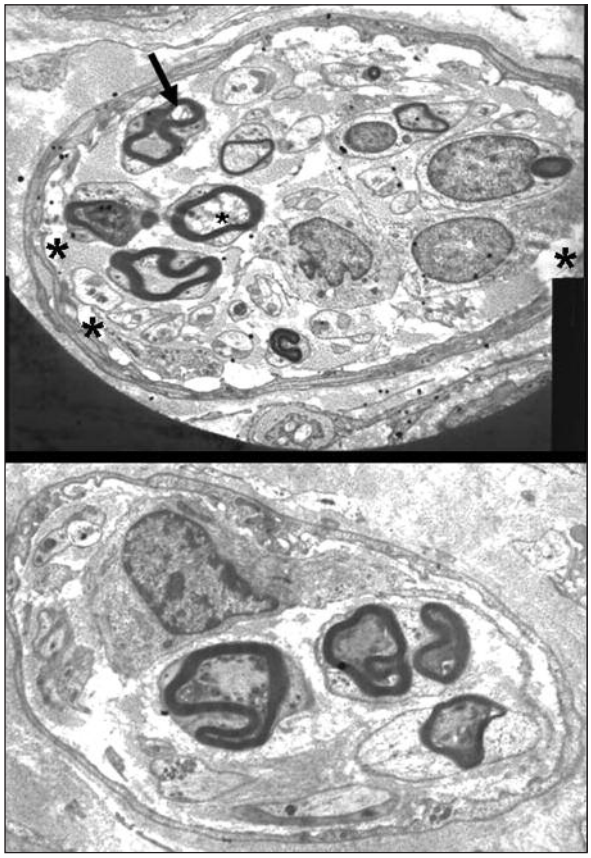

FIGURE 6: a. Peripheral nerve in the trauma group, edema in perineurium (*), axonal degeneration (arrow) (x 2500, uranyl acetate and lead citrate). **b.** Peripheral nerve in the melatonin group (x 2500, uranyl acetate and lead citrate).

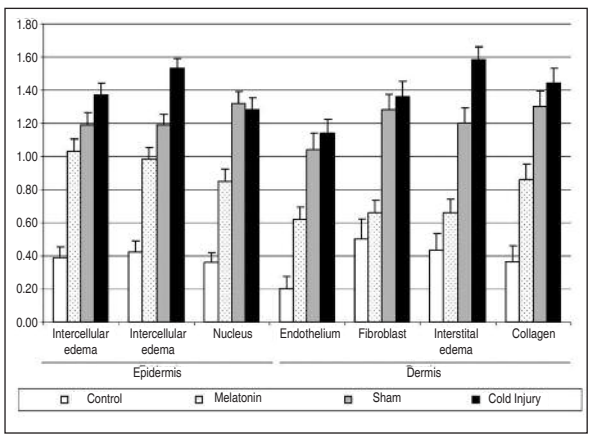

FIGURE 7: Chart showing the ultrastructural effects of melatonin.

present study, although some of the endothelial cells of the trauma group seemed normal, there was margination and clumping of the nuclei in the majority of the cells. Edema and vacuolization were present in the cytoplasm of these cells. In contrast, the endothelial cells of the melatonin group were normal in appearance. Marzella and Kulka also reported separation between the keratinocytes, subepidermal vesicles and vacuolization.[3,22] Bourne et al analyzed the microvascular changes in frostbite injury and remarked that the severe endothelial cell damage was an initial event consequent to frostbite injury and followed by vascular dilation, vascular incompetence and erythrocyte extravasation.[23]

Knize et al induced frostbite in rabbit ears by a method that produced predictable tissue loss [1] They mentioned that edema was caused by increased intravascular hydrostatic pressure due to postcapillary obstruction and decreased intravascular osmotic pressure, secondary to loss of plasma proteins. As frostbitten rabbit ears are analogous to a composite skin graft, their survival is endangered by the same factors. In the trauma group of the present study, intra-cytoplasmic edema and vacuoles in the cells of the stratum corneum layer of epidermis, nuclei and between the collagen fibers in the dermis layer were evident. These findings can be explained with the same mechanism as mentioned by Knize et al.[1] Weatherley-White et al also studied cold injury in rabbit ears experimentally and showed that there was progressive degeneration of cell membranes and nuclei throughout all layers of the ear.[8]

The treatment of frostbite is a topic frequently addressed in many studies. Widely varying models of treatment have been evaluated for severe cold injury. Snider et al emphasized that the administration of intra-arterial reserpine and tolazoline might be of benefit in the treatment of frostbite.[7] Salimi et al found that streptokinase treatment prolonged the tissue reperfusion time.[6] Knize et al compared a non-ionic detergent and a low molecular weight dextran in preventing tissue damage and found that the non-ionic detergent was more effective in preventing tissue loss.[1]

Melatonin, known as an extremely efficient free radical scavenger with lipophilic and hydrophilic properties that enable it to protect against oxidative stress throughout the body, has been shown

to have an effect on spinal cold injuries.[11,12] This agent was also experimentally used in acute cardiac injuries and cerebral edema.[10,12] Investigators reported that melatonin treatment improved recovery in these tissues. Lim et al used melatonin to study the survival of reimplanted rabbit auricular composite grafts. Their study demonstrated that melatonin significantly affected the survival of reimplanted auricular composite grafts.[14] Some actions of melatonin as a potential supportive pharmacologic agent in burn patients was studied by Maldonado and Sener, but effects on frostbite are still unknown.[20,24] Findings of the present study showed that melatonin had a protective effect against edema in the epidermis layer and prevented irregularity and disarrangement of the collagen fibers in the dermis layer after cold injury.

CONCLUSION

Melatonin's low toxicity and its ability to penetrate all morphophysiologic membranes could make it a highly beneficial molecule. The present study attempted to show that melatonin reduced trauma scores and demonstrated that a significant level of cytoprotection could be achieved after cold injury. Thus, this oxygen free radical scavenger may be a considerable therapeutic agent in the treatment of cold injury of the skin. Further studies are required to definitively establish the effects of melatonin in frostbite.

Acknowledgement

We would like to thank Corinne Can for English revision.

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