Original Article

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Effects of topical melatonin and vitamin E in a rat ischemic wound model

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Summary

Objective: Reactive oxygen species are known to increase on a chronic wound background. We therefore investigated the possible efficacy of the topical administration of melatonin and vitamin E, known to have radical scavenging properties, in the ischemic wound model.

Methods: Forty Sprague-Dawley type adult male rats were divided into 4 groups as normal wound, ischemic group, and with vitamin E or melatonin applied to the ischemic wound. Bipedicular flap surgery to the shaved back of the rats was used to induce ischemia. A full-thickness skin lesion was produced by punch biopsy in each animal 3 days after this procedure. The punch biopsy procedure was performed without the flap surgery in the normal wound group. The vitamin E and melatonin administration continue twice a day for a total of 7 days after the wound was formed.

Results: Hydroxyproline (OH-proline) and malonyldialdehyde (MDA) levels and superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) enzyme activities were measured on the wound tissues removed by excisional biopsy at the end of the procedure. The MDA levels were significantly decreased in the groups receiving vitamin E and melatonin compared to the ischemic wound group. Vitamin E application also significantly increased OH-proline levels in the ischemic wounds. The antioxidant enzyme activities were not seen to be affected by the treatment procedures used.

Conclusion: We concluded that the collagen synthesis decreased together with increased oxidative stress in the induced ischemic wound model; topical vitamin E application could reverse both states and melatonin could not support collagen synthesis although it had an antioxidant effect.

Key words:
Ischemic wound; Melatonin; Vitamin E; Wound healing

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Introduction

Wound healing is a well-organized repair process appearing following surgical procedures or trauma. Any problems in this organization due to various factors may prolong the healing period. Such chronic cases pose a big problem for the patient and the physician. Thousands of people are affected every year causing millions of dollars in healthcare spending. Diabetic, pressure and venous ulcers are the three main examples of unhealing wounds [1].

The healing process begins with inflammation right after the injury and continues with new tissue formation and the maturation stage. The chemotactic factors secreted during the early stage of the inflammation attract many neutrophils and macrophages from the circulation to the wound area. The contribution of these cells arriving at the wound region and the fibroblasts already in the area lead to collagen synthesis and contraction and finally wound closure. Wound healing on a background of no circulatory or metabolic problems is concluded rapidly with its own active process [2].

The main factor disturbing and prolonging wound healing is disturbance of tissue oxygenation. This leads to a disturbance in extracellular matrix repair by inhibiting collagen synthesis, which is oxygen-dependent and one of the most important steps of healing [3]. Similarly, the healing period is prolonged in the ischemic wound model defined by Schwarz *et al* that we have used in our study [4].

Melatonin is a hormone produced mainly by the pineal gland and secreted in the dark of the night. It is known to be associated with many biological events such as biological rhythms, sexual maturation, reproduction sleep, mood and immunity. It has also been shown to have an antioxidant effect in various in vivo and in vitro studies [5]. The antioxidant property of melatonin has many aspects as it can scavenge reactive molecules such as superoxide anion, hydroxyl radical and peroxynitrite and can also stimulate the expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and

glutathione peroxidase (GSH-Px) [6]. Vitamin E has also been shown to prevent oxidative stress by scavenging free radicals and is a potent fat-soluble antioxidant vitamin [7].

In this study and we tried to determine potential healing effects of substances with known antioxidant efficacy such as melatonin and vitamin E when used topically on this continuing important health problem.

Materials and methods

Study design

The Experimentation Ethics Committee of our institution approved the experimental procedures of the present study. A total of 40 adult male Sprague-Dawley rats bred in our laboratory were used. Rats were 12 weeks of age and weighed 200-250 g. They were housed in a temperature-controlled environment (22-24ºC) with free access to water and lights on from 08.00 to 20.00. All animals were fed a commercial diet during the course of the experiment. They were randomly divided into four study groups:

Normal wound (negative control) group: group where a wound was created on healthy skin with normal blood supply.

Ischemic wound (positive control) group: group where a wound was created on skin with a disturbed blood supply due to flap surgery.

Vitamin E group: group receiving 5 mg vitamin E ointment for each created ischemic wound.

Melatonin group: group receiving 0.1 mg melatonin ointment for each created ischemic wound.

Ointment preparation

Vaseline was melted at 50ºC. The heat was reduced while adding the active compound and this combination was then mixed well until a uniform mixture is obtained. Preservatives were not used because of the short application time. After preparation, the ointments were kept in jars at room temperature. Physical stability (appearance, odor, color) was under control during the study.

Surgical method

Preparation of ischemic skin area: the wound model of Schwarz *et al* [4] firstly employs creation of a skin flap with a pedicule on either side to produce an ischemic base. The rats were given intraperitoneal ketamine (85 mg/kg) plus xylasine (12.5 mg/kg) [8] anesthesia and secured on their bellies with their backs (dorsal regions) upwards to produce this bipedicule flap. The rectangular area from the two scapulae to the crista iliacae at the dorsal region was softened with surgical soap and then shaved to eliminate hair from the work area. The bare region was disinfected by cleaning with Betadine solution (Isosol, Hayat Corporation, Istanbul, Turkey). Skin incisions along the two long edges lying parallel from the scapulae to the crista iliacae to include the panniculus carnosus were then made with a No.21 blade under sterile conditions. A flap approximately 4 x 10 cm in size that was attached to the skin by the upper and lower edges (pedicules) was next elevated by dissecting the subcutanous tissue starting from these guide incisions. The edges of this bipedicular flap were sutured with 4/0 atraumatic silk (Dogan Corporation, Trabzon, Turkey). Antisepsis was provided in the flap area and surroundings by spraying Opsite (Smith & Nephew Healthcare, Hull, UK) at the end of the procedure.

Wound creation: the animals with bipedicular flaps were left alone for three days. They were anesthetized again on the third day and the wound creation procedure started under sterile conditions following skin cleaning with Betadine solution. A total of 6 full-thickness skin wounds were created over the flap region with a 6 mm punch biopsy instrument (Acupunch, Acuderm Inc., Fort Louderdale, FL, USA) (Fig.1).

Vitamin E and melatonin application

Treatment administrations were started 24 hours after wound formation and continued twice a day for a total of 7 days. The control groups were administered ointment without any active ingredient.

Vitamin E (α-tocopherol acetate; Sigma T3001) was administered at an amount of 5 mg per wound and melatonin (N-Acetyl-5-methoxytryptamine; Sigma M5250) at 0.1 mg. The targeted total daily topical administration was 240 mg/kg (animal weight) for vitamin E and 5 mg/kg for melatonin.

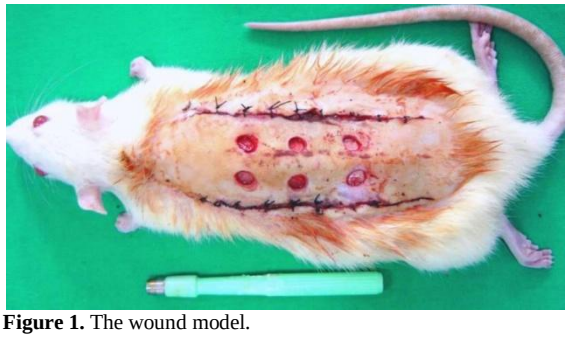

Figure 1. The wound model.
Six Full-thickness wounds were constituted by 6 mm punch on the bipedicule flap.

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Tissue preparation

Five hours after the 7-day course of melatonin and vitamin E application, the animals were anesthetized again and all wound tissues (6 each) excised from each animal, staying within the wound borders as much as possible, and using a scalpel and fine surgical scissors. The excised tissues were placed into eppendorf tubes and put into liquid nitrogen for fast freezing.

The frozen tissues were then homogenized in phosphate buffer by means of the homogenizer (Heidolph Diax 900; Heidolph Elektro, Kelhaim, Germany). Finally, the supernatants were divided into 2-3 parts, put in separate tubes, and stored at −70ºC.

Biochemical analysis

The protein content of tissues was measured according to the method of Lowry *et al* with bovine serum albumin as the standard [9].

Lipid peroxidation levels were measured with the thiobarbituric acid reaction by the method of Ohkawa *et al* [10]. This method was used to obtain a spectrophotometric (Helios Epsilon, Thermo Electron Corporation, Madison, WI, USA) measurement of the color produced during the reaction to thiobarbituric acid with malondialdehyde (MDA) at 535 nm. MDA levels were expressed as nmol/g-protein.

Superoxide dismutase activity was assayed using the nitroblue tetrazolium (NBT) method of Sun *et al* [11]. In this method, NBT was reduced to blue formazan by superoxide, which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The calculated SOD activity was expressed as U/mg-protein.

Glutathione peroxidase activity was measured using the method described by Paglia & Valentine [12] in which GSH-Px activity was coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was spectrophotometrically followed up at 340 nm at 37ºC. The final GSH-Px activity was presented as U/mg-protein.

Hydroxyproline (OH-proline) activity was measured using the method described by Reddy [13]. The hydrolysates were obtained by incubating with hydrochloric acid and 2N NaOH was added. All samples were exposed to chloramine-T for 25 minutes at room temperature. Development of the chromophore was achieved by addition of Ehrlich's reagent and incubating the samples at 65°C for 25 minutes. The hydroxyproline concentrations were calculated by a standard curve using standard solutions of l-hydroxyproline. The hydroxyproline content was expressed as µg/g-protein.

Chemicals

All chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) and all organic solvents from Merck KGaA (Darmstadt, Germany). All reagents were of analytical grade, were prepared each day (except the phosphate buffer) and stored in a refrigerator at +4ºC. The reagents were equilibrated at room temperature for 0.5h before use when the analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at +4ºC for 1 month.

Statistics

All the numeric data were first analyzed using the Kruskal-Wallis test to determine differences between the groups and the Mann-Whitney U test was employed to analyze two groups consecutively. The results were expressed as the mean ± standard error of the mean (SEM) and $P<0.05$ was considered statistically significant.

Results

Collagen formation

OH-proline results are provided in Fig.2. The OH-proline levels of the ischemic wound group were found to be significantly lower than the normal wound group ($P<0.01$). Vitamin E application significantly increased this decreased OH-proline level ($P<0.01$). Melatonin application had no effect on the OH-proline level.

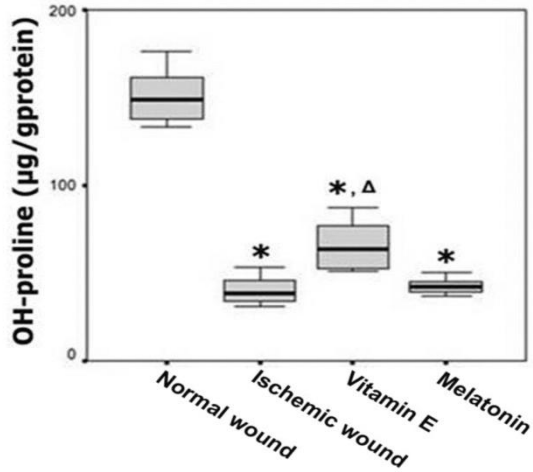

Figure 2. Hydroxyproline levels of wound tissue.
OH-proline levels increased significantly with vitamin E application but not with melatonin. *Significantly lower than the normal wound group ($P<0.01$). ΔSignificantly higher than the ischemic wound group ($P<0.01$).

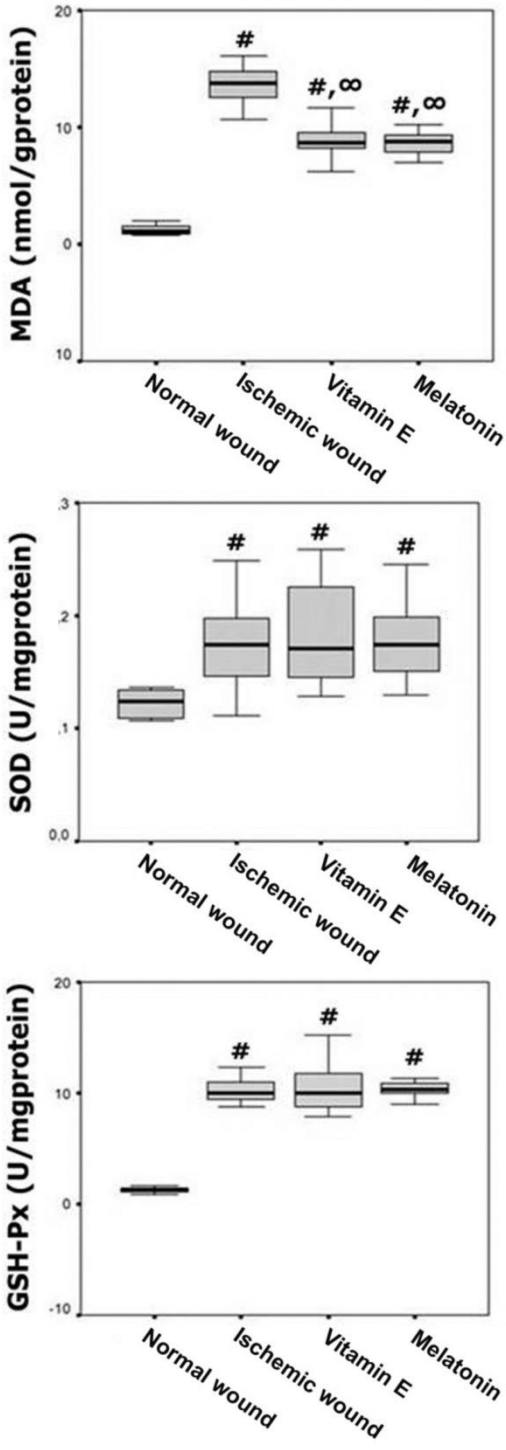

Figure 3. Wound tissue oxidant and antioxidant indices.
The increased MDA levels in ischemic wound tissue decreased significantly both with vitamin E and melatonin. There was no significant effect regarding antioxidant enzyme activities. #Significantly higher than the normal wound group (P<0.01). ∞Significantly lower than the ischemic wound group (P<0.05).

Oxidant/antioxidant parameters

The MDA levels were found to be significantly higher in the ischemic wound group compared to the normal wound group (P<0.01). Both vitamin E and melatonin application significantly decreased these increased MDA levels (P<0.05). However, the wound tissue MDA levels of both treatment groups were still higher than the normal wound group (P<0.01).

The SOD and GSH-Px activities measured in the normal wound tissue were significantly higher than all other groups (P<0.01). Vitamin E and melatonin applications did not produce a change in ischemic wound tissue regarding SOD and GSH-Px enzyme activities (Fig.3).

Discussion

Wound healing on an ischemic basis is problematic and prolonged [14]. The inadequate blood flow can lead to chronic wounds, depending on the degree of ischemia. Ischemia for any reason causes the formation of reactive oxygen species (ROS) that lead to tissue damage. The ischemia and the resultant ROS's that play a role in the pathogenesis of chronic wounds cause decreased protein synthesis, increased leukocyte infiltration and increased metalloproteinase activity in the tissue. Wound healing cannot follow its normal sequence as a result of the disturbed extracellular matrix formation and prolonged inflammatory response due to these pathological changes caused by ischemia [15-17].

It is known that antioxidant SOD and GSH-Px enzyme levels increase in the presence of oxidative stress [18]. The high levels of oxidant/antioxidant parameters (MDA, SOD and GSH-Px) in the ischemic wound group in the current study indicated the presence of an oxidative stress environment. Steiling *et al* have also observed an increase in SOD and GSH-Px levels together with oxidative stress in wound tissue in the wounds they created in mice. The increased antioxidant enzyme expression during healing was interpreted as for protection from the increased ROS production [19].

Lipid peroxidation is the best known of the radical reactions characterized by biological damage and mostly appears following the oxidation of the fatty acid side chains of membrane phospholipids [20]. One of the final products to appear as a result of these chain oxidative reactions is MDA and it has therefore generally been used as an indication of lipid peroxidation in molecular studies. The decrease in the MDA levels that were

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higher in the ischemic study groups than the normal wound groups following bipedicular flap surgery in the groups applied vitamin E or melatonin in our study indicates that these two agents may stop the progress of lipid peroxidation reactions. We also observed in this way that melatonin and vitamin E can show their known antioxidant properties through topical administration as well [6, 21].

We noted that the increased SOD and GSH-Px activity in the ischemic wound group in our study was not affected by melatonin and vitamin E applications. This may be due to the MDA levels that were still high in the treatment groups compared to the control group, even after showing a significant decrease. The MDA levels were up to 10 times higher in the ischemic wound group compared to the normal wound group but still 6-7 times higher in the treatment groups compared to the normal wound group animals.

One of the commonly used methods to evaluate wound healing is to determine the collagen level in the healing wound. A common method to evaluate the collagen amount is to determine the level of OH-proline that is found in large amounts in collagen structure and enters little into the structure of other proteins [22]. Looking at the OH-proline levels in the current study, we see that the normal wound group levels are higher than both the ischemic wound group and the two treatment groups. The melatonin treatment did not cause a significant difference in the ischemic wound group regarding OH-proline. Vitamin E administration significantly increased OH-proline levels when compared to the ischemic wound group. We found contradicting results in previous studies with some reporting that the effect of vitamin E on collagen synthesis is mostly beneficial while some report unfavorable effects. One of the studies reporting such unfavorable effects is the Jenkins *et al* study showing that vitamin E administration decreased scar formation in their patients who had been grafted for burn treatment [23]. Another study has reported a similar result stating that vitamin E decreased wound tensile strength by disturbing collagen synthesis in experimental animals with the delayed wound healing model [24]. One of the studies reporting beneficial effects is the Taren *et al* study where intraperitoneal vitamin E administration at various doses showed increased wound tensile strength with increased doses in a model where radiation was administered on the wound [25]. Another study by Musalmah *et al* showed that vitamin E administration accelerated wound closure by increasing collagen synthesis. This effect of vitamin E was thought to be due to its free radical scavenging effect in this study [21].

The increased OH-proline levels with vitamin E administration in the current study may have been due to its radical scavenging feature as stated by Musalmah *et al* [21]. However, melatonin does not give a result parallel to that of vitamin E despite being known as a very potent antioxidant, indicating that the result cannot be solely the result of the radical scavenging characteristics. Vitamin E may have had other effects besides scavenging ROS. For example, vitamin E has been shown to inhibit superoxide formation in polymorphonuclear cells and also to prevent the adhesion of these cells to the endothelium [26, 27]. Another study has shown that vitamin E suppresses ROS production, interleukin-1β production and endothelial adhesion of monocytes, whether activated or not [28, 29].

However, melatonin administration has been shown to decrease the amount of collagen in general in wound model studies regarding the effect of melatonin administration on collagen synthesis. Drobnik *et al* observed healing in the wounds they created on the backs of rats and evaluated the amount of collagen at the end of 4 weeks. They used various doses of melatonin (3, 30 and 100 μg/100 g body weight) and found the collagen amount to decrease at the 30 μg dose but not in the other groups. The same study found an increased collagen amount in the wound tissues of animals where melatonin secretion was suppressed via pinealectomy [30]. Another study by the same team where they evaluated the effect of melatonin on normal skin collagen level showed a significant decrease in the amount of collagen in normal tissue in the group receiving melatonin without a pinealectomy. The normal skin collagen amount had increased in the pinealectomy group and the collagen levels increased again when these animals were administered melatonin [31]. Bulbuller *et al* have reported in another study where the effect of pinealectomy was evaluated that melatonin delayed wound healing and decreased collagen synthesis [32].

All the studies listed above on the effects of melatonin on wound healing had one important point in common: the melatonin had been administered systemically. The collagen synthesis decreasing and wound healing delaying effect of melatonin is probably due to its oncostatic and antimitotic properties [33-35]. The topically applied melatonin in our study did not have a positive or negative (increasing or decreasing) effect on OH-proline levels although it showed antioxidant

efficacy through decreased MDA levels. This relative inconsistency with other reports may be due to the difference in the route of administration (systemic and topical) as systemically administered melatonin is a hormone with many effects affecting many tissues [36]. It may also show its effect on the wound healing process by affecting various systems. It is however only possible to clarify the matter with studies where both the systemic and topical routes are used.

In conclusion, we observed in this study that one of the most important reasons for difficult wound healing in wounds created in areas with disturbed circulation was oxidative stress and that chronic wounds with increased oxidative stress could respond well to topical vitamin E application. Despite its oxidative stress decreasing effect, topical melatonin can be said to have no effect on healing as it could not support collagen formation. It will be beneficial to conduct new studies to compare systemic and topical use and determine the relevant dose-response curves.

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