Phototherapy With LED Light Modulates Healing Processes in an In Vitro Scratch-Wound Model Using 3 Different Cell Types

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BACKGROUND An effective way of modulating wound healing processes, including proliferation and apoptosis, is low-level light therapy. Because of several disadvantages of lasers, light-emitting diodes (LEDs) could be more feasible light sources.

OBJECTIVE To evaluate and compare the effects of blue and red light from LEDs on different cell types in an in vitro scratch-wound model.

METHODS Monolayers of C2C12 myoblasts, NIH/3T3 fibroblasts, and BICR10 keratinocytes were injured by mechanical scraping. Cells were illuminated on 5 consecutive days for 10 minutes by LED at 470 or 630 nm. Effects of light on in vitro wound healing were evaluated by analyzing time to closure, proliferation, apoptosis, and necrosis rates.

RESULTS Illumination substantially affected cell viability and cell growth. Blue light strongly decreased proliferation and augmented apoptosis in all 3 cell types and increased necrosis rates in C2C12 and NIH/3T3 cells. In contrast, red light did not alter apoptosis in either cell type but promoted proliferation in all 3 cell types with significant effects in C2C12 and NIH/3T3 cells and shortened time to closure in all 3 cell types.

CONCLUSION Light-emitting diode light illumination could be a therapeutic option and positively affect wound healing processes. By choosing appropriate wavelengths, variable effects can be achieved.

P. Dungel cooperates with Relux Lichtmedizintechnik GmbH which markets the LED device Repuls Tiefenstrahler for use in orthopedics, rehabilitation centers and sports medicine. The other authors have indicated no significant interest with commercial supporters.

A variety of therapeutic options for skin wounds have been established, including wound debridement, application of growth factors, pressure regulation, the use of skin equivalents, and specific wound dressings. In addition to these already established treatments, low-level light has been shown to be a promising means of enhancing wound repair processes and could be a medical option to reduce scar formation after surgery.[1] Especially in chronic wounds such as ulcer wounds and ischemia, different studies have shown low-level laser therapy (LLLT) to be a viable treatment option.[2,3] Historically, the first beneficial effects of LLLT were noticed by Mester and colleagues,[4] who found a more rapid closure of ulcer wounds when treated with ruby laser.

In vitro studies suggested that low-power lasers may enhance wound healing processes. Several groups

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showed that helium–neon laser irradiation stimulates the migration and cell proliferation of fibroblasts and keratinocytes with prospects of accelerated wound closure.[5,6] In vivo studies in rat skin wound models demonstrated a significant time reduction in different processes of wound healing, such as the inflammation phase, the reepithelization of wounds, and maturation phase.[7] Low-level laser therapy also showed beneficial effects in the healing of bones, nerves, and tendons. Photostimulation with laser light enhanced neovascularization in damaged Achilles tendons and stimulated the growth of the trabecular area in a rat model.[8,9]

The majority of these studies, however, were performed with lasers in the red or near-infrared spectrum. Laser devices may cause significant patient discomfort, continue to be expensive, can produce heat, and apply light only on a narrow spot. Recently, light-emitting diodes (LEDs) have been introduced into the field of light treatment and seem to be a more feasible source for the therapy of many indications. Light-emitting diodes are small robust devices that emit a narrow band of electromagnetic radiation, which can range from ultraviolet (UV) to visible and infrared wavelengths.[10] Klebanov and colleagues[11] compared the effects of noncoherent LEDs and coherent lasers on wound healing and concluded that they are quite similar.

Wound healing can be subdivided into 4 distinct, yet overlapping phases: hemostasis, inflammation, proliferation, and remodeling.[12] Particularly during the proliferation phase, local cells are involved in the wound healing process. Although fibroblasts synthesize extracellular matrix, basal epithelial cells (e.g., keratinocytes) migrate over the granulation tissue to close the wound surface over time. In the case of myogenesis and muscle regeneration after muscular damage (e.g., laceration and contusion), myoblasts must migrate into the wounded area. For all cell types involved in the wound healing process, proliferation and migration are key factors necessary to perform their specific tasks.

To study the effects of LED irradiation on wound healing in vitro, the authors chose the classic scratch-wound assay. This wound model has been used for decades to study wound healing processes and cell migration. In this assay, a confluent monolayer is "injured" by creating a gap defined in width, and the healing of the remaining monolayer is observed under defined conditions. The end point for "healing" of the in vitro wound was the first closure of the cell layer gap. Although the scratch-wound assay has been used to identify signal molecules and signaling pathways for cell migration and wound healing, the predominant mechanisms underlying the expansion of cells in the surviving monolayers are still debated.[13] Despite the ongoing discussion about the interaction of these mechanisms, it is beyond controversy that this "healing" occurs because of cell migration, spreading, and proliferation.

To investigate and compare the effects of LED light on different cell types, fibroblastic, myoblastic, and keratinocytic cell lines were chosen for this study. These cell types are the dominant cell types involved in skin/surface wound healing. The fibroblastic cell line NIH/3T3 and the myoblastic cell line C2C12 used in these experiments have already been described in wound healing studies.[6,14] In addition, keratinocytes are known to also take part in wound healing. Thus, they were included in this study (keratinocytic cell line BICR10).

The majority of LLLT studies currently reported were performed with a single cell type and lasers as a light source. Studies comparing the effects of LLLT on different cell types are limited.[15,16] As a consequence, there is a need to compare the effects of LLLT on different cell types with LEDs as a light source.

Materials and Methods

If not indicated otherwise, all reagents used in this study were purchased from Sigma-Aldrich (Vienna, Austria).

Cell Culture/Scratch-Wound Assay

NIH/3T3 and BICR10 cell lines were purchased from ECACC (European Collection of Cell Cultures, United Kingdom). The mouse C3H muscle myoblast

precursor cell line C2C12 (#ACC565) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

NIH/3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) (Lonza Ltd., Basel, Switzerland) in plates coated with 0.2% gelatine solution. C2C12 cells were cultured in DMEM containing 5% FCS supplemented with 2 mM glutamine. BICR10 cells were cultured in DMEM containing 10% FCS, supplemented with 2 mM glutamine, and 0.4 μg/mL hydrocortisone. For all cell types, DMEM without phenol red was used. C2C12 cells were seeded at 3.4×10^4 cells/cm^2, NIH/3T3 at 4.4×10^4 cells/cm^2, and BICR10 at 2.4×10^4 cells/cm^2. All cell types were seeded in rows of 3 wells per 6-well plate until 90% of confluence was reached (i.e., 2 days after seeding for all cell types).

Subsequently, a wound of 1 cm in width was made in the confluent monolayers using a cell scraper. Cell debris was removed by washing the cells once with DMEM. Subsequently, 3 mL of medium was added to each well. At this point, the plates were ready for LED therapy. The medium was changed twice a week.

Low-Level Light Therapy by LED

To study the influence of LED irradiation on cell proliferation, wells containing cellular gaps were illuminated every week on 5 consecutive days for 10 minutes. Illumination of the 3 adjacent wells containing cells was performed at a distance of 10 cm using LEDs (LEDline2; Philips Austria GmbH, Vienna, Austria) of 2 different wavelengths. Reflectors around the LEDs allowed an even illumination of all wells. The daily dose provided was 30 J/cm^2.

Light-emitting diodes had the following characteristics: 470 nm (blue), 1 W; 630 nm (red), 1 W. Light-emitting diode calibration was performed with a USB 2000 spectrometer (Ocean Optics, Dunedin, FL) (Table 1). The specific power supply conditions were defined for each light source by using a regulated DC power supply (PW18.1.8Y; Kenwood Electronics Deutschland GmbH, Bad Vilbel, Germany). Thus, irradiance intensity was normalized to 50 mW/cm^2.

TABLE 1. Characteristics of Light Sources and Experimental Setup Used for the LED Treatments

	Red LED	*Blue LED*
Wavelength, nm	630	470
Potency, W	1	1
Dosages		
Intensity, mW/cm^2	50	50
Energy fluence yield, J/cm^2	30	30
Duration treatment, consecutive days	5	5
Irradiation time, minutes daily	10	10

For the determination of time to gap closure, illumination was performed as described above (Figure 1). Subsequently, the time until the cells bridged the gap was noted for each well. Illumination continued until bridges were formed in every well. Blue light restrained cell proliferation. Thus, in this case, illumination was stopped after 6 weeks even when no bridge was formed.

Plates were observed on an Axiovert 200M inverse microscope (Carl Zeiss Jena GmbH, Jena, Germany) once a day until first closure of the gap up to a maximum of 6 weeks.

For further performed proliferation analyses, cells were illuminated for 5 consecutive days and analyses were performed on Day 8.

Proliferation/MTT-assay

Cell proliferation was evaluated by means of standard MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) assay. Thus, cell culture medium was aspirated and the medium containing 650 mg/mL MTT was added to each well. Cells were incubated for 1 hour in the incubator (37°C, 5% CO_2). Then, the medium was aspirated and the MTT-formazan precipitate was dissolved in 1 mL of dimethylsulfoxide by shaking mechanically in the dark for 20 minutes. Aliquots of 100 μL of each sample were pipetted in 96-well plates. The absorbance at 550 nm was read immediately thereafter on an automatic microplate reader (Spectra Thermo; TECAN Austria GmbH, Vienna, Austria). Optical

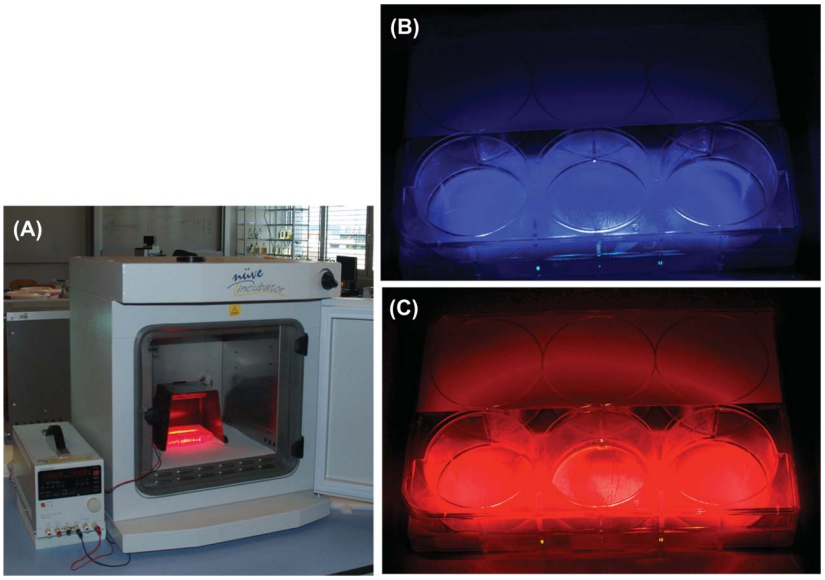

Figure 1. Light-emitting diode irradiation placed below the cell culture plates and maintained inside the incubator during the entire treatment time (A). Blue LED 470 nm (B) and red LED 630 nm (C).

density values are corrected for unspecific background. All samples were tested in triplicates.

Apoptosis/Necrosis Rate

Apoptosis and necrosis of cells were analyzed by flow cytometry with Annexin-V-FLUOS staining kit (Roche Diagnostics, Vienna, Austria) according to the protocol provided by the manufacturer. Briefly, the cell layer was carefully detached, centrifuged at 1,000*g* at room temperature and washed with phosphate-buffered saline. The washed cell pellets were resuspended in 100 μL incubation buffer (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid buffer) and stained with 2 μL fluorescein isothiocyanate–labeled recombinant human Annexin V. To discriminate necrotic cells, each sample was incubated with 2 μL of propidium iodide at room temperature for 15 minutes. Then, additional 400 μL of binding buffer were added. Flow cytometry was carried out on a FC 500 MPL (Beckman Coulter Cytomics; Nyon, Switzerland), where at least 7,000 events were analyzed. Samples were evaluated in triplicates.

Statistical Analysis

All data are presented as mean ± SD. Differences between treated and nontreated cells were analyzed using analysis of variance followed by post hoc testing with the Tukey test. A $p < .05$ was considered statistically significant. The calculations were made using GraphPad software (GraphPad software, Inc., La Jolla, CA).

Results

Proliferation

Proliferation was determined by MTT assay. There was a significant decrease in proliferation in C2C12 and BICR10 cells after the application of blue LED light (n = 6, $p < .05$) (Figure 2). In NIH/3T3 cells, the results for the blue light illumination group and the control group were not significantly different.

In contrast, red LED light illumination caused an increase of proliferation rates in C2C12 and NIH/3T3 cells. No significant effect was observed in the BICR10 cell group (Figure 2).

Time to Closure

Optical investigations showed that red light treatment exerts positive effects in comparison to non-illuminated controls in all 3 different cell types (Figure 3), by reducing the time to closure significantly (n = 6, for C2C12 and NIH/3T3 cells; $p < .05$ and for BICR10 cells; $p < .01$). Blue light, regardless of the cell

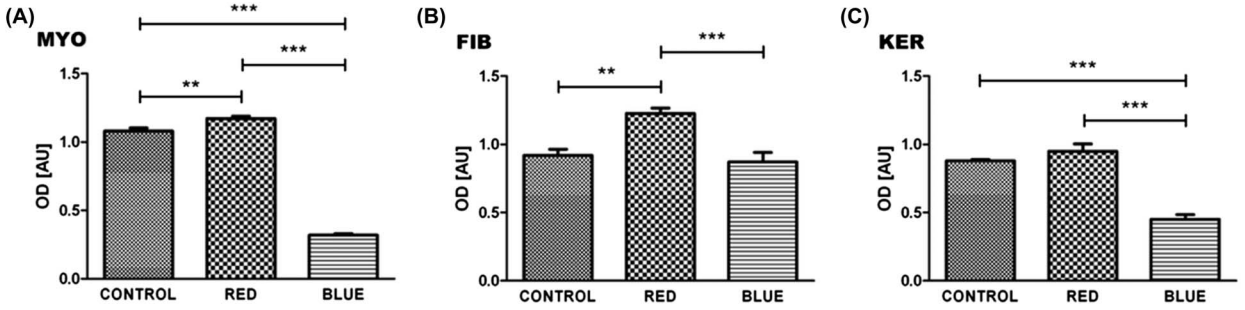

Figure 2. Proliferation of the myoblastic cell line C2C12 (A), the mouse embryonic fibroblast cell line NIH/3T3 (B), and the human keratinocytes cell line BICR10 (C). A 1-cm wound was made in the well of a 6-well plate using a cell scraper. Proliferation was measured by a conventional MTT assay. Red and blue mean treatment with red and blue LEDs, respectively, on 5 consecutive days per week for 10 minutes at a distance of 10 cm and an intensity of 50 mW/cm^2. Values of mean ± SD are indicated. ***Significant difference of $p < .001$, **significant difference of $p < .01$.

type, prevented any bridging of the gaps for 6 weeks, thus experiments were terminated at this time point (indicated in the figures with bars capped with triangles).

Apoptosis Rate

Illumination with blue LED light significantly increased the percentage of apoptotic cells compared with the control group ($p < .001$) in all cell types (Figure 4). Furthermore, no statistically significant effect of red LED light illumination on the percentage of apoptotic cells compared with the nonilluminated control groups could be observed.

Necrosis Rate

Treatment of C2C12 and NIH/3T3 cells with blue LED light strongly induced necrosis (Figure 5). In C2C12 cells, a statistically significant ($p < .01$) decrease in the necrosis rate was observable with red light. Except for the keratinocytic cell line BICR10, no significant difference between blue LED light, red LED light, and the control group could be seen.

Discussion

Low-level light therapy has been shown to promote tissue repair processes of cutaneous wounds, tendons, nerves and bones. Some investigators reported that coherent laser and noncoherent LED illumination have very similar effects on wound healing.[11] Because LEDs are patient-friendly and highly selective devices for light-based therapy for many medical indications,[10] the authors investigated the effects of LED illumination on the wound healing capacity of 3 different cell types at 2 different wavelengths, 470 nm (blue light) and 630 nm (red light). In the authors' study, red light illumination enhanced in vitro "wound healing" of myoblastic (C2C12), fibroblastic (NIH/3T3), and keratinocytic (BICR10) cell lines that

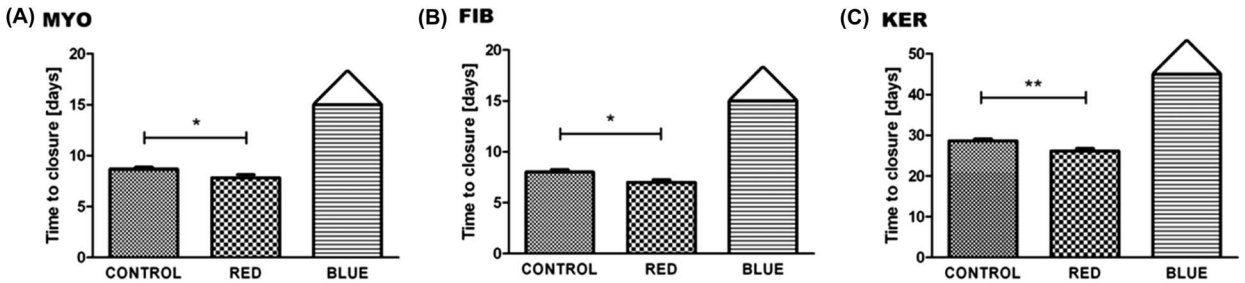

Figure 3. Time to closure values of the myoblastic cell line C2C12 (A), the mouse embryonic fibroblast cell line NIH/3T3 (B), and the human keratinocytes cell line BICR10 (C). A 1-cm wound was made in the well of a 6-well plate using a cell scraper. Time to closure was investigated by optical observations of the gaps in the cell monolayers. Red and blue mean treatment with red and blue LEDs, respectively, on 5 consecutive days per week for 10 minutes at a distance of 10 cm and an intensity of 50 mW/cm^2. Values of mean ± SD are indicated. Regardless of the cell type, no bridging of the gaps was detectable after 6 weeks for the blue light-treated wells, and thus time to closure was not determinable (indicated with bars capped with triangles). **Significant difference of $p < .01$, *significant difference of $p < .05$.

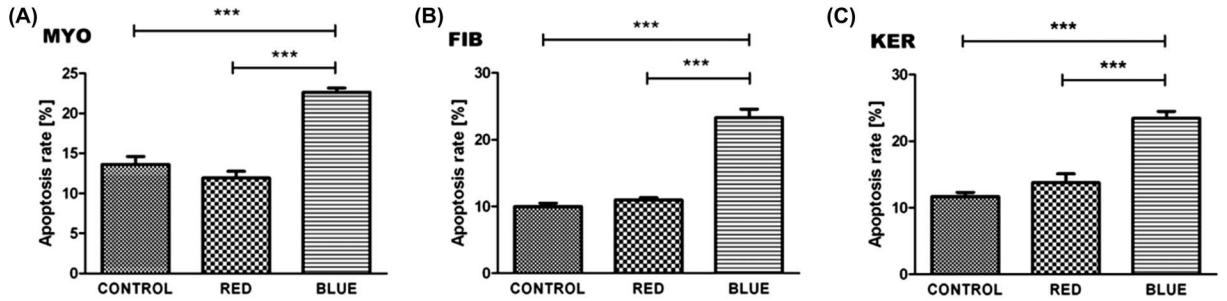

Figure 4. Apoptosis rates of the myoblastic cell line C2C12 (A), the mouse embryonic fibroblast cell line NIH/3T3 (B), and the human keratinocytes cell line BICR10 (C). A 1-cm wound was made in the well of a 6-well plate using a cell scraper. Red and blue mean treatment with red and blue LEDs, respectively, on 5 consecutive days per week for 10 minutes each at a distance of 10 cm and an intensity of 50 mW/cm^2. After first bridging of the 1-cm gap was observable, apoptosis was measured using Annexin V staining determined by flow cytometry. Indicated are the values of mean ± SD. ***Significant difference of $p < .001$.

is apparent in a reduced time to closure. In C2C12 and NIH/3T3 cells, this effect was accompanied with increased proliferation. These results are in line with observations from Seo and colleagues.[17] The authors compared different LED wavelengths on activity and migration of ligament cells. They concluded that irradiation with red LED wavelengths clearly stimulated proliferation in cultured cells. Wound closure depends on 2 cellular effects: proliferation and migration. One of the first processes in wound healing is reepithelialization, with keratinocytes migrating across the granulation tissue from deep within the dermis and the basal cells of the wound edge. After the migration phase of cells beyond the wound edge, cells have to proliferate to repopulate the denuded area.

In BICR10 keratinocytes, time of closure was also shortened by red light treatment, but no significant effect on proliferation could be observed. This could be explained by an enhanced migration rate instead. These results call for further investigation of altered cell migration behavior because of LED light illumination.

Blue light has been shown to be able to positively affect biologic systems in vitro and in vivo. Dungel and colleagues[18] investigated the influence of blue LEDs on the respiratory activity of mitochondria. They showed that the visible light of short wavelengths efficiently facilitates the recovery of mitochondria through nitric oxide (NO) release.

In an animal study, Mittermayr and colleagues[19] used blue laser light irradiation to improve local tissue perfusion in a controlled manner, stimulating NO release from NO-Hb complexes. By enhancing perfusion, blue laser irradiation could be a therapeutic technique to enhance wound healing after surgical interventions.

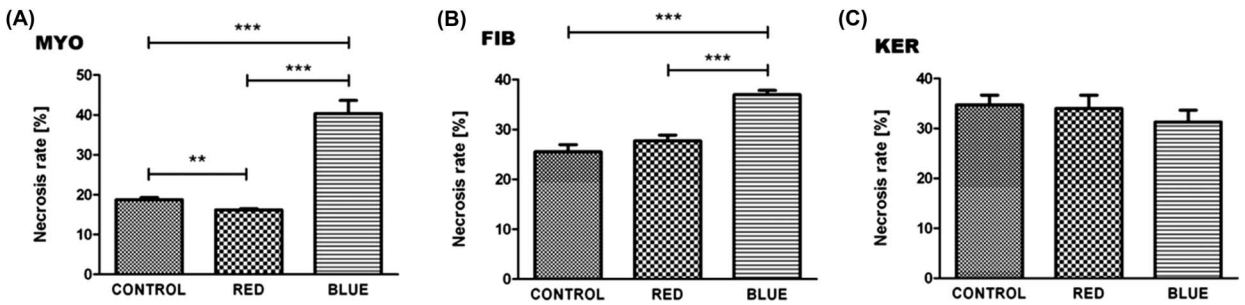

Figure 5. Necrosis rates of the myoblastic cell line C2C12 (A), the mouse embryonic fibroblast cell line NIH/3T3 (B), and the human keratinocytes cell line BICR10 (C). A 1-cm wound was made in the well of a 6-well plate using a cell scraper. Red and blue mean treatment with red and blue LEDs, respectively, on 5 consecutive days per week for 10 minutes each at a distance of 10 cm and an intensity of 50 mW/cm^2. After the first bridging of the 1-cm gap was observed, necrosis was measured using propidium iodide staining determined by flow cytometry. Indicated are the values of mean ± SD. ***Significant difference of $p < .001$, **significant difference of $p < .01$.

However, in this in vitro study, in which the perfusion effects of in vivo settings are not at work, no positive effects of blue light compared with red light in terms of time to closure could be observed. Even after 6 weeks of light treatment, no bridging of the created gaps could be observed. Accordingly, proliferation was heavily decreased, indicating a repressive effect on cell metabolism. Blue light significantly enhanced apoptosis rates in all cell types and necrosis rates in C2C12 and NIH/3T3 cells. Interestingly, the necrosis rate of BICR10 keratinocytes in the blue light group was not altered compared with the other cell types. This observation is similar to studies dealing with oxidative stress induced after exposure to UV-A and UV-B light. In 1 of these studies, it was shown that the survival ability of dermal fibroblasts was lower compared with epidermal keratinocytes after exposure to UV light because of an apoptotic process occurring in dermal fibroblasts, but not in epidermal keratinocytes.[20] Additionally, other studies also provided clear evidence that keratinocytes respond differently to oxidative stress induced by UV light compared with the other cell types such as fibroblasts, indicating cell type–specific, anti-oxidant defense programs.[21,22]

In a recent publication, Liebmann and colleagues[23] studied the effect of wavelength and different light doses on keratinocytes and skin-derived endothelial cells. They observed that blue light irradiation (453 nm) is nontoxic up to a distinct dose (500 J/cm^2) and demonstrated that blue light irradiation up to 453 nm photolytically generates NO from nitrosated proteins, which is known to initiate differentiation in skin cells. In concert with the authors' findings in this study, proliferation rates are remarkably reduced by irradiation with blue LEDs. As a consequence, treatment with blue LEDs could be used to treat hyperproliferative skin conditions by reducing cell proliferation (e.g., inhibition of extensive scar formation). Interestingly, in a recent publication, Capon and colleagues[1] demonstrated in a prospective, comparative clinical trial that 810-nm diode-laser treatment, performed immediately after surgery, can also be used to prevent and reduce scars in plastic surgery.

Further studies must be designed to study the effects of different parameters, such as time of irradiation, wavelength, pulsation versus continuous light or energy dose in various cell types.

In this study, the authors showed that the effects of LED light irradiation do not only depend on the wavelength of light used but also differed between cell types. To conclude, illumination with LEDs of different wavelengths represents a promising alternative therapy for various medical applications, especially in the area of wound healing.

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