Wound healing and the effect of pineal gland and melatonin

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Abstract

Wound healing is a complex phenomenon that is controlled by local and general regulatory mechanisms. The aim of the paper is to analyze recently-published data devoted to the regulation of wound repair by melatonin.

The effect of melatonin has been reported in different wound types healed with various mechanisms. The action of the pineal indoleamine is dependent on the used dose, time of application and target organ. Moreover, melatonin influences different phases of wound repair such as inflammation, by regulating the release of inflammatory mediators, cell proliferation and migration, by influencing angiogenesis, and the proliferation of fibroblasts, as well as the synthesis phase, by regulating collagen and glycosaminoglycan accumulation in the wounded milieu. Thus, healing of the skin wound, myocardial infarction, bone fractures and gastric ulcer is influenced by melatonin.

In patients with low levels of melatonin (elderly or β-blocker treated patients), its regulatory effects are expected to be impaired. Thus, the need for melatonin supplementation in those patients is postulated in the study.

Key words:
Angiogenesis; Collagen; Fibroblasts; Glycosaminoglycans; Inflammation; Melatonin

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Introduction

Wound repair incorporates mechanisms that have evolved during million of years to re-establish the homeostasis which was unbalanced by the injury of the tissue. A wound is a complex system that consists of many interdependent components. They comprise cells (myofibroblasts, inflammatory cells, epithelial cells, endothelial cells), intercellular transmitters (hormones, cytokines, mediators of inflammation), synthetic products (collagen, proteoglycans), enzymes and their inhibitors (metalloproteinases, tissue inhibitor of metalloproteinase). The complexity of the wound requires the system governing of the healing processes to work in accord with all components. The system transducing the information consists of the intracellular signaling, intercellular local factors and the systemic reaction (neuroendocrine, immune response) [1]. Moreover, the environmental stimuli may influence the repair process.

Healing

Repair is composed of five overlapping phases: hemostasis, inflammation, cell migration and proliferation, synthesis of protein, scar contraction and remodeling.

Hemostasis comprises blood vessel contraction, platelet adhesion, aggregation and fibrin polymerization. Aggregating platelets secrete the contents of their granules: platelet-derived growth factor (PDGF), α- and β-transforming growth factors (TGF-α, TGF-β) and fibroblast growth factor 2 (FGF-2). These growth factors released by platelets stimulate the healing process. PDGF is the chemotactic factor for neutrophiles, monocytes and fibroblasts as well as an agent which stimulates the extracellular matrix synthesis [2]. TGF-β stimulates collagen synthesis, collagenase secretion, angiogenesis, fibroblast proliferation and differentiation of fibroblasts into myofibroblasts [3].

Polymerization of fibrin leads to formation of clots, the porous gel-like meshwork that contains trapped cells and proteins (fibronectin). Fibronectin is attached to the fibrin fibres of the clot [4]. One or two days after wounding, the hialuronic acid synthesized in the wound by trapped blood cells is attached to the fibrin gel and stabilizes its physical structure. The fibrin–fibronectin meshwork stabilizes the platelet plug, serves as a provisional matrix for the wound and a scaffold for migration of cells, as well as binding circulating cytokines [5]. The enzymes secreted by migrating cells are able to modify the provisional matrix.
Inflammation is intended to facilitate delivery of the inflammatory cells to the site of injury. The inflammatory cells remove infectious factors, cell debris and damaged matrix components from the wounded milieu. Moreover, they are the source of the cytokines regulating the repair processes. Neutrophils enter the wound within 24 hours after injury, and after 48 hours, an influx of monocytes to the wound is observed. The cells are transformed into macrophages that release growth factors (PDGF, TGF-β). Later, T-lymphocytes enter the wound and their concentration peaks from the 5th to 7th days after injury. Both the macrophages and T-lymphocytes play a role in regulation of the healing process.

Inflammatory mediators released from the mast cells accelerate the healing process. Disodium cromoglycate, the mast cell stabilizer, is responsible for inhibition of the superficial wound healing as well as decreasing the collagen content of the wound [6]. Histamine however, accelerates healing and increases collagen and glycosaminoglycan content in the granulation tissue [7].

Toll-like receptors (TLR) recognize the molecular patterns associated with pathogens. However, even in the absence of microbial pathogens, the TLR could be activated by the parts of the extracellular matrix damaged during injury [8]. Binding to the TLR by a ligand is responsible for activation of the innate immune system. Both suppression of the inflammatory reaction and reduction of the infarct size has been observed in TLR4-deficient mice [9].

The complement cascade is activated during the inflammatory phase of the repair process of the peripheral wounds [10] and myocardial infarction [11]. The complement factors of C3a and C5a are chemotactic compounds for neutrophils and monocytes.

Reactive oxygen species (ROS) can participate in regulation of repair, however, excessive production of ROS inhibits healing. In the skin wounds, ROS are secreted by leukocytes migrating to the wound, as well as injured epithelial cells that generate a hydrogen peroxide gradient in the margins of the wounded tissue. This gradient attracts migrating leukocytes to the healing milieu [12]. ROS are essential in the killing of phagocytosed microbes and trigger the cytokine cascade through activation of the nuclear factor-κB (NF-κB) [13]. Hydrogen peroxide induces re-epithelialization, stimulates synthesis of collagen types I, III, IV and influences the conversion of fibroblasts into myofibroblasts [14]. However, excessive oxidative stress exerts damaging effects on the wound; it induces fibroblast senescence and impairs its contraction [15]. Some beneficial results of antioxidant intervention have been described in wound healing [15]. In addition, antioxidant therapy reduces the size of the myocardial infarction [16].
The phase of **cell migration and proliferation** is composed of three main processes: Influx to the wound by fibroblasts responsible for the synthesis of the extracellular matrix, re-epithelialization and angiogenesis. Fibroblasts from the wound margin enter the wounded milieu. Furthermore, transformation of undifferentiated cells into fibroblasts is also possible in the wound. Following this, differentiation of the fibroblasts into myofibroblasts is induced by TGF-β1 [17, 18]. The myofibroblasts are found in the skin wound from 4th to 6th day after injury, but after 2-3 weeks these cells are eliminated by apoptosis. In the scar of myocardial infarction, the myofibroblasts have been seen several months after infarction in rats, and even 19 years after coronary artery occlusion in humans. The myofibroblasts contain peripheral myofilaments organized parallel to the cell membrane, and positively stain for vimentin and actin [19]. Myofibroblasts synthesize more collagen in comparison with fibroblasts [17] and are responsible for contraction of the wound [18].

During intensive healing the oxygen pressure is lowered in the wounded milieu. These conditions activate hypoxia inducible factor (HIF) expression. HIF is the transcription factor regulating the expression of vascular endothelial cell growth factor (VEGF), which stimulates angiogenesis. An elevated oxygen level in the wound blocks activity of HIF and reduces synthesis of VEGF [20].

Reepithelialization involves migration of the basal cells from the margins of the wound to the site of injury. The migration of epithelial cells continues until the cells form a monolayer surface covering the wound. The migration is stopped by contact inhibition.

**Figure 3.** Cell migration and proliferation phase is consisted of fibroblast migration into the wound, reepithelialization and angiogenesis.

**Protein synthesis and wound contraction:** collagen is synthesized in the wound by fibroblasts and myofibroblasts. Collagen types I and III predominate in the granulation tissue. The collagen synthesis starts on the 4-5th days after injury and maximal rate of collagen production is observed for 2 or 4 weeks of healing. Comparing with the intact skin, an excess of collagen type III is produced in granulation tissue at the beginning of the repair process. In the myocardial infarction scar, the highest amount of type I collagen was observed between the 21th and 28th days after infarction but a peak of type III collagen content was seen between the 11th and 28th days of healing [21]. Non-fibrillar type IV collagen forms the basement membranes in the wound. The synthesis of collagen comprises intracellular and extracellular phases, leading to fibril formation in the extracellular space. Its key-regulatory synthetic step is the proline and lysine hydroxylation phase, which requires enzymes.
(prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl hydroxylase) and cofactors (oxygen, vitamin C, ferrous ion and α-ketoglutarate).

Hydroxylation of the collagen defines the crosslinking of its fibres and thus, the tensile strength of the collagen molecules and scar. Hydroxyproline is a marker used in methods assessing the quantity of collagen [22]. Maturation of the collagen is related to cross-link formation. During this process, the solubility of the collagen in neutral salt solutions or diluted acid solution decreases. Formation of cross-links involves oxidative desamination of the ε-amino groups in lysine or hydroxylysine. This reaction is catalyzed by lysyl oxidase. During maturation of the collagen fibres the increased number of cross-links runs parallel with elevation of the tensile strength of the tissue.

Fibroblasts or myofibroblasts within granulation tissue synthesize proteoglycans that are composed of a protein core with attached glycosaminoglycans (GAG). The concentration of GAG in the peripheral wound increases from day 3, reaches a peak on the 4th to 8th week after injury and then gradually decreases until the 24th week after wounding [23]. In a myocardial infarction scar, the GAG level increases to a peak in the 6th week after myocardial infarction and then decreases to the 12th week after coronary artery occlusion [24]. The meaning of the transient elevation of GAG in the wound is not known. GAG regulate the water/electrolyte balance in the extracellular space, determine the action of growth factors [25] and enzymes and possibly also exert an influence on gene expression [26]. Moreover, GAG bind collagen by electrostatic forces under physiological conditions. Collagen fibrillogenesis is also regulated by proteoglycans [27].

Wound contraction is responsible for the reduction of its volume. The margins of the wound are drawn closer to each other. Gabbiani suggests that myofibroblasts anchored to the collagen net are the motor of contraction. The force of contracting myofibroblasts is transduced through the collagen net linked to the margins of the wounds [28].

Remodeling of the wound: in the remodeling process, the two processes of collagen destruction and synthesis run parallel to each other. The collagen fibers are cleaved by metalloproteinase (MMP). The activity of MMP is regulated by the secretion of the tissue inhibitors of metalloproteinase (TIMP) [29]. In the mature scar, the collagen fibres are thicker, better organized and crosslinked and the tensile strength of the scar increases. The excess of type III collagen is reduced. As the maturation of the scar progresses, the number of cells is lowered due to apoptosis [30], and the water content and proteoglycan level are reduced.

Regulation of the wound healing

The regulatory mechanisms of repair comprise the endocrine, nervous and immune systems.

Endocrine regulation: glucocorticoids retard the healing process by inhibiting fibroblast proliferation as well as by slowing down collagen synthesis. These hormones influence collagen gene expression by decreasing the mRNA level of type I procollagen α1 and α2 chains. Glucocorticoids increase degradation of collagen mRNA by controlling latent RNase. The degradation is specific for the collagen mRNA [31]. Moreover,
glucocorticoids inhibit HIF-1 synthesis, wound contraction and exert an antiinflammatory effect.

Impairment of the healing process related to the age of patients is reversed by estrogen application [32]. Estrogens stimulate fibroblast migration directly by increasing PDGF secretion, the main mitogen for fibroblast, as well as increase collagen deposition in the wound. Moreover, estrogens decrease L-selectin expression, reduce both excessive neutrophil migration to the wound and elastase secretion. Estrogens are involved in regulation of several gene expressions important in matrix production, protease inhibition and epidermal function. Conversely, testosterone inhibits repair; an androgen receptor blockade or castration has been found to encourage healing [32].

Impairment of a healing process in diabetes is related to hypoxia as well as formation of advanced glycation end-products. Moreover, inadequate angiogenesis and increased protease activity have been found in diabetic wounds [33]. Reduced collagen solubility and its increased stability have been observed in experimental diabetes. Biomechanical studies have suggested an augmented number of cross-links in the collagen by reporting increased stiffness and strength of the skin.

Growth hormone is widely accepted an anabolic agent that increases the synthesis of collagen. Augmented incorporation of proline by granulation tissue under the influence of growth hormone has been found. Moreover, application of the growth hormone increases collagen deposition in the wound and elevates the biomechanical strength of the scar [34]. Neuropeptide growth hormone-releasing hormone (GHRH) was found to stimulate dermal wound healing and to accelerate proliferation of the skin-associated fibroblasts [35]. The beneficial effect of triiodothyronine on wound healing has been confirmed [36].

Synthesis of collagen by cardiac fibroblasts in culture is augmented by angiotensin II. This effect is dependent on the stimulation of AT₁ receptors [37]. Both fibroblast proliferation and collagen synthesis in the myocardial infarction scar is also enhanced by endothelin. A blockade of endothelin receptor A (ETₐ) resulted in decreased procollagen α₁(I) and procollagen α₁(III) gene expression in the myocardial infarction. This effect was accompanied by inhibition of the TGF-β₁ gene expression. The blockade of the ETₐ receptor was linked with enhanced expansion of myocardial infarction [38].

The nervous system is involved in the regulation of inflammation as well as wound healing. Several neuropeptides involved in repair of the wounds are released from the nerve endings. Substance P, secreted by sensory neurons, stimulates leukocyte migration and is involved in macrophage activation. An acceleration of wound healing after administration of substance P to diabetic mice has been observed [39]. Administration of neuropeptide Y (NPY) accelerates healing when retardation of the wound repair was reported in NPY knockout mice [40]. Calcitonin gene-related peptide (CGRP) is known as the factor regulating both inflammation and angiogenesis. In CGRP knockout animals, delayed wound repair was accompanied by a low level of VEGF and reduced angiogenesis [41]. In the diabetic mice, application of the nervous growth factor (NGF) accelerates epithelialization and improves nerve regeneration [42]. Moreover, NGF is the chemotactic compound for keratinocytes, fibroblasts and inflammatory cells.

The immune system is involved in the regulation of the healing process. Activated macrophages in the wound secrete several molecules: PDGF, TNF-α, IL-1, and IL-6. These cytokines induce release of the keratinocyte growth factor (KGF) by fibroblasts, which influences re-epithelialization. Moreover, macrophages secrete a family of three cytokines: TGF-β₁, TGF-β₂ are involved in scar formation, while a high concentration of TGF-β₃ is related to scarless repair in the fetal wounds. TGF-β₁ is the chemoattractant molecule for the fibroblasts and induces secretion by their connective tissue growth factor (CTGF) that accelerates proliferation of the cells in an autocrine manner. Moreover, TGF-β₁ induces transformation of the fibroblasts into myofibroblasts [17]. Macrophages secreting both metalloproteinase and TGF-β are stated to be potent regulators of the extracellular matrix composition [43]. Application of antimacrophage sera caused depletion of macrophage number in the wound, lowered fibrosis and decreased number of fibroblasts in the wounded milieu [44].

T lymphocytes were found to play a regulatory role in the wound. They are the source of cytokines (IL-1, IL-2, TNF-α, EGF, and TGF-β) regulating the healing process. The γδ-T cells are the subset of lymphocytes expressing the γδ receptor for antigens. The removal of γδ-T cells from the mouse wound results in inhibition of the healing of the wound as well as decreased number of the migrating macrophages in the wounded milieu. The γδ-T cells synthesize TNF-α and express KGF-1 and KGF-2 genes [45].
The pineal gland

Light dark variations are translated in the pineal gland into chemical signals. Thus in the dark phase, the pineal gland secretes melatonin, which is known as the “chemical expression of darkness” [46]. At night, the level of melatonin ranges from 100-400 pM. Light blocks the secretion of the pineal indoleamine and during the day, the pineal indoleamine level decreases below 30 pM. The main steps of melatonin synthesis comprise acetylation of serotonin by arylalkylamine N-acetyltransferase (NAT) and then a methyl group is transferred to N-acetylsertotonin by the enzyme hydroxyindole-O-methyltransferase (HIOMT) [47, 48]. Regulation of the enzymes synthesizing melatonin determines the rhythm of pineal indoleamine secretion [47]. Due to its lipophylic properties, melatonin is able to passively diffuse through the cell membrane. The half-life of melatonin is 30 min. Melatonin is eliminated from the blood mainly by the liver through a process of pineal indoleamine metabolization to 6-hydroxy-melatonin and than excretion in the urine [49].

Two mammalian melatonin receptors, MT1 and MT2, have been described. Both MT1 and MT2 receptors are linked with a G protein. Stimulation of MT1 inhibits the forskolin-induced cAMP synthesis [50]. Activation of MT2 receptor inhibits both cAMP and cGMP formation, and stimulates protein kinase C (PKC) activity [51]. The involvement of retinoid nuclear receptors in the mediation of melatonin action suggested by some authors [52] is still controversial. Calmodulin is considered as the target for melatonin and is supposed to be the intracellular receptor for the pineal indoleamine. The binding of melatonin to calmodulin is responsible for inhibition of the neuronal nitric oxide synthase in the brain of rats [53].

Since melatonin is a free radical scavenger and a stimulator of antioxidative enzyme activity [54-56], it is known to protect tissues from injuries caused by ROS. Examples of ischemia/reperfusion injury [57] and ionizing radiation damage [58] which have been reduced by melatonin have been described. Melatonin also influences the function of the nervous, immune and endocrine systems and, in this way, is involved in the coordination and transfer of the information in the body.

Melatonin effect on collagen content in the wound

Excessive fibrosis of the retroperitoneal space was observed by Cunnane and coworkers in pinealectomised rats [59]. This result was supported by studies showing methysergide-induced fibrosis in humans [60, 61]. Methysergide is known as an antagonist of serotonin, which is the substrate for melatonin synthesis. Thus, retroperitoneal fibrosis, as well as heart and pulmonary fibrosis, in patients treated with methysergide were supposed to be effects of melatonin synthesis inhibition [59]. Furthermore, inhibition of formation of prostaglandin E1 (PGE1) has been found in pinealectomised rats. PGE1 is known as an inhibitor of collagen synthesis [62]. All the above data supports the hypothesis regarding pineal gland involvement in fibrosis regulation formulated by Cunnane et al [59].

Evening injections of melatonin retards the repair of full-thickness circular skin wounds and increases the wound surface area compared with a control [63]. Thus, all applied doses ranging from 10 µg/100 g to 100 µg/100 g body weight (b.w) increased wound surface area from 7th to 14th days after injury. On the 14th day after wounding, while the healing of the control rats was almost completed, the wound surface area was still high in the melatonin-treated animals. Pinealectomy, however, has the opposite effect to melatonin and has been found to reduce the surface area of the wound, accelerating repair [63]. A pinealectomy essentially reduces melatonin level in the blood. To study the contents of the extracellular matrix in the wounded tissue, sponges were implanted subcutaneously in the lumbar region of rats. Evening application of melatonin (30 µg/100 g b.w) reduced the level of collagen in the granulation tissue of the sponge, whereas a pinealectomy expressed the opposite effect to melatonin and elevated the collagen content [63-65]. Moreover, a 30 µg/100 g b.w dose of melatonin administered to the pinealectomised rats reversed the effect of the pinealectomy and normalized collagen content in the granulation tissue.

Additionally, reduction of collagen level and inhibition of full-thickness circular wound healing was observed in rats kept in continuous darkness [63]. In the continuous darkness, the rhythm of melatonin secretion persists. However, an increasing background level of melatonin was found [63]. Melatonin was proved to have no effect on the unpolymerised collagen (salt soluble) level in the granulation tissue [65].

The final effect of melatonin is dependent on the time of the indoleamine application. Thus, a morning injection of melatonin increases level of collagen in the wound, while evening administration of the pineal indoleamine lowers it...
[66]. This experiment suggests that the tissue response to melatonin varies during the day.

Bulbuller and coworkers [67] showed that evening melatonin application (30 µg/100 g b.w) decreased hydroxyproline level in intestine anastomotic wounds in rats. This result was linked with reduced mechanical resistance of the intestinal wounds (bursting pressure). Pinealectomy, however, increased the level of hydroxyproline, but melatonin applied to the pinealectomized rats reversed the effect of pineal gland removal and lowered both the level of hydroxyproline in the wound as well as the bursting pressure. In the incisional skin wound of the same experimental model, melatonin decreased hydroxyproline content in the scar and lowered the breaking strength of the wound. Pinealectomy, oppositely to melatonin, elevated hydroxyproline level in the incisional wound. This effect was accompanied by increase of the breaking strength. Application of melatonin (30 µg/100 g b.w) to the pinealectomised rats lowered hydroxyproline level and decreased the breaking strength of the incisional wounds. Moreover, histopathological examination showed retardation of the wound epithelization by melatonin in both intact and pinealectomised rats. Pinealectomy, however, improved epithelization in incisional skin wounds [67]. In an ischemic wound model in rats, melatonin (100 µg/100 g b.w) did not modify hydroxyproline content in the wounded milieu [68]. The hydroxyproline concentration in a dorsal burn injury followed by full thickness midline skin incision was not influenced by melatonin at a dose of 10 mg/kg b.w applied for two days [69].

A dimethylnitrosamine injury of the liver occurs, followed by fibrosis, when melatonin at a dose of 100 mg/kg reduces the hydroxyproline level in the liver [70]. Moreover, melatonin has also been found to reduce fibrosis, as measured by the hydroxyproline content of the livers of rats with with ethanol-induced injury. [71].

Pugazhenthithi and coworkers [72] showed that melatonin at a dose of 1.2 mg/kg applied intra-dermally improved the healing process of full-thickness incisional wounds; an improvement of the quality of scarring from the 14th to 21st days after injury was reported. Histological analyses showed that the collagen fibres of melatonin-treated wounds were more mature and resemble fibres in the intact skin, which are more random in structure [72].

The regulatory influence of the pineal gland on collagen content is not limited only to the injured tissue but was observed also in the intact skin [73]. Thus, while melatonin applied in the evening, decreased the level of collagen in the skin and the pinealectomy exerted an opposite effect, melatonin application (30 µg/100 g b.w) to the pinealectomised animals normalized collagen level in the intact skin [73].

To explain whether the observed in vivo effect of melatonin is the result of the direct influence of the pineal indoleamine on the cells synthesizing collagen in the wound, in vitro experiments were carried out. The cells were isolated from the granulation tissue of the wound model with a polypropylene net implanted subcutaneously. The isolated cells had elongated shape and formed whirl-like structures in culture. Electron microscope studies showed mainly fibroblasts in the culture with some myofibroblasts. Melatonin at a concentration of 10⁻⁷M increased collagen accumulation in the culture but lower concentrations were ineffective. The inhibitory effect of melatonin observed in vivo cannot be explained by direct influence of the pineal indoleamine on fibroblasts in the wound. Thus, indirect effects of melatonin via regulatory systems are supposed to be responsible for final effect observed in vivo. Although melatonin (100–400 µg/ml) was observed to have an inhibitory effect on the proliferation of fibroblasts isolated from normal and sclerodermic skin, lower concentrations of the pineal indoleamine were found to stimulate the proliferation of fibroblasts [74].

The presence of melatonin membrane receptors in fibroblasts has been suggested by some experiments. Melatonin-induced augmentation of Cu/Zn-superoxide dismutase and glutathione reductase expression in human corneal fibroblasts is inhibited by lusindol, the nonselective melatonin receptor antagonist [75]. Moreover, lusindol reduces the stimulatory effect of melatonin on lipid metabolism in the cells [76]. Stable expression of both MT₁ and MT₂ was shown in NIH-3T3 cells (mouse embryonic fibroblasts) [77]. Melatonin was also found to interact with nuclear and cytoskeletal structures and, by this mechanism, is thought to modify cell function [78].

The results presented above clearly suggest that the pineal gland and its secretory product, melatonin, inhibit accumulation of the collagen content in the healing tissue. This effect has been observed in different types of wound such as full thickness circular wounds [63], sponges inserted subcutaneously [63-65], skin incisional wounds, intestinal anastomotic wounds [67] and dimethylNitrosamine [70] or ethanol [71] induced
liver fibrosis. Thus, the effect of melatonin on the healing process is not dependent on the mechanism of healing and the type of the wound. In the case of incisional wounds healed by first intention, or a sponge implanted subcutaneously, resembling healing by the second intention, melatonin was reported to have similar effects on the collagen content. These effects observed in wounds were mainly described for lower doses of melatonin. The same lowering effect of melatonin on collagen accumulation was also seen in liver injury when pharmacological doses of melatonin were applied (10 mg/100 g b.w). The experiments also defined the dose of melatonin that reverses the effects of pinealectomy and normalizes the collagen content in the wound of pinealectomised animals comparing to controls [65, 67, 73]. That dose of melatonin (30 μg/100 g b.w) could be assumed as equivalent to the amount of melatonin expressing physiological effects. Moreover, melatonin improved collagen maturation and assembly in the full thickness skin wounds.

**Melatonin action in healing of myocardial infarction**

Expansion of myocardial infarction, aneurysm formation or rupture of infarcted area could be dependent on the mechanical features of the scar that are mainly defined by the collagen fibres. Thus, assessment of the regulatory mechanisms of collagen accumulation in the infarcted area is thought to indicate a new target for therapy of myocardial infarction and its complications.

The doses of melatonin applied in the evening, which were shown to increase the collagen level in the myocardial infarction scar, were found to be comparable to those that were effective in the peripheral wounds. Unlike melatonin treatment, pinealectomy induced by surgical removal of the pineal gland decreased collagen level in the scar. Treatment of the pinealectomised animals with 60 μg/100 g b.w melatonin reversed the effect of pineal gland removal and normalized collagen content in the myocardial infarction scar. A dose of 30 μg/100 g b.w was not effective in reversing the effect of the pinealectomy [79].

Treatment of rats with metoprolol given in the evening caused a ‘pharmacological pinealectomy’. Secretion of melatonin from pinealocytes depends mainly on β-adrenergic stimulation, and so a blockade of β-adrenoreceptors by β-blocker application in the evening was reported to cut off nocturnal elevation of melatonin [80]. A morning injection of metoprolol served as the control for the pineal independent effects of β-blockers and did not modify the nocturnal concentration of melatonin [81]. However, metoprolol application in the evening reduced collagen content in the scar of myocardial infarction in rats. This effect in rats was seen to be identical to that of a surgical pinealectomy. Morning application of the metoprolol did not change the collagen content in the infarcted area [79]. Moreover, melatonin (60 μg/100 g b.w) application to the rats with pharmacological pinealectomy (evening application of metoprolol) reversed the effect of pinealectomy and normalized the collagen content in the scar.

An increasing number of reports shows a direct influence of melatonin on the cells synthesizing collagen. To clarify the possible mechanism of melatonin action, myofibroblasts were isolated from the myocardial infarction scar. The isolated cells displayed a polyhedral shape and grew in a formless manner in the culture. Melatonin at concentrations of 10⁻⁸ and 10⁻⁷ M increased the level of collagen content in the culture. Thus, the elevation of collagen level in vivo by melatonin could be explained as the effect of direct action of the pineal indoleamine on myofibroblasts in the scar [79].

Application of the pineal indoleamine to the intact rats did not change the expression of both α₁(I) and α₁(III) procollagen genes in the myocardial scar and myofibroblast cultures [82]. These results suggest that elevation of the collagen level in the myocardial infarction scar is regulated during the posttranscriptional steps. However, in the pinealectomised rats, melatonin application increases the expression of the two collagen types. Thus, in spite of fact that collagen content is increased by melatonin in both intact and pinealectomised rats [79], the mechanism of the pineal indoleamine action is different in the two experiment models [82].

The differences of melatonin action in intact and pinealectomised animals are thought to be due to the different patterns of melatonin secretion as well as the varied sensitivities of the tissues to pineal indoleamine in intact rats and animals with a removed pineal gland. The application of melatonin to the pinealectomised animals is thought to not replicate the physiological pattern of pineal indoleamine release. Moreover, after pinealectomy, a transient elevation of melatonin receptor density and increased binding of iodomelatonin to the crude membranes of the spleen were observed. Melatonin is suggested to interfere with the common mechanism of the both α₁(I) and α₁(III) procollagen...
Melatonin effects in skeletal system

The ovariectomised rats were used to study melatonin influence on postmenopausal osteoporosis. Serum melatonin level decreased after ovariectomy and negatively correlated with the serum concentration of cross-linked type I collagen carboxyterminal telopeptide (ICTP) and urinary levels of calcium and hydroxyproline. All these three parameters are known as the bone resorption markers that increase after ovariectomy. Melatonin is suggested to be responsible for the changes of bone mass in postmenopausal osteoporosis [84].

Melatonin (30 mg/kg) has been proved radiologically and histologically to have a beneficial effect on the healing of bone fractures in rats and was seen to reduce the severity of an injury caused by ROS in a bone fracture model [85].

Melatonin was also reported to elevate collagen type I synthesis by both normal human bone cells (HOB-M) and human osteoblast cells (SV-HFO), [86]. The maximal stimulatory concentration ranged from 50 to 100 µM. However, the efficiency of stimulation is dependent on the cell type. The carboxyterminal propeptide of type I procollagen (PICP), the marker of collagen synthesis, was increased by 983% in HOB-M cells and 139% in SV-HFO cell culture. Moreover, melatonin stimulates proliferation of both HOB-M and SV-HFO cell lines. The most effective concentration of melatonin was evaluated as 50 µM. Thus, melatonin was concluded to be a potent stimulator of bone formation [86].

Angiogenesis in the wound

Intradermal administration of melatonin increases new blood vessel formation on the third day after injury but no later neovascularisation changes were observed. These results were supported by data showing that melatonin treatment starts increasing VEGF concentration on the first day after injury but the statistically significant peak of this growth factor is achieved on the 7th day of healing [72]. Ganguly and coworkers [87] showed on two models of wound healing (rat corneal micropocket assay and indometacin-induced gastric lesions in mice) that melatonin increases angiogenesis in the healing wounds. Moreover, the pineal indoleamine reversed the antiangiogenic effects of indometacin in both wound models. The melatonin-induced promotion of angiogenesis was linked with increased levels of VEGF and matrix metalloproteinase-2 (MMP-2). These results were linked with MMP-14 upregulation and decrease of the TIMP-2 [87].

Regulation of an inflammatory process in the wound

Melatonin regulates inducible nitric oxide synthase (iNOS) activity during the healing process. Thus, inhibiting pineal indoleamine iNOS activity and lowering iNOS protein level during the inflammatory phase were reported. The opposite changes were seen in the proliferative step. The iNOS activity and NO content in the wound are suggested to influence angiogenesis and granulation tissue formation. Furthermore, melatonin increases the activities of the cyclooxygenase-2 (COX-2) and arginase. The upregulation by melatonin of the levels of heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2) may play a role in the regulation of the inflammatory process [72].

Melatonin administered at a dose of 20 mg/kg accelerates gastric ulcer healing. This effect is linked with stimulation of COX-2 and elevation of gastroprotective prostaglandin synthesis. Furthermore, stimulation of constitutive NOS was also observed. The effect of melatonin is inhibited by the MT2 receptor blockade [88].

The antioxidative effects of melatonin have been found in the healing of the wound. Malonyldialdehyde level was markedly reduced by melatonin in the ischemic wound tissue but activities of superoxide dismutase (SOD) and glutathione peroxidase were not influenced by the pineal indoleamine [68]. Pharmacological doses of melatonin decreased lipid oxidation and increased the sulphhydril group content of total proteins and glutathione in the myocardial infarction scar. These observations suggest a reduction of oxidative stress effects in the infarcted area by pineal indoleamine [83]. By lowering lipid oxidation and reducing the augmentation of sulphhydril groups in proteins, melatonin is thought to influence cell membrane
stabilization as well as protein function. Melatonin was also reported to reduce both lipid and protein peroxidation in gastric ulcers. In addition, it inhibits hydroxyl radical generation as well as expression of SOD-2 [89].

Summary and conclusion
The presented results clearly demonstrate the regulatory influence of melatonin on the healing of the different types of wounds. This effect is seen in various phases of healing; melatonin exerts its action on the inflammatory phase, cell migration and proliferation (angiogenesis) as well as extracellular matrix component synthesis (collagen and GAG accumulation). In addition, the final effect of melatonin is dependent on the applied dose, the time of its application as well as the target tissue. The present data does not clearly describe the mechanism of melatonin action. However, its direct effect on the cells in the wounded milieu is postulated. An indirect action of melatonin via general regulatory mechanisms can not be excluded. Experiments with both surgical and pharmacological pinealectomies report that decreased secretion of endogenous melatonin may influence the healing of both peripheral wounds and myocardial infarction. Similar effects could be expected in patients with low levels of melatonin (β-blockers treated and elderly patients). Thus, melatonin supplementation in those patients is postulated. Moreover, melatonin should be considered as a therapeutic compound in some diseases such as liver fibrosis, myocardial infarction, osteoporosis and bone fractures.

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