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THE EFFECT OF THERMAL INJURY AND MELATONIN ON INCISIONAL WOUND HEALING

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ABSTRACT

Background: *Oxygen - free radicals are generated during inflammatory reactions and cause tissue damage when overproduced. The wounds, especially burn injuries which comprise several events, result in generation of reactive oxygen species and impairment of cellular functions as in wound healing process. This experimental study was done in order to investigate whether 20% body surface area, third degree burn injury creates systemic impairment in wound healing. Additionally, our aim was to evaluate the effects of melatonin on incisional cutaneous wound healing.*

Methods: *Fifty adult Wistar-Albino rats were included in the study. A group of animals were subjected to dorsal burn injury followed by full-thickness midline skin incision, 2 cm in length on the abdominal region which was primarily sutured. Melatonin was administered on incisional wounds and breaking strength, hydroxyproline, thiobarbituric acid reactive substance values and antioxidant enzyme activities in the wounded tissue were determined at day 7; to examine firstly the influence of thermal injury on systemic wound healing and secondly, whether melatonin possesses improving effects.*

Results: *No detrimental effect of 20% burn injury on unburned cutaneous incisional wound healing was determined. There was not any difference in breaking strength, hydroxyproline, thiobarbituric acid reactive substance and glutathione peroxidase values, except for significantly elevated catalase and superoxide dismutase activities in melatonin-treated animals comparing to the control group.*

Conclusion: *This preliminary study disclosed that exogenous melatonin, at a dose of 10mg/kg for two days, exerted few variation in antioxidant status during wound repair. Nevertheless, half-life of melatonin is short and further studies are required, to investigate longer duration or higher dosage of administration which may be beneficial for cutaneous wound healing.*

Key words: *Burn, melatonin, reactive oxygen species, wound healing*

INTRODUCTION

Wound healing is a complicated process which is composed of a cascade of many cellular and biochemical mechanisms involving phases of hemostasis, inflammation, proliferation and remodelling. During inflammation, neutrophils migrate into the wound site and release several mediators followed by cellular activation. Oxygen-free radicals or reactive oxygen species (ROS) are one of these products, generated due to excessive delivery of oxygen to tissues, and reported to be responsible for the decrease in wound strength after incision.[1,2] There is evidence that overproduction of ROS, particularly the most reactive radical hydroxyl, is followed by the release in extracellular space and causes tissue damage.[3-5] Thus, wound healing might benefit from the drugs that act by inhibiting neutrophil-derived ROS.

The wounds of burn injuries comprise various local and systemic events initially. Ischemia-reperfusion, stimulates inflammatory cells to generate ROS in many tissues, especially after burns.[3,6,7] Cellular functions are impaired due to systemic inflammation in the post-burn period.[8,9] In addition, cutaneous burn results in many abnormalities of the endocrine system that contribute to impairment of wound repair. Overactivity of adrenal axis, leading to elevated cortisol levels, might counteract regular wound healing in several organs.[10,11] Systemic lipid peroxidation in the post-burn period causes impairment of several tissues and circulating cells.[8,12] In the present study, it was hyphotesized whether 20% body surface, third degree burn injury creates systemic impairment in cutaneous wound healing process. Moreover, we intended to investigate the effects of melatonin, being a popular formulation for its antioxidant properties recently,[13,14] on incisional cutaneous wound healing in both normal and burned animals. In addition, a treatment approach with melatonin could be attained by this animal model.

MATERIALS AND METHODS

Experimental design

Fifty adult male Wistar-Albino rats which weight between 230 and 290 gr, were used in the study. The guiding principles in the use and care of laboratory animals were adhered during the experiment. Animals were housed in a 20 °C room and offered rat chow and water ad libitum. They were kept in dark:light cycle (DL=12:12 hours). Thus, lights were turned off at 8:00 pm and turned on at 8:00 am for achieving satisfactory photoperiod. After light ether inhalation, general anesthesia was performed using ketamine hydrochloride, 30 mg/kg intraperitoneally. In order to accomplish 20%-third degree burn, hot boiling water (98°C) was applied on the back of the animals during a period of ten seconds.[8,15,16] Sham burned rats were exposed to room-temperature water.[10] For those rats which were subjected to burn injury, 4 ml physiological saline was applied intraperitoneally for immediate resuscitation.

Two series of experiments were established: In the first arm of the study (experiment 1), dorsal and abdominal regions of the animals were shaved and they were randomly allocated in 3 groups. In Group 1 (n=10) only a full-thickness midline skin incision, 2 cm in length was made on the abdominal region and primarily sutured with 4/0 polypropylene. In Group 2 (n=10) the animals were subjected to dorsal burn injury followed by abdominal skin incision. In Group 3 (n=10) dorsal sham burn and abdominal skin incision were made. Biopsy samples of burned skin with brown eschar were taken to confirm the depth of the burn on post-burn day 4.

In the second arm of the study (experiment 2), the rats were divided into 3 groups of ten in each and abdominal shaving was performed. Group 1 (n=10) served as control group and comprised same animals in experiment 1, with only skin incision on the abdomen. In Group 2 (n=10) and Group 3 (n=10), in addition to abdominal skin incision, either subcutaneous injection of melatonin (N-acetyl-5-methoxytryptamine, Sigma Chemical Co., St. Louis, MO) 10mg/kg body weight (BW) solvated in vehicle, or vehicle (ethyl alcohol 2% in physiological saline, in a dose of 5ml/kg) was administered respectively. Melatonin and vehicle injections were performed the following day after skin incision (day 1), in the morning between 8:00 am and 9:00 am. On day 2, melatonin and vehicle injections were repeated once in Groups 2 and 3 in the morning.

Follow-up procedures

The animals were reanesthetized with ether terminally and killed at the end of the experiment, 7 days after the incisional wounds were made. A rectangular tissue segment covering the incision was excised. Breaking strength (BS) (kg) values in this tissue segment were evaluated with a tensiometer (Ethicon®). The tissue was cut into two pieces for biochemical analysis.

Biochemical analysis

The biochemical specimen was examined by a blind investigator. Tissue samples were washed with physiological saline and stored at -70 °C until the measurements.

a) Hydroxyproline (HP)

HP levels (mg/mg dry weight) were carried out in dry tissue of the excised samples through the method described previously by Woessner.[17]

b) Thiobarbituric acid reactive substance (TBARS) level and antioxidant enzyme (AOE) activities

TBARS (nmol/mgprotein) was measured according to the double heating method of Draper and Hadley.[18] Determination of glutathione peroxidase (GSH-Px) activity (U/mgprotein) was performed with the method described by Paglia and Valentine.[19] Superoxide dismutase (SOD) activity (U/mgprotein) was detected by the degree of inhibition of the reaction which xanthine reacts with xanthine oxidase to generate superoxide radicals.[20] Catalase (CAT) activity (k/mgprotein) was measured by using the method of Aebi.[21] Abbott Aeroset (USA), an autoanalyser, was used to determine the concentration of protein and the activities of SOD and GSH-Px, while the spectrophotometer of Shimadzu UV-1601 (Japan) was used for measuring the rest of the parameters.

Microbiological assessment

Cultures of blood were taken into Brain Heart Infusion Broth in order to rule out blood stream infection. Cultures from the wound site were also prepared using 5% Blood Agar and Eosin Methylene Blue Media. Standard microbiological assessments were performed for wound site and blood cultures at the end of 48 hours and 7 days, respectively.

Statistical analysis

In statistical analysis, Kruskal-Wallis Analysis of Variance and Mann Whitney-U tests were used and non-parametric values were expressed as median (min-max). P values less than 0.05 were considered statistically significant.

RESULTS

None of the animals died during the experiment. Macroscopic examination of the wounds at day 7 was consistent with minimal wound dehiscence in 5 animals in the burn injury group and 2 in vehicle-treated group. In the histopathologic analysis of burned tissue, a third degree burn was determined. Microbiological assessment revealed no pathogenic bacteria neither in the wound site nor in blood cultures.

Table 1. Comparison of the burn groups with control in experiment 1. Values were expressed as median (min-max).

	Group I (control) n=10	Group II (burn injury) n=10	Group III (sham burn) n=10
Breaking strength (kg)	0.22 (0.08-0.25)	0.17 (0.09-0.26)	0.15 (0.12-0.3)
Hydroxyproline (mg/mg dry weight)	134 (71.8-193.6)	151.3 (95.8-204.7)	139.4 (74-180)
Superoxide dismutase (U/mgprotein)	0.62 (0.45-1.74)	0.49 (0.41-0.82)	0.59 (0.42-0.93)
Glutathione peroxidase (U/mgprotein)	0.44 (0.36-0.51)	0.42 (0.35-0.49)	0.45 (0.41-0.99)
Catalase (k/mgprotein)	1.10 (0.68-1.39)	0.93 (0.61-1.57)	0.84 (0.76-2.05)
Thiobarbituric acid reactive substance (nmol/mgprotein)	3.05 (2.03-16.20)	2.35 (1.65-10.18)	2.79 (1.54-15.99)

P value is not found significant in any pairwise comparisons versus control (Mann Whitney-U).

No difference was detected in any of the groups compared with the control, in means of BS, HP, TBARS levels and AOE activities, in the first arm of the study (Table 1). Same results were attained in experiment 2, except for the significantly elevated CAT and SOD activities in melatonin-treated animals comparing to the control group (Table 2).

Table 2. Breaking strength, hydroxyproline, thiobarbituric acid reactive substance values and antioxidant enzyme activities in melatonin and vehicle-treated groups in Experiment 2. Values were expressed as median (min-max).

	Group I (control) n=10	Group II (melatonin) n=10	Group III (vehicle) n=10
Breaking strength (kg)	0.22 (0.08-0.25)	0.12 (0.08-0.24)	0.17 (0.09-0.24)
Hydroxyproline (mg/mg dry weight)	134 (71.8-193.6)	140 (92.8-169.8)	160 (122.5-213.4)
Superoxide dismutase (U/mgprotein)	0.62 (0.45-1.74)	1.16 (0.64-3.10)*	0.81 (0.40-1.27)
Glutathione peroxidase (U/mgprotein)	0.44 (0.36-0.51)	0.45 (0.29-0.53)	0.44 (0.38-0.52)
Catalase (k/mgprotein)	1.10 (0.68-1.39)	1.32 (1.15-1.70)*	0.92 (0.79-1.35)
Thiobarbituric acid reactive substance (nmol/mgprotein)	3.05 (2.03-16.20)	5.12 (1.85-27.25)	2.91 (1.65-4.59)

*p<0.05 versus control (Mann Whitney-U)

DISCUSSION

Burn injury initiates inflammatory reactions resulting in complex metabolic aberrations in organs, relevant to wound healing. Skin is one of the target organs that is easily exposed to chemical or mechanical injury which promotes the production of ROS. Numerous studies support the role of ROS in post-burn systemic organ dysfunction.[7,8,12,22] Excessive generation of lipid peroxides results in diffusion to the other organs and causes tissue damage which could be reversed by antioxidants.[7,23]

There are several reports that verify experimental burn models involving different sizes. Cetinkale et al[8] reported that cell-mediated immunity was disturbed in rats exposed to 30% of the body surface area burn. The burn model involving 20% body surface was reported to avoid mortality and exclude additional problems that adversely affect the study.[15,16,24] Nevertheless, it was reported that 5% body surface area scald burn does not cause significant damage in the zone of stasis.[25]

Kawakami et al[26] suggested that 20% full-thickness burn increased interleukin-6 levels of the unburned skin in mice. Paradoxically, Gruber et al[9] proposed that thermal injury results in quantitative increase but qualitative impairment in oxidative capability of peripheral leukocytes. In the present study, it was aimed to facilitate impairment in wound healing, in unburned skin of the 20% burned

animals and assess the effects of melatonin on wound repair. Thus, a 2 cm midline skin incision was performed after burn injury. Minimal injury model was used because larger burned animals demonstrate unforeseen outcomes with unmanagable metabolic abnormalities and fatality whereas smaller burn injuries do not impair systemic functions.[10,26] Although half of the burned animals showed wound dehiscence in gross appearence, we could not demonstrate detrimental effects of 20% burn injury biochemically, on the unburned cutaneous incisional wounds compared with the control. In addition, the antioxidative defence system might not be directly affected by the systemic impairment which is expected to be created with burn injury, in this 20% burn model. Since the initial hypothesis in the present study is not supported by the results, further administration of melatonin has not been performed in the current experiment.

Dealing with the second arm of the study, there is increasing evidence that lipid peroxides are generated at the injury sites and impair wound healing due to cytotoxic effects.[4,22] The degree of oxidative injury depends on the balance between oxidant/antioxidant system. 27 In the studies by Foschi[3] and Hogstrom,[28] superoxide anion was found to inhibit the healing of the wound and this damage could be prevented by oxygen-free radical scavengers. Allopurino,[18,28] aprotinine[3], superoxide dismutase,[3,22] and catalase[8] have been tried in healing due to their antioxidant effects.

The pineal hormone, melatonin, is a potent scavenger of hydroxyl and peroxyl radicals and activates endogenous antioxidant defence potential.[5,13,14] Melatonin has also been reported to protect cells from neutrophil-induced cytotoxicity by oxygen-derived free radicals.[29] To our knowledge, few studies investigated the effects of melatonin on cutaneous wound healing and its role in collagen metabolism; this issue still remains a question.[30,31] Recently, it was reported that the interference of melatonin with arachidonic acid metabolism, might result in inhibition of 5-lipoxygenase activity and stimulation of prostaglandin E1 synthesis.[31] Dose-dependent inhibitory action of melatonin on wound repair was proposed by Drobnik et al[30] and melatonin was found to decrease collagen content in a wound model of sponge-induced granuloma. Another investigation of the same authors revealed that collagen accumulation in the intact skin is under the control of the pineal gland[32] However, it was also reported that the effect of melatonin depends on the time of the application, so that morning injections increase the level of collagen in a wound[33] Hence, in the current experiment, melatonin injections were administered in the morning, between 8:00 am and 9:00 am for regular collagen production during wound healing.

Previous studies showed that dosage and duration of the exogenous melatonin administration is variable and depends on the purpose of usage. Suggested dose required to exert antioxidant properties was considered 10mg/kg BW, in animal experiments.[5,14,34] Moreover, exogenous melatonin at a dose of 10mg/kg BW, was shown to have immunopotentiating effects after trauma-hemorrhage[35] Melatonin, 2.5mg/kg BW, was reported to produce protective effects in a rat model of spinal cord injury.[29] Although there is no previous evidence about the dose and duration of application in cutaneous wound healing, the present study indicates that treatment with exogenous melatonin 10mg/kg BW, once daily for two consecutive days, does not influence BS, HP and TBARS levels.

Melatonin is known to demonstrate its antioxidant effects by scavenging free radicals and stimulating the activity and synthesis of AOE. Antolin et al[36] investigated that melatonin increases mRNA levels of Mn-SOD and CuZn-SOD, which catalyse the reaction of superoxide to hydrogen peroxide. Moreover, melatonin was reported to increase the synthesis of GSH-Px,[34,37] the essential enzyme that modulates the reduction of hydrogen peroxide directly to water, lowering toxic hydroxyl radicals that will be generated.[5,14] In the current experiment, only SOD and CAT activities were elevated in melatonin-treated animals comparing to the control group, while GSH-Px activity was not significantly different. This might be attributed to the stimulating effect of melatonin on the activity of AOE. In contrast, while melatonin was expected to increase GSH-Px activity, we may suggest that this could be counterbalanced by the mediators secreted during wound healing.

It is well-known that scavengers should be delivered to the site of injury at therapeutic concentrations and their half-life should be long enough to exert potential benefit[7] Recent data indicated that scavengers are large molecules with short duration of activity and their penetration to the site of injury might have been limited[25] In addition, melatonin was found to reduce lipid peroxidation in a dose-dependent manner.[14,38] Although our results did not support any significant effect of melatonin with the dose and

duration given in the current experiment, it may be suggested that melatonin injection directly to the wound site for optimal concentration at the zone of injury needs to be investigated. Since the half-life of melatonin is proposed to be short,[39,40] it is possible that longer duration of administration can improve wound repair. This preliminary study should precede further studies performed with higher doses and longer duration of melatonin application to evaluate its effects on wound healing.

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