Light therapy by blue LED improves wound healing in an excision model in rats

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A B S T R A C T

Background: Low level light therapy (LLLT) is an attractive alternative to enhance wound healing. So far most studies are performed with red or infrared irradiation. However, we recently showed that blue light (470 nm) can significantly influence biological systems, improving perfusion by release of nitric oxide from nitrosyl complexes with haemoglobin in a skin flap model in rats. Here, we compared the effects of blue and red low level light by light-emitting diodes (LEDs) on in vivo wound healing in an excision wound model in rats.

Methods: Circular excision wounds were surgically created on the dorsum of each rat. Excisions on either the left or right side were illuminated post-OP and on five consecutive days for 10 min by LED at 470 nm or 630 nm with an intensity of 50 mW/cm², while protecting the contralateral side from exposure. In the control group, neither side was illuminated. On day 7 post-OP, we analysed planimetric and histological parameters, as well as expression of keratin-1, keratin-10 and keratin-17 on mRNA level.

Results: Illumination substantially influenced wound healing. Blue light significantly decreased wound size on day 7, which correlated with enhanced epithelialisation. Light also affected mRNA expression. Both wavelengths decreased keratin-1 mRNA on day 7 post-OP, while keratin-10 mRNA level was elevated in both light treated group compared to control. Keratin-17 mRNA was also elevated in the red light group, but was unchanged in the blue light group.

Conclusion: In contrast to previous studies, we showed that also blue light significantly influences wound healing. Furthermore, our data suggest that light therapy can play an important role in normotrophic wound healing by affecting keratin expression. Illumination would provide an easily applicable, safe and cost-effective treatment of surface wounds.

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Introduction

Skin wound healing is a complex and multiphase process. Despite present therapeutic options such as wound debridement, pressure regulation, application of growth factors, the use of skin equivalents and specific wound dressings, healing of cutaneous wounds costs much time, effort and money.

Low-intensity light has been documented as a promising method for various medical problems including wound repair processes. Low level laser therapy (LLLT) as a therapeutic method was introduced by Mester and colleagues, who noted beneficial effects of ruby laser on wound healing, resulting in faster closure of refractory and persistent ulcers.

Since then several in vitro studies showed the efficiency of LLLT. Helium–neon laser irradiation activates human monocytes and increases the rate of keratinocyte migration and proliferation. Studies with human fibroblasts demonstrated pronounced cell proliferation and migration of fibroblasts, raising the hope that wound closure also in vivo may be accelerated. Indeed, LLLT proved to have positive effects in rat skin wound models leading to earlier resolution of the inflammatory phase, faster re-epithelialisation and acceleration in maturation phase.

LLLT, in some publications also called photostimulation, promoted the tissue repair processes of wounds and increased the quantity of collagen fibres, both in diabetic and non-diabetic rats. Also healing of tendons, nerves and bones can be positively affected by light treatment as shown in several small animal models. LLLT at different intensities promoted neovascularisation in damaged Achilles tendons of rats after partial rupture and increased vascularisation and the number of hypertrophic osteoclasts during tibia wound healing in rats.
In large animal models, such as pigs, whose skin resembles that of humans and has comparable wound-healing capacities, laser light improved dermal remodelling by increasing dermal thickness, collagen band width, and cellular hypertrophy. Clinical studies on the influence of LLLT on wound healing, including the remodelling phase, demonstrated the positive effects of irradiation on the healing of post-operative aseptic wounds and on burn wounds, concerning macroscopic appearance, pruritus and pain.

Most of these studies were performed with red or infrared laser irradiation, however other wavelengths may also have a positive effect on wound healing. We recently showed that blue light irradiation, however other wavelengths may also have a positive effect on wound healing. We recently showed that blue light therapy applied: Group 1 treated with blue LED, Group 2 treated with red LED, and Group 3 was not illuminated.

Despite the positive consequences of lasers on wound healing this kind of devices are expensive, require high energies and may cause significant patient discomfort.

Recently, light-emitting diodes (LEDs) have been presented as a comfortable, potentially highly selective light source for the therapy of many indications. LEDs are small, robust devices that emit a narrow band of electromagnetic radiation ranging from ultraviolet to visible and infrared wavelengths. LEDs usually generate low-intensity light in the milliwatt range, and can be configured on small chips or connected to small lamps. Klebanov et al. compared coherent laser and non-coherent light-emitting diodes and found that they both had very close effects on wound healing.

The aim of this study was to investigate the effects of red (630 nm) and blue (470 nm) light from LED lamps on in vivo wound healing in an excision wound model in rats.

Materials and methods

The Animal Protocol Review Board of the Vienna City Government approved the experimental protocol. All experimental procedures were performed under the conditions described in the guide for the care and use of laboratory animals of the National Institute of Health (publication NIH 86-23, revised 2007). Male Sprague Dawley rats weighing 300–350 g were initially anaesthetised in an inhalation box provided with isoflurane (2.5 vol.%), oxygen (300 mL/min), and air (3 L/min). Anaesthesia was maintained by intraperitoneal (i.p.) injection of a mixture of 110 mg/kg ketamin and 12 mg/kg xylazin.

Rats were shaved and under sterile conditions two circular full-thickness excision wounds were created on the dorsum of each rat, including the panniculus carnosus.

Wound depth was standardised by reference to viewing of the muscle plane. As analgesic treatment, rats received 1.25 mg/kg butorphanol and 5 mg/kg meloxicam subcutaneously (s.c.) on the operating day and three days post-OP as well as 15 ml Ringer solution s.c. for fluid resuscitation. Wounds were covered with a transparent film dressing (Opsite, Smith and Nephew, England) and fixed with a second dressing (Fixomull-stretch, Beiersdorf, Germany), sparing the wound area, so illumination through the transparent Opsite foil was possible. Bandages were changed on days 3 and 5 or replaced when necessary.

Treatment with low level light therapy by LED

Animals were divided into three groups (n = 6) according to the therapy applied: Group 1 treated with blue LED, Group 2 treated with red LED, and Group 3 was not illuminated.

Rats were anaesthetised by isoflurane via an inhalation mask as specified time points and photographed with adjacent ruler. These images were then further analysed by a planimetric software (Lucia G®, Version 4.8, Laboratory Imaging Ltd., Czech Republic).

Histology and morphometric analysis

Excised tissue was fixed in neutral buffered formalin and subsequently embedded in paraffin. 3 μm tissue sections were cut, deparaffinised in an increasing series of ethanol up to xylene, and rehydrated in a downgraded alcohols series. Slides were stained for evaluation of wound healing. One half of each sample was fixed in 10% buffered formalin solution for histological and immunohistological procedures, the other half was snap-frozen immediately in liquid nitrogen and stored at −70°C for PCR analysis.

Planimetical analysis

Excision wounds were photographed using a digital camera after surgery and on day 3 as well as on day 7 post-OP. Furthermore, for precise measuring of residual open wound areas, excision wounds were traced on a transparent acrylic sheet at specified time points and photographed with adjacent ruler. These images were then further analysed by a planimetric software (Lucia G®, Version 4.8, Laboratory Imaging Ltd., Czech Republic).

Fig. 1. Effect of low level light therapy by LED on wound size. Excision wounds were illuminated on five consecutive days with red (630 nm) or blue (470 nm) light of 50 mW/cm². Control wounds were not illuminated. Wound areas were evaluated immediately after surgery (baseline) and on days 3 and 7 post-OP. Digital pictures of the wounds were analysed by planimetric software (Lucia G®).

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with hematoxylin and eosin (HE staining). Morphometric analysis was performed using the microscope and corresponding image processing software (Axiovision, Carl Zeiss, Germany). The depth of granulation tissue as well as the length of epithelial coverage were measured.

**Quantitative PCR analysis**

Expression of keratin-1 (Krt1), keratin-10 (Krt10) and keratin-17 (Krt-17) were investigated by semi-quantitative reversed transcriptase (RT) PCR. Briefly, snap-frozen samples were solubilised in TRI Reagent (PeqLab) by a ball mill (VWR) and total RNA was isolated according to the TRI Reagent protocol provided by the manufacturer. For RT-PCR, 1 μg of RNA was used for cDNA synthesis. PCR was performed with 100 nM primers, 10% DMSO and TaqPolymerase (PeqLab). The PCR product was run on a 1% agarose gel and quantitative analysis was done by band intensity read out (MultiImage Light Cabinet, Alpha Innotec). All genes were normalised to GAPDH.

**Statistical analysis**

All data are presented as means ± SEM. Statistical analysis for in vitro experiments was performed by one-way ANOVA test followed by the Tukey test. Significance was based on a value of \( p < 0.05 \).

**Results**

Wound size was determined after surgery and on days 3 and 7 post-OP. In the course of ongoing wound healing, wound areas decreased in all groups compared to the initially wound size. However, low level light therapy by LED markedly influenced this parameter. While there was no difference on day 3 post-OP, on day 7 the wound area was 50% smaller (\( p < 0.05 \)) in the blue light group compared to not illuminated control (Figs. 1 and 2). The difference was even greater compared to the red light group. In contrast, red light seemed to delay wound closure, although these finding were not significant. No significant difference could be detected regarding the degree of granulation (Fig. 3). However, light effected re-epithelialisation as there was a strong trend to enhanced epithelialisation. This effect was stronger in the blue light group (Fig. 4).

**Fig. 2.** Typical macroscopic results of low light therapy with blue LED (BL) versus not illuminated controls (NL) on wound size. Excision wounds were illuminated on five consecutive days with red (630 nm) or blue (470 nm) light of 50 mW/cm². Control wounds were not illuminated.

**Fig. 3.** Effect of low level light therapy by LED on granulation. Excision wounds were illuminated on five consecutive days with red (630 nm) or blue (470 nm) light of 50 mW/cm². Control wounds were not illuminated. On day 7 post-OP excised tissues were prepared on microscope slides and stained with hematoxylin and eosin (HE staining). Depth of granulation tissue was analysed using a microscope and corresponding image processing software (Axiovision, Carl Zeiss, Germany).

**Fig. 4.** Effect of low level light therapy by LED on epithelial coverage. Excision wounds were illuminated on five consecutive days with red (630 nm) or blue (470 nm) light of 50 mW/cm². Control wounds were not illuminated. On day 7 post-OP excised tissues were prepared on microscope slides and stained with hematoxylin and eosin (HE staining). Length of epithelial coverage was analysed using a microscope and corresponding image processing software (Axiovision, Carl Zeiss, Germany).

**Fig. 5.** Effect of low level light therapy by LED on expression of keratin-1 normalised to GAPDH. Excision wounds were illuminated on five consecutive days with red (630 nm) or blue (470 nm) light of 50 mW/cm². Lab controls are samples of healthy skin. On day 7 post-OP total RNA was isolated from excised tissues and mRNA of keratin-1 analysed by semi-quantitative RT-PCR.

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Discussion

In recent years, large interest has been directed towards the benefits of laser light as a treatment modality for various medical conditions, including wound repair processes. Different biological effects have been observed after light irradiation, but reports are still controversial. A number of cases have demonstrated the positive influence of LLT on the healing of cutaneous wounds, tendons, nerves and bones, manifested in promotion of the tissue repair process. Recently, LED have been presented as a comfortable, potentially highly selective light-based therapy for many indications. As some investigators reported that coherent laser and non-coherent LED radiation have very close effects on wound healing, we tried to influence wound healing by low level LED light of different wavelengths. In this study we investigated the effect of LLLT by LED in an excision wound model in rats.

Wound repair is a very complex biological process, that occurs in different stages and involves multiple cell types. The normal healing cascade begins with haemostasis and fibrin deposition, which leads to an inflammatory cell cascade, characterised by neutrophils, macrophages and lymphocytes within the tissue. This is followed by the attraction and proliferation of fibroblasts and collagen deposition (2–10 days after injury), and finally remodeling by collagen cross-linking and scar maturation.

Regarding epidermal injury both wound contraction and re-epithelialisation from the margins of the wound play an important role in wound closure. Wound epithelialisation is an essential feature of a healed wound, achieved by keratinocyte proliferation and migration over an extracellular matrix. In our study treatment of the wounds with blue but not red light led to a significant decrease in the wound area as shown by planimetric analysis. The rise in temperature caused by illumination was marginal and the same for both LEDs, thus cannot account for the differences between the two groups. Moreover, Toyokawa et al. showed in a similar excision model that a 2.5 °C increase during 14 days did not enhance wound healing, while (infrared) irradiation did.

Although blue light does not penetrate tissue as deep as red light, our data with blue light are comparable to the reports of Dahiya et al. and Whelan et al., who studied the influence of red laser light on dermal remodelling in a pig model and of Klebanov et al. who showed positive effects of LED (630 nm) in decreasing the wound area in a rat model. When evaluating wound areas, however, one should keep in mind that contraction mechanism as opposed to true re-epithelialisation can play a major role in wound healing in rats because of the anatomical peculiarity of rats (elastic skin and panniculus carnosus).

Furthermore, blue light showed a strong trend to positively influence epithelialisation, correlating with the data from Vidinsky and colleagues, who investigated the healing effects of a diode laser (670 nm) on surgical wounds in a rat model and found earlier regression of the inflammatory phase, faster completion of re-epithelialisation and acceleration in maturation phase. Also Herascu et al. reported that irradiation (904 nm) stimulated epithelialisation of post-operative aseptic wounds.

Although other reported that light can also influence the quality of granulation tissue we found no significant difference concerning the depth of granulation tissue in our model. However, as already mentioned, there is no consent regarding standardised parameters and wavelengths and intensities differ between the studies.

Effects of low level light on the gene expression of selected keratins were another parameter we were interested in. As part of the epithelial cytoskeleton, keratins are important for the mechanical stability and integrity of epithelial cells and tissues. One of the regulatory function of keratins is the influence on intracellular signalling pathways, that are involved in protection from stress, apoptosis and in wound healing. We therefore investigated the effects of low level light from LED on the expression of Krt-1, Krt-10 and Krt-17 by PCR.

Epidermal injury leads to an induction of keratins like Krt-16 and Krt-17, which occurs on the expense of Krt-1/10. While on the wound edge Krt-17 is accumulated, Krt-1 and 10 are downregulated. In our study we observed a significant increase of Krt-17 mRNA in the red light group, while there was no difference in the blue light group. This correlates with the data regarding wound closure. The data suggest that in the blue light group wound healing is nearly completed, wounds are closed and therefore Krt-17 downregulated, while in the red light group wound healing still continues.
Krt-1 data fits into this picture showing a significant reduction of Krt-1 mRNA in the red light group, but not any more in the blue light group. In contrast to most literature, in our model Krt-10 did not parallel to Krt-1, but was increased in both light groups. However, there are reports that Krt-10 is up-regulated post-wounding. As Krt-10 is a marker for keratin differentiation it suggests that re-epithelialisation is not yet completed in both groups.

To sum up, blue light by LED proved to exhibit positive effects on wound healing parameters. One of the mechanisms may be the influence of blue light on NO metabolism. Our previous studies on wound healing parameters as well as on keratins mRNA expression.

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