

## The effects of topical melatonin on oxidative stress, apoptosis signals, and p53 protein expression during cutaneous wound healing

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**Abstract:** Elimination of reactive oxygen species (ROS) can be an important strategy to improve healing of wounds. ROS have an effect on proliferation and cell survival signaling, which results in alteration of apoptotic pathways in cells. Melatonin has antioxidant properties on skin wounds. In our study, we investigated the effects of topical melatonin (3%, w/w) on apoptosis and p53 protein expression together with parameters of oxidative stress in a cutaneous excision wound model. Bcl-2 protein levels in wound tissue at the end of days 3, 7, and 14 were significantly increased, while caspase-3 activity and p53 protein expression in wound tissue at the end of days 3, 7, and 14 were also reduced with melatonin treatment during wound healing. On days 3 and 7 after the wound, malondialdehyde level was reduced and glutathione was increased with melatonin treatment. Melatonin decreased myeloperoxidase levels and increased hydroxyproline levels in wound tissue at the end of day 7. However, melatonin had no significant effect on percentage of wound closure. Considering our results, topical melatonin displays antioxidant, antiapoptotic, and p53-inhibitory effects, but these effects are not sufficient for the acceleration of wound closure.

**Key words:** Apoptosis, Bcl-2, p53, oxidative stress, topical melatonin, wound healing

### 1. Introduction

Wound healing is a complex and well-designed repair process that occurs after any injury, such as surgical procedures or trauma. The process is divided into three serial phases: inflammation, tissue formation, and tissue remodeling (Wu and Chen, 2014). Apoptosis is important to the wound healing process, especially in removing inflammatory cells and inhibiting scar formation. The early phase of inflammation is characterized by the invasion of neutrophils, macrophages, and lymphocytes to the wound area. The fibroblasts then migrate and synthesize extracellular matrix components. Inflammatory cells must be removed in order to begin this next phase of wound healing. Remodeling of granulation tissue during the wound healing process is also accompanied by the apoptosis of fibroblasts (Rai et al., 2005; Wu and Chen, 2014). Dysregulation in these apoptotic processes may result in abnormal wound healing, such as hypertrophic scars and keloid formation (Greenhalgh, 1998), or may delay wound healing (Deveci et al., 2005; Blakytyn and Jude, 2006). p53 is generally known as a tumor suppressor and can induce apoptosis via transcription-dependent or -independent mechanisms (Lippens et al., 2009).

Reactive oxygen species (ROS) that arise after cutaneous injury may worsen the healing process and induce apoptosis (Hameedaldeen et al., 2014). Low and normal levels of ROS play important roles in wound repair and signal transduction for reepithelialization and proliferation of cells, such as the collagenase activity and the epidermal growth factor signaling. However, higher levels of ROS can cause oxidative stress and may damage intracellular macromolecules such as DNA, lipids, and proteins. Therefore, regulating oxidative stress and the inflammatory response is important during the cutaneous wound healing process (Schäfer and Werner, 2008; Hameedaldeen et al., 2014). The utilization of antioxidants has been considered as an effective therapeutic approach in wound healing (James et al., 2001; Sharifi et al., 2012).

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone produced primarily by the pineal gland and secreted in the dark during night. It modulates sleep, reproduction, circadian rhythm, and immunity. Additionally, melatonin is a potent antioxidant and directly detoxifies oxygen and nitrogen-based reactants (Reiter et al., 2003; Sener et al., 2009). It affects the activity and the levels of cellular mRNA of antioxidant enzymes including

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superoxide dismutase, glutathione peroxidase, and glutathione reductase (Reiter et al., 2003). Melatonin may stimulate and regulate gene transcription of these enzymes via its receptors (Tomas-Zapico and Coto-Montes, 2005). Melatonin can cross all biological membranes, and thus it can indicate protective effects against oxidative stress. Melatonin reaches the nucleus of the cell and protects essential intracellular structures, including DNA, from oxidative damage (Fischer et al., 2008a). The human skin is capable of producing melatonin. Melatonin is involved in skin functions such as hair growth, pigmentation physiology, and control of melanoma. Melatonin receptors (MT1 and MT2) are expressed in different levels depending on cell types of skin and modified by environmental factors, such as, exposure to UV radiation (Slominski et al., 2005).

Novel treatment choices to modify the wound healing process are being investigated to provide suppression of inflammation and infection, promotion of the wound healing process, and prevention of scar formation. For the investigation of an appropriate therapy to cure wounds, the evaluation of various factors during the process of wound healing and their interactions with each other should be considered. To our knowledge, the effects of topical melatonin on apoptosis parameters and p53 protein expression have not been investigated in the cutaneous excision wound healing process. In the present study, the effect of topical melatonin on apoptosis (Bcl-2 and caspase-3) and p53 protein expression together with parameters of oxidative stress has been investigated in a cutaneous excision wound model.

## 2. Materials and methods

### 2.1. Animals

A total of 36 adult Sprague Dawley rats (16 weeks of age; 250–300 g body mass) of both sexes were obtained from the Marmara University Experimental Animal Implementation and Research Center (DEHAMER). The rats were maintained on a 12/12-h light/dark cycle and constant temperature ( $22 \pm 1$  °C). They were fed with standard rat chow and were allowed free access to water, but they were fasted during the 12 h before the experiment. All procedures for experimental protocols of the present study were approved by the Marmara School of Medicine Animal Care and Use Ethical Committee (protocol number: 14.2013).

### 2.2. Experimental groups

After wound formation, rats of either sex within each group were randomly divided into treatment ( $n = 6$ ) and control ( $n = 6$ ) groups and they were placed individually in separate cages. The study was carried out in three time periods: days 3, 7, and 14 after wounding.

### 2.3. Wound model and experimental protocol

Rats were anesthetized by intraperitoneal injection of ketamine at 10 mg/kg body weight. Hair of the dorsal surfaces of rats was trimmed with an electrical clipper before the formation of wounds and cleaned with cleansing antiseptic solution of chlorhexidine (5 mg/mL). Two circular wounds were established on the dorsal thoracic area of each rat by performing full-thickness excisions using a biopsy punch instrument with 0.6 mm in diameter. Wounds formation day was accepted as day 0.

Simple ointment (Basiscreme™, Genesis, Turkey) was applied to the control group and 3% (w/w) melatonin ointment was applied to the treatment group (~3 mg/wound), administered to each wound twice a day (Sener et al., 2006, Ozler et al., 2011) after wound creation during the experimental periods. Rats were sacrificed at the end of the experiment under anesthesia with ketamine and wound tissue samples were collected for the evaluation of oxidant/antioxidant status, collagen content, and apoptotic changing. The skin samples were homogenized five times at 8,000 rpm for 30 s in an MagNA Lyser homogenizer (Roche Diagnostics GmbH, Germany). Between each cycle the tubes were set on ice for 2 min.

### 2.4. Wound closure

Wound contraction is a centripetal movement of the wound edges towards the center. Sizes of wounds were measured on days 3, 7, and 14. The shape of each wound was drawn on transparent paper on days 3, 7, and 14, for which 1-mm<sup>2</sup> graph paper was placed to measure the wound surface area. The percentage of wound closure was calculated by using the following formula (Sharifi et al., 2012):

$$\% \text{ of wound closure} = (\text{initial wound size} - \text{specific day wound size}) / \text{initial wound size} \times 100.$$

### 2.5. Hydroxyproline assay

To determine collagen synthesis in wound tissues, hydroxyproline assay was performed according to a previously described method (Reddy and Enwemeka, 1996). Briefly, wound tissues were homogenized in saline and then were hydrolyzed with 2 N NaOH for 30 min at 120 °C. After hydrolyzation, samples were blended with buffer containing chloramine-T reagent and were oxidized for 20 min at room temperature. The addition of Ehrlich's reagent developed a reddish-purple chromophore complex and absorbance was measured at 550 nm with a spectrophotometer. Results were expressed as mg hydroxyproline/g tissue.

### 2.6. Malondialdehyde and reduced glutathione assays

Wound samples were homogenized with cold 150 mM KCl buffer in order to measure malondialdehyde (MDA) and reduced glutathione (GSH) levels. Tissue MDA levels were measured as thiobarbituric acid (TBA) reactive

substance formation determining lipid peroxidation as per a previously described method (Buege and Aust, 1978). Skin homogenates were treated with 1 mL of TBA reagent and incubated for 15 min in a boiling water bath and then cooled. The absorbances were measured at 530 nm. Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  and results are expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure (Beutler, 1975). Briefly, after the precipitation of protein and centrifugation at  $1000 \times g$  for 10 min, 0.5 mL of supernatant was added to 2 mL of 0.3 M phosphate buffer, and then 0.2 mL of a dithiobisnitrobenzoate solution (0.4 mg/mL) was added and the absorbance at 412 nm was measured. GSH levels were calculated using an extinction coefficient of  $13.600 \text{ M}^{-1} \text{ cm}^{-1}$ . Results are expressed as  $\mu\text{mol GSH/g tissue}$ .

### 2.7. Myeloperoxidase activity

Myeloperoxidase (MPO) is released by activated polymorphonuclear leukocytes (PMNs). To assess accumulation of tissue PMNs in inflamed tissues, MPO activity is frequently measured (Sener et al., 2006). The skin MPO levels were determined by the method of Hillegas et al. (1990). Wound samples were homogenized in 50 mM potassium phosphate buffer containing 0.5 % hexadecyl trimethyl ammonium bromide. Samples were frozen and thawed three times, processed with sonications (10 s, 25 °C), and centrifuged at  $41,400 \text{ g}$  for 10 min. Supernatant was mixed with reaction buffer containing o-dianisidine dihydrochloride and 20 mM hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) solution in 50 mM phosphate buffer. One unit of MPO activity was calculated as the change in absorbance at 460 nm for 1 min. Results were expressed as U/g tissue.

### 2.8. Caspase-3 activity

Determination of caspase-3 activity was performed using the caspase-3 cellular activity assay kit (Calbiochem, USA) according to the manufacturer's protocols. Wound samples were lysed with cold lysis buffer for 10 min. After the lysis samples were centrifuged at  $10,000 \times g$  for 10 min at 4 °C. Samples (40  $\mu\text{L}$ ) were added to plate wells, and then 50  $\mu\text{L}$  of reaction assay buffer was mixed into each well and incubated for 10 minutes at 37 °C. A final concentration of 200 mM acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) substrate was added and incubated for 1–2 h at 37 °C. The colorimetric release of p-nitroaniline (pNA) from the Ac-DEVD-pNA substrate was measured at 405 nm using a microplate reader (EPOCH, BioTek Instruments, Inc., USA). The results were expressed as nmol pNA/mg protein.

### 2.9. Western blot analysis for p53 and Bcl-2

The expressions of p53 and Bcl-2 protein were also measured by western blot. Samples' protein concentrations

were determined using the Bradford method (Bradford, 1976). Samples were boiled in 2X Laemmli buffer (Sigma, USA) and loaded on 12% SDS-PAGE. Nitrocellulose membrane was used for transferring to protein and membrane was blocked with 5% milk powder for 2 h at room temperature. The membrane was then incubated with primary antibodies (1:500 anti-p53, anti Bcl-2, and anti-b-actin; Santa Cruz Biotechnology, USA) overnight at 4 °C and horseradish peroxidase-conjugated secondary antibody (1:2500; Santa Cruz Biotechnology) was used at room temperature. The membrane was finally developed with ECL chemiluminescence reagent and exposed to film. The samples were quantified using Image J software (NIH, USA). Signals were normalized with respect to  $\beta$ -actin.

### 2.10. Statistical analysis

All data from the groups were recorded using GraphPad Prism 4.0 (GraphPad Software, USA). Two groups were compared with Mann-Whitney U tests. Results are expressed as means  $\pm$  standard deviation (SD). The differences were considered significant at  $P < 0.05$ .

## 3. Results

### 3.1. Wound closure and hydroxyproline formation

On days 3, 7, and 14, there was a difference in percentage of wound contraction between the control and melatonin groups (Figures 1A and 1B). These differences were not significant ( $P > 0.05$ ). After day 14 of injury, wound closure in the melatonin group was 98%. The control group had 94% wound closure. Melatonin did not significantly accelerate the wound closure rate in normal rats.

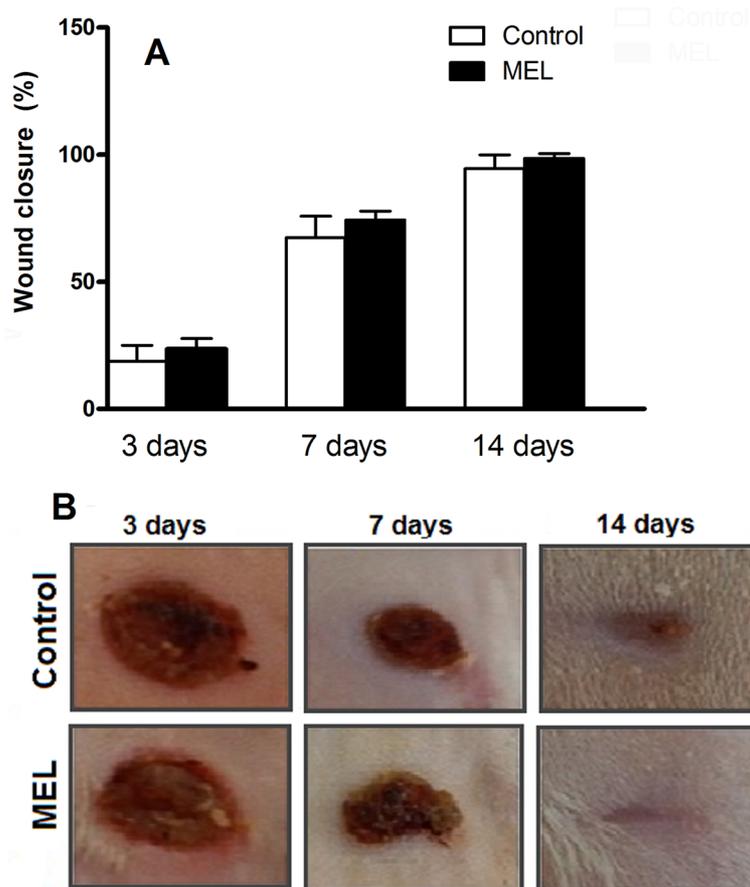
At the end of day 7, the hydroxyproline content in granulation tissue was significantly increased in the melatonin treatment group versus the control group ( $P < 0.05$ ) (Figure 2A). On days 3 and 14, no significant difference was detected in hydroxyproline levels between the two groups.

### 3.2. Oxidative stress markers

At the end of days 3 and 7, MDA levels were significantly reduced in the melatonin treatment group (Figure 2B,  $P < 0.05$ ). At the end of day 14, MDA levels of the melatonin-treated group were similar to those of the control group rats.

At the end of days 3 and 7, GSH levels were significantly increased in the melatonin treatment group (Figure 2C,  $P < 0.05$ ). At the end of day 14, GSH values in the melatonin group were higher than in the control group, but this increase was not statistically significant ( $P > 0.05$ ).

Melatonin also significantly reduced MPO levels compared to the control group on day 7 (Figure 2D,  $P < 0.05$ ). At the end of day 14, MPO levels of the melatonin group were near the control levels.



**Figure 1.** (A) The percent of wound closure in control and melatonin groups (MEL) at different days. (B) Photographic representation of closure rate in control and melatonin groups in different time periods.

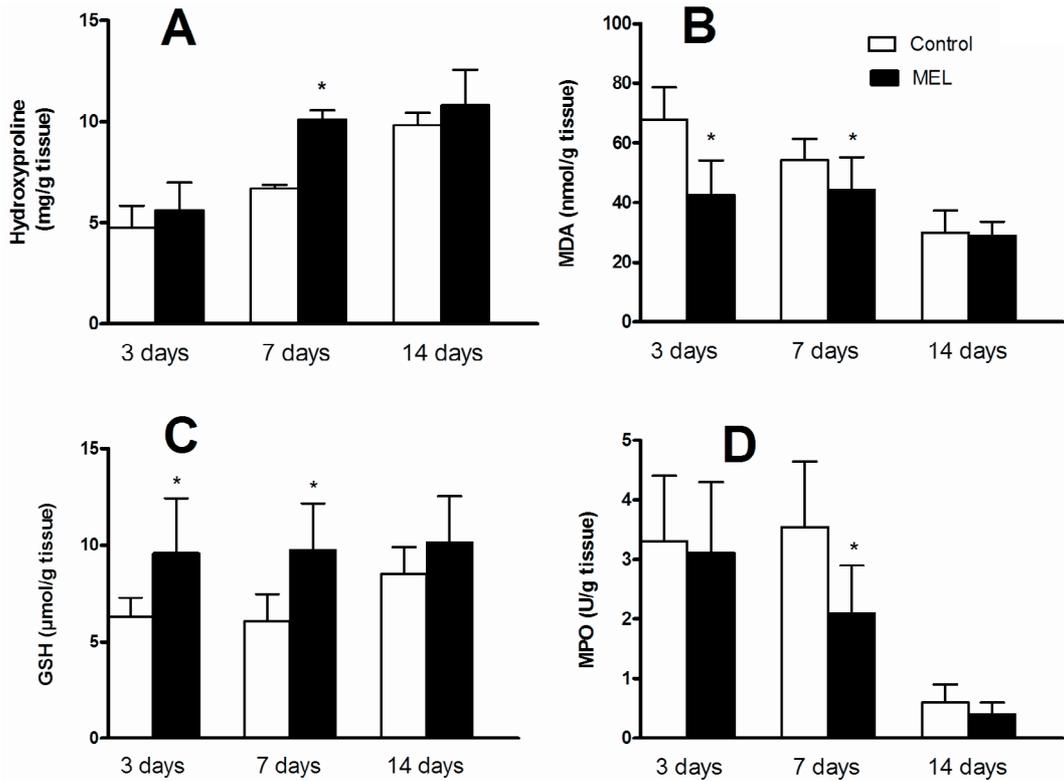
### 3.3. Apoptosis markers and p53 protein expression

The level of apoptosis in the wound tissue of control rats was greater than that in the wound tissue of melatonin-treated rats. Topical melatonin decreased the level of apoptosis after skin injury (Figure 3). As shown in Figure 3A, caspase-3 activity was significantly reduced on days 3 and 7 in the group treated with melatonin ( $P < 0.05$  and  $P < 0.001$ , respectively). At the end of day 14, caspase-3 activity measurements of the melatonin group were still lower than those of control rats ( $P < 0.001$ ). Bcl-2 and p53 protein expression were determined by western blotting. Figure 3B represents the western blot images of control and melatonin groups. The expression of Bcl-2 protein was significantly upregulated at the end of day 3 and 7 (Figure 3C), whereas the expression of p53 protein was significantly downregulated at the end of days 3, 7, and 14 in the melatonin-treated wounds when compared with those in the control wounds (Figure 3D). On days 3, 7, and 14, Bcl-2/p53 was  $0.96 \pm 0.02$ ,  $0.56 \pm 0.03$ , and  $1.03 \pm 0.13$  in the control group and  $2.04 \pm 0.03$ ,  $2.84 \pm 0.3$ , and  $1.67$

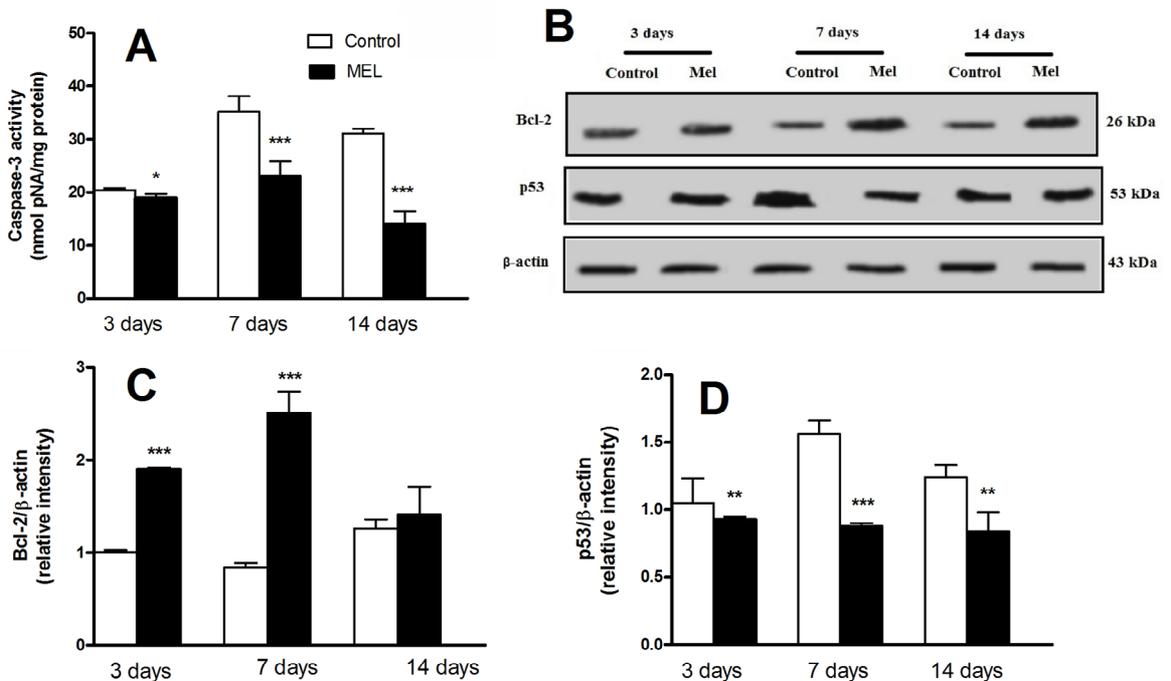
$\pm 0.47$  in the treatment group (data not shown in figures). According to these results, Bcl-2/p53 was markedly increased on day 7 after the administration of melatonin.

### 4. Discussion

Topical administration of melatonin in wound healing has been investigated in different wound models (Drobnik, 2012). Most studies pointed out that the effect of melatonin on wound healing was dependent on the type of wound model and the route, dosage, and time of administration (Drobnik and Dabrowski, 1996; Drobnik, 2012). Pinealectomy, which decreases melatonin level in the blood, has been found to reduce the surface area of the wound and accelerate wound repair (Drobnik and Dabrowski, 1996). Bulbulla et al. (2005) observed that subcutaneous melatonin application decreased collagen synthesis and epithelium proliferation and indicated undesirable effects on incision and anastomotic wound healing in normal and pinealectomized rats. However, positive improvement in the wound healing process



**Figure 2.** (A) Hydroxyproline, (B) malondialdehyde (MDA), (C) reduced glutathione (GSH) levels, and (D) myeloperoxidase (MPO) activity in the wounds of control and melatonin (MEL)-treated rats. Results are presented as mean ± SD. \*: P < 0.05 versus control group.



**Figure 3.** (A) Caspase-3 activity in the wound tissue of control and melatonin-treated rats. (B) Representative western blot images of groups. (C) Expressions of Bcl-2 and (D) p53 protein in the wounded tissue of control and melatonin (MEL)-treated rats. \*: P < 0.05, \*\*: P < 0.01, \*\*\*: P < 0.001 versus control groups.

has been detected due to topically and systematically administered melatonin on chronic wound healing in rats with pinealectomy (Ozler et al., 2010) and also on pressure ulcer in rats (Sener et al., 2006).

Melatonin has oncostatic and antimetabolic properties (Viswanathan and Schernhammer, 2009) and systemic administration of melatonin may affect many tissues. However, melatonin's limited access to skin would be expected after systematic administration of melatonin. Melatonin with topical application can penetrate into the stratum corneum, where it accumulates because of its marked lipophilic chemical structure (Tomas-Zapico and Coto-Montes, 2005). Therefore, in the present study, melatonin was administered topically two times daily (morning and evening). In our study, although topical melatonin increased hydroxyproline content on day 7 in skin, when compared with the control and treatment group on day 14, a similar wound closure rate was detected in both groups. Melatonin did not delay wound healing at day 14, but on the other hand, it did not markedly provide acceleration in wound closure, either.

Management of inflammation and oxidation is crucial during the process of wound healing. Although ROS in low and normal levels can be involved in regulation of repair, excessive formation of ROS may lead to postponed wound healing by the means of destruction of lipids, proteins, and extracellular matrix elements (Hameedaldeen et al., 2014). The positive effect of antioxidants on cutaneous wound healing via inhibition of ROS has been presented in several studies (James et al., 2001; Reiter et al., 2003; Sharifi et al., 2012). In the present study, when compared with control group, topical administration of melatonin provided suppression of MDA levels in injured skin while increasing GSH levels.

Endogenous melatonin may have antiinflammatory effects (Cuzzocrea et al., 1999). In our study, skin injury caused increase in MPO activity, an index of PMN accumulation, while treatment with topical melatonin caused reductions in MPO activity on day 7. Increased MPO activity in wound tissue suggests neutrophil accumulation and contributes markedly to tissue damage (Sener et al., 2006). Ozler et al. (2011) showed that topically applied melatonin (5 mg/kg) in ischemic wounds had neither a positive nor a negative effect on hydroxyproline levels. However, it showed antioxidant efficacy through decreasing MDA levels (Ozler et al., 2011). Although melatonin was administered at an amount of 3 mg per wound in the present study, in the study conducted by Ozler et al. (2011), melatonin was administered at an amount of 0.1 mg per wound. Similar to our results, topical administration of melatonin (5 mg melatonin per rat and twice a day) decreased both MDA and MPO level in injured skin in rats with pressure ulcers (Sener et al.,

2006). According to results shown in the present study, topically administered melatonin could suppress oxidative stress and inflammation in the wound tissue.

In the present study, caspase-3 activation and Bcl-2 and p53 protein expression were detected as apoptotic markers. Apoptosis occurs through several independent pathways that are initiated by diverse extracellular (via death receptor) and intracellular factors. The intrinsic pathway centers on the mitochondria, which contain key apoptotic factors. One of the major regulators of the intrinsic pathway is the pro- and antideath member, the Bcl-2 family. Bcl-2 regulates mitochondrial physiology and caspase activation (Lippens et al., 2009). Caspases are a family of cysteine proteases that play essential roles in apoptosis. Caspase-3 is a key factor in apoptosis execution and a point of junction for the intrinsic and extrinsic apoptotic pathways (Fan et al., 2005). p53 acts to integrate multiple stress signals into a series of diverse antiproliferative responses and can induce apoptosis. Cytoplasmic p53 can bind directly to several antiapoptotic members of the Bcl-2 superfamily by neutralizing their antiapoptotic activity (Lippens et al., 2009).

The increase in ROS would also induce apoptotic processes during wound healing. It was stated that enhancement in apoptosis in injured skin would also explain the problems seen during wound healing in diabetes mellitus (Blakytyn and Jude, 2006). Devenci et al. (2005) observed that augmentation in oxidative stress would also increase apoptosis of keratinocytes when cultured in hyperglycemic media, indicating delayed wound healing when compared to normoglycemic media.

It has been shown that melatonin has differential actions on apoptosis in normal and cancer cells (Sainz et al., 2003). In most studies, it was shown that melatonin possessed antiapoptotic effects on normal skin cells. However, other studies focused on isolated cell populations and UV or thermal-induced skin injury (Fischer et al., 2008b; Kim et al., 2001). In one in vivo study, melatonin maintained muscle restoration after muscle injury and inhibited apoptosis through regulation of apoptosis-related signaling pathways (Stratos et al., 2012). Studies on Bcl-2 and p53 protein expression in wounded tissue were observed and it was found that there was a negative relation between levels of Bcl-2 and p53 protein in normal wound healing processes. Nagata et al. (1999) followed apoptosis and p53 protein expression in full-thickness burn wounds of guinea pigs from 0.5 to 28 days. It was observed that apoptosis and p53 protein expression rose in both the inflammatory (0.5–2 days) and proliferation (2–14 days) stages of wound healing, but apoptosis and p53 protein expression diminished during the remodeling stage (14–28 days). Kane and Greenhalgh (2000) also observed that

there was an increase in Bcl-2 and a decrease in p53, which allows cellular proliferation for tissue repair after injury. As time passes, Bcl-2 levels decrease while p53 levels increase to end the inflammatory process and downregulate the proliferative response. According to researchers, in diabetic mice, this negative relationship between Bcl-2 and p53 may be lost. However, it was reported that inhibition of p53 would also improve wound healing. Vollmar et al. (2002) demonstrated that pifithrin- $\alpha$  (PFT- $\alpha$ ) could transiently inhibit p53. This results in acceleration of early epithelization and neovascularization during cutaneous wound healing by promoting leukocytes, increasing cell proliferation, and reducing apoptotic cell death.

Similar to previous studies, our study also observed that the Bcl-2 protein expression in the control group had a negative relation with p53 protein expression. After injury, the expression of p53 protein increased while the expression of Bcl-2 reduced on day 7. The expression of Bcl-2 increased on day 14 when compared to Bcl-2 levels on day 7. Adherence to inverse association between levels of Bcl-2 and p53 protein was also seen on day 14, and decrease in levels of p53 protein was observed when compared with p53 protein levels on day 7.

Melatonin markedly reduced the expression of p53 protein when compared with the control group, and the expression of p53 protein levels remained close to each other in the melatonin group on days 3, 7, and 14. In this way, melatonin caused deterioration of the negative relationship observed between Bcl-2 and p53 during the normal wound healing process. Increase in ROS can also cause downregulation of Bcl-2 (Chang et

al., 2002). According to results obtained in the present study, melatonin detoxified ROS and inhibited apoptosis by reversing the decline in oxidative stress induced Bcl-2 levels. In the present study, after injury occurred, activation of caspase-3 was increased on day 7. However, activation of caspase-3 was decreased on day 14. Melatonin caused the upregulation of Bcl-2 protein on days 3 and 7. Melatonin also markedly inhibited the activation of caspase-3. This decrease in activation of caspase-3 could be explained by upregulation of Bcl-2 by melatonin. It has been also shown that melatonin suppresses caspase-3 activity via Bcl-2 induction in several cell types (Yoo et al., 2002; Baydas et al., 2005).

In conclusion, the evidence of apoptosis was decreased by melatonin via the downregulation of expression of p53 and detoxification of ROS in injured skin tissue. Although effects such as antiapoptosis and inhibition of p53 of melatonin were demonstrated in the results of the present study, melatonin could not significantly accelerate wound closure in our study conditions. Further studies should be planned to define the associations among the suppression of p53 expression, the reduction of apoptotic cell death, and wound closure with topical administration of melatonin.

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