Acta Dermatovenerol Croat 2023;31(2):63-71 ORIGINAL SCIENTIFIC ARTICLE

The Impact of Blue Light Irradiation on Keratinocytes in Vitro

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ABSTRACT

Background: This study examined the effects of irradiation with blue light on HaCaT keratinocytes. As irradiation with blue light is known to be antimicrobial, it offers a promising alternative therapy for contaminated wounds. There is evidence that red light promotes wound healing, but the potential benefits of irradiation with blue light have not yet been adequately investigated.

Methods: The rate of wound closure in sterile and contaminated cells was measured using an *in vitro* scratch assay wound-healing model. Additionally, cell viability after treatment was determined using a Sulforhodamine B (SRB) assay.

Results: In both the sterile and contaminated groups, treated cells showed delayed wound closure when compared with cells not irradiated with blue light. Additionally, treatment with blue light resulted in poorer viability in the treatment groups.

Conclusion: Although irradiation with blue light may offer a promising alternative therapy for reducing bacterial colonization, our data indicate that re-epithelization may be negatively influenced by blue light. Further research is needed to clarify possible wound healing applications.

INTRODUCTION

There is sufficient evidence of the positive effects of light treatment on wound healing for a range of tissues, demonstrating enhanced healing of wounds (1-3), ligaments (4), tendons (5,6), bone (7,8), and cartilage (9,10) using phototherapy. While the effects of red light are already well-known, the potential effect of blue light is not yet fully understood. Irradiation with red light results in enhanced proliferation and migration of fibroblasts, with higher collagen synthesis (5,11,12) and release of growth factors and interleukins, such as basic fibroblast growth factor (bFGF) (13) and fibroblast growth factor 7 (FGF-7) (14). Blue light, on the other hand, seems to have immune modulating (15,16) and antimicrobial properties, as irradiation reduces bacteria such as *Pseudomonas aeruginosa* (17,18), *Propionibacterium acnes* (19), *Salmonella enterica* (20), *Staphylococcus aureus* (18), and methicillin-resistant *Staphylococcus aureus* (20-24), as

well as releasing Interleukin 10 (IL-10) (25) and reducing Interleukin 6 (IL-6) (26). Additionally, it has been reported that blue light is anti-inflammatory (27) and increases angiogenesis (28).

Those findings suggest that irradiation with blue light might serve as a promising alternative wound healing therapy and could be implemented in daily wound therapy, especially for wound infections, with only minor side effects. While there is sufficient evidence of the suppression of bacterial growth by blue light irradiation, its influence on wound healing, and especially on re-epithelization, remains unclear. Because of its anti-microbial effects, it would seem likely that blue light can also promote wound healing by reducing bacterial colonization and providing a better wound healing milieu.

The aim of the present study was to investigate the effect of irradiation with blue light on re-epithelization and cell viability. To this end, we measured the rate of wound healing *in vitro* in sterile and contaminated wounds using a scratch assay wound-healing model, as well as examining cell viability following irradiation.

MATERIALS AND METHODS

Cell culture

HaCat cells were grown in 175 cm^2 cell culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin and phenol red at 37 °C and 5% CO_2. When the cells became confluent, all medium was removed; cells were washed twice with phosphate-buffered saline without Ca^{2+} (PBS) and then detached using 0.25% trypsin in buffered ethylenediaminetetraacetic acid (EDTA). Detachment was stopped with DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin, and phenol red. Following centrifugation and resuspension, cells were counted in a Neubauer chamber before plating.

Scratch assay

The scratch assay is a well-established and reproducible technique that is commonly used to investigate cell proliferation and migration (29). For present purposes, 200,000 cells were seeded in 24 well plates with 1 mL DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin, and phenol red. After three days of incubation, when cells had reached complete confluency, the cell monolayer was wounded using a sterile 10-100 µl pipette tip, creating a straight scratch line in the cell monolayer. Afterwards, cells were washed several times with PBS until all remaining cell debris was removed. Finally, 1 mL new DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin, and phenol red, either sterile (n=3) or contaminated with *Achromobacter* (n=5), was added, and the scratch was photographed at ×4 magnification (t0 photo). As there was no additional medium change after this step, cytokines and growth factors that may have been released by the irradiated cells were not removed with the supernatant. As subsequently described, the cells were irradiated each day. After 24 and 48 hours (for sterile medium also additionally after 72 hours), further images of the same viewframe were captured. The size of the wound in these images (percentage of cell-free / uncovered area in the image as a whole) was determined by TScratch, a computer program designed for automated analysis of monolayer wound healing assays (30). Wound closure was calculated for each day following the start of the study (e.g., Wound $size_{t24}$ % – wound $size_{t0}$ % = wound $closure_{day1}$ pp [percentage points]). On terminating the scratch assay, cell viability was investigated using SRB staining. On completing the experiments, all control groups underwent microbiological tests (standard wound swab examination with microbiological cultures at the Institute of Medical Microbiology and Hygiene, University of Tübingen). As only *Achromobacter* species could be detected before (medium sample) and after incubation (different t0 min treatment groups), the possibility of further contamination was excluded.

Blue light irradiation

For irradiation therapy, we used the DermoDyne® UV-free irradiation device (DermoDyne HealthCare, Berlin), which is used to treat skin disorders such as atopic dermatitis, psoriasis, eczema, and alopecia areata (16,31). We have previously shown that irradiation with this device has antimicrobial effects (unpublished data). DermoDyne® emits blue light with a wavelength of 400-450 nm (28.9 J/cm^2) in a pulse rate of 2 to 5 s (for our trials, a pulse rate of 5 s was used) at a glass surface on the top of the device, on which the cell culture plates were placed. As the device also releases heat, well plates were placed at a 2 cm distance from the light source. The control group (0 min group) was protected from the light by aluminum foil and placed on the device to ensure the same thermal influence as the treatment groups. To investigate whether this thermal energy has any impacts on the cells, another control group was further incubated without being placed on the device (incubator group/Baseline BL). Treatment groups were irradiated with blue light for 10, 20, or 30 minutes. The 10-minute group was wrapped in aluminum foil following

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irradiation and placed on the device for another 20 minutes until irradiation of the 30-minute group had finished. The 20-minute group underwent the same procedure for 10 minutes, which meant that cells were protected from the light but not from potential heat exposure. This procedure was repeated each day after the scratch assay of all sterile and contaminated cell culture medium samples. Color changes of phenol red as a result of acidification due to bacterial contamination (as commonly used in cell culture) was used to assess differences in bacterial contamination in addition to microscopical observations.

Sulforhodamine B staining

The Sulforhodamine B (SRB) assay is an established technique developed by Skehan *et al.* (32) as a cytotoxicity assay for anticancer drug screening. Because SRB binds to surface proteins under acidic conditions, it can be used to document viability / cytotoxicity.

After the scratch assay, cells were washed once with 1 mL PBS and then covered with 500 µl Ethanol (99%), as reported. All well plates were then stored at -20 °C for further investigation. Ethanol was removed; the cells were washed once with tap water, and the plates were then air-dried in a temperature-controlled room (room temperature 21±1 °C; relative humidity 36-42%; no additional warming). Cells were covered with 250 µl SRB solution (4% SRB with acetic acid) and incubated while protected from light. The SRB solution was removed after 30 minutes of incubation; the remaining unbound SRB was washed four times with acetic acid solution until fully removed. The bound SRB was resolved with 500 µl 10 mM unbuffered TRIS solution. Finally, absorbance was measured at λ=565 nm (SRB) and λ=690 nm (impurities), and $OD_{690\ nm}$ were subtracted from $OD_{565\ nm}$. The 0 min group was designated as a control (100% viability). Viability assays use normal cell metabolism to detect cells viability. As normal eukaryotic cells (like the HaCat cell line we used for our experiments) and prokaryotic cells (like bacteria) have basically the same metabolic mechanisms, it is not valid to determine the cell viability of only the HaCat cell line if the cells have already been contaminated. Viability results would then only measure the "combined" viability of both HaCat cells and bacteria and would not provide precise information about whether HaCat or bacterial cells have been damaged due to irradiation. For this reason, SRB staining was performed only with sterile samples, as bacterial metabolism and protein synthesis in contaminated scratch assays are likely to interfere with SRB measurements and are therefore unreliable.

Heat development

As excessive heat exposure could harm the cells, we measured the surface temperature of the device and that of a well plate filled with cell culture medium. As in the previous scratch assays, the plates were placed at exactly the same distance from the device. Measurements were performed using the same digital laser thermometer for an irradiation period of 30 minutes at 0, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 15, 20, 25, and 30 minutes (n=1).

Statistics

All data are presented as means with standard deviation (SD). Data were analyzed using Kruskal-Wallis Test with Dunn0s multiple comparison test to analyze differences between groups.

All analyses were performed using the GraphPad Prism statistical software package (Version 6, GraphPad Software, La Jolla, USA).

RESULTS

Wound closure in sterile wounds

To investigate the effect of blue light on re-epithelization, we analyzed wound closure in a scratch wound healing assay using HaCaT keratinocytes.

Day 1

Although the control groups showed noticeable wound closure (incubator group 13.05±4.24 percentage points at day 1; 0 min group: 8.09±2.72 pp at day 1), irradiation with blue light resulted in much slower wound healing. While ten minutes of light therapy each day slowed re-epithelization (2.29±4.80 pp at day 1), irradiation for 20 minutes daily or longer resulted in slight enlargement of the scratch wound (20 min: -1.17±1.86 percentage points at day 1; 30 min: -0.78±1.80 pp at day 1).

Day 2

Similar results were obtained on the second day. Once again, both control groups showed obvious acceleration of wound closure (incubator group: 11.25±0.37 pp at day 2; 0 min group: 15.65±2.22 pp at day 2) while treatment groups exhibited slower healing (10 min group: 3.25±7.09 pp at day 2; 20 min group: 0.052±1.760 pp at day 2, 30 min group: 0.13±1.11 pp at day 2).

Day 3

On the third day, control groups (incubator group: 8.68±1.78 pp at day 3, 0 min group: 15.17±6.51 pp at

day 3) once again showed faster wound closure than treatment groups (10 min: 3.01±5.08 pp at day3, 20 min: -0.78±1.63 pp at day 3, 30 min: -0.34±0.25 pp at day 3). As on the first day, some of the treatment groups exhibited enlargement of the scratch wound.

Viability following irradiation

The 0 min group was set as reference/control (100.00±16.73%). All treated groups showed lower viability than the control group (10 min: 59.85±17.72%; 20 min: 38.51±6.77%; 30 min: 60.42±10.78%). Viability of the incubator groups was also lower than in the control group (56.37±9.02%) (Figure 1).

Wound closure in contaminated wounds

As treatment with blue light has been reported to have antimicrobial effects, we investigated wound closure using cells cultivated in an unsterile medium that tested positive for *Achromobacter* species (contamination of medium). Microbiological testing indicated greater bacterial growth following incubation in the scratch assay samples (+++) than in the cell culture medium sample used for those contaminated scratch assays (+).

In general, wound closure was much slower in contaminated scratch assays than in sterile scratch assays. While sterile control groups exhibited only gradually decreasing wound closure, this was very low from the outset in contaminated probes (13.05 pp and 11.25 pp mean value in the sterile incubator group versus 5.30 pp and 2.13 mean value in the contaminated incubator group).

Day 1

All contaminated groups showed slower wound closure when compared with sterile groups. In the presence of bacteria, control groups displayed values of 5.30±1.43 pp at day 1 (incubator group) and 4.19±2.12 pp at day 1 (0 min group); treatment groups exhibited much slower wound closure on the first day (10 min: 0.20±1.00 pp at day 1; 20 min: -0.26±0.57 pp at day 1; 30 min: -0.38±0.55 pp at day 1). Some treatment groups exhibited enlargement of the scratch wound following irradiation.

Day 2

Similar results were obtained on the second day. Both control groups showed clear positive wound closure (incubator group: 2.13±2.36 pp at day 2; 0 min group: 1.67±1.01 pp at day 2), while treatment groups exhibited wound closure that was negative (10 min: -0.04±0.73 pp at day 2) or only weakly positive wound closure (20 min: 0.48±0.78 pp at day 2; 30 min: 0.49±0.38 pp at day 2) (Figure 2).

Reduction of bacteria due to irradiation

While groups treated with blue light showed only low contamination, both control groups exhibited strikingly increased contamination under the microscope. The medium was cloudy and had turned orange, indicating acidification due to high bacterial contamination. Irradiation with blue light clearly reduced bacterial contamination in all samples (Figure 3).

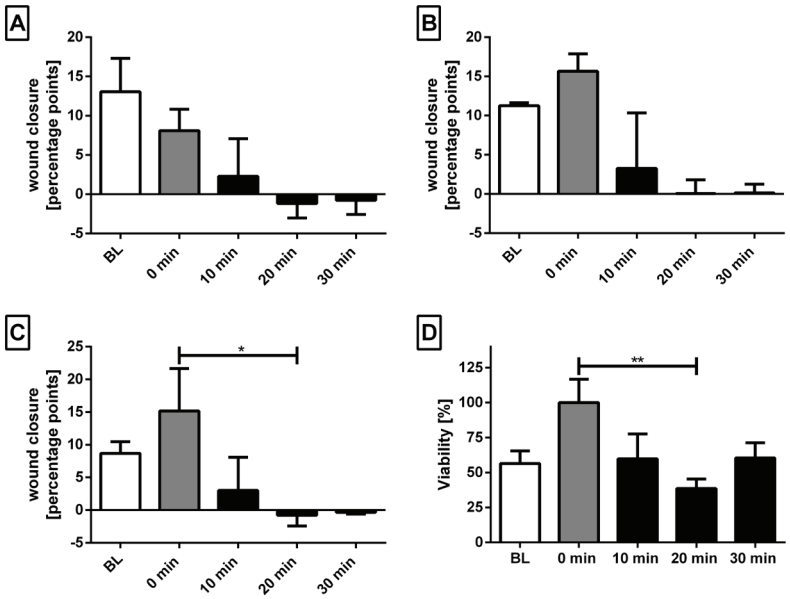

Figure 1. Extent of wound closure over time and cell viability following different durations of irradiation in sterile scratch assays. Wound closure: (A) day 1; (B) day 2; (C) day 3. Viability: (D). (BL = Baseline/incubator group) (n = 3)
*p≤0.05; **p≤0.01

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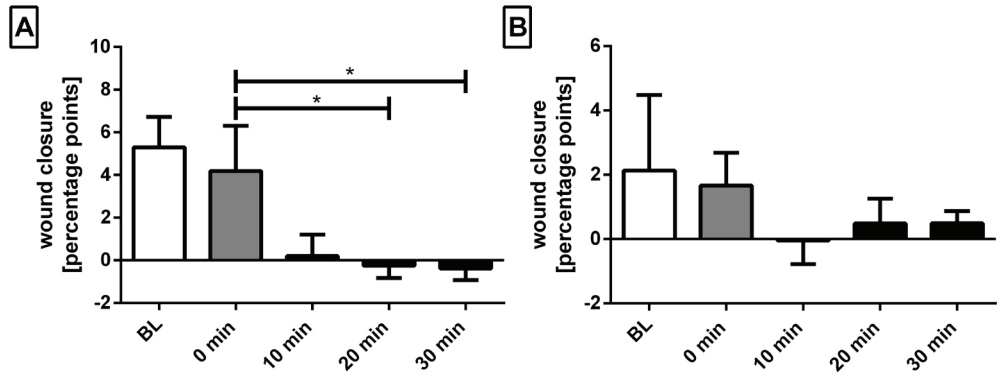

Figure 2. Extent of wound closure over time and cell viability following different durations of irradiation in contaminated scratch assays. Wound closure: (A) day 1; (B) day 2. (BL = Baseline/incubator group) (n = 5)
*p≤0.05

Heat development

To exclude potential damage due to heat development, we measured the surface temperature of the device and of a well plate placed at exactly the same distance from the device as in previous scratch assays.

While the device warmed up to 60.3 °C (maximum measured value at 30 min), the temperature of the well plate never rose above 37 °C (dashed line). The maximum measured temperature was 33.6 °C after an irradiation time of 20 minutes (Figure 4).

DISCUSSION

Chronic wounds and pronounced wound healing disorders represent an increasingly common and significant issues and are often linked to wound contamination or infection (33). Previous studies have indicated the potential of blue light irradiation for wound healing (5,11) and for treatment of wound infections (27). In consequence, phototherapy is a promising treatment for contaminated wounds in daily nursing care. Despite this, advantages and limitations are still not completely understood. In this context, we evaluated the effect of blue light on re-epithelization in vitro.

As re-epithelization is the decisive step in the process of wound healing, we used the human HaCaT keratinocyte cell line for these wound healing experiments. HaCaT cells have been reported to be nontumorigenic and maintain full epidermal differentiation capacity (34).

For irradiation therapy, we used the DermoDyne® UV-free irradiation device (DermoDyne HealthCare, Berlin), which is used to treat skin disorders such as atopic dermatitis, psoriasis, eczema, and alopecia areata (16,31). As the device has already been established in clinical practice for these diseases, our investigation serves to clarify what happens to the top layers of skin during this form of light therapy.

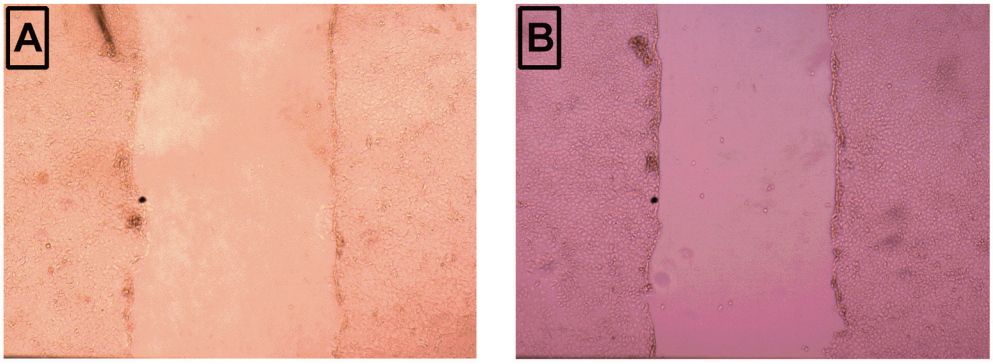

Figure 3. Representative images of scratch assay with contaminated cell culture medium after 24 h. In control groups (A, here baseline BL group), the medium was cloudy even after 24 hours of incubation and additionally turned orange over time due to massive bacterial growth. Because of the turbidity of the cell culture medium, the borders of the scratch wound became unclear and less definable than at the outset. Treatment groups (B, here irradiation time 30 min) showed significantly less contamination, as shown by the more normal purple color of the cell culture medium and the clearly defined borders of the scratch wound.

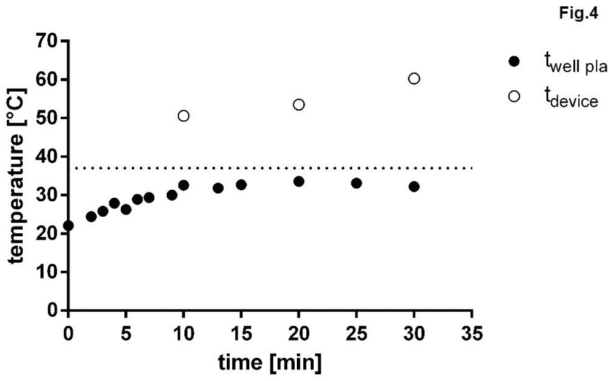

Figure 4. Heat development during irradiation with blue light. While the temperature of well plates (black circles) never rose above 37 °C (dashed line), the device itself heated up much more (empty circles).

To improve our knowledge about the quantitative reduction of bacteria, we initiated a study in order to perform a quantitative evaluation of the reduction of common burn wound pathogens. This would allow us to demonstrate that irradiation with DermoDyne® showed high antimicrobial effects (unpublished data).

The effect of blue light irradiation on normal skin has been previously assessed in other studies. While no inflamed or sunburn cells were detected following irradiation, there was an increase in perinuclear vacuolization of keratinocytes, but no significant change was observed in p53 expression (15).

Our data confirm previous findings in relation to the antimicrobial properties of irradiation with blue light. When compared with control groups, the irradiated groups exhibited much lower bacterial contamination.

However, our findings indicate that *in vitro* irradiation with blue light decreases re-epithelization and may reduce cell viability. As well plate temperatures never rose above 37 °C, thermal damage due to irradiation can be excluded as an explanation for slower wound closure. Instead, it seems more likely that the irradiation with blue light is responsible for this effect.

In the sterile scratch assays, wound closure was slightly recurrent over time, probably due to a physiological medium/nutrient consumption of the cells. Additionally, we found that irradiation with blue light had a dose-dependent negative effect on the cells. An irradiation time of 10 minutes resulted in slower wound healing and poorer viability, indicating that even low doses of blue light could harm keratinocytes. Longer exposure to blue light even resulted in enlargement of the scratch wound, possibly as a consequence of cell detachment, indicating that this treatment might aggravate wound healing disorders or interfere with re-epithelization. All treatment groups exhibited lower viability when compared with the control group (0 min group), as did the baseline group, possibly due to optimal growth conditions resulting in higher cell numbers as well as a lack of nutrition supplements in the cell culture medium after 72 hours. It was notable that the incubator group also exhibited viability at a lower level similar to all treatment groups. The high wound closure rate in this group on day 1 indicates that the optimal conditions in this incubator may have led to higher cell metabolism with relatively elevated medium consumption. This would have reduced the remaining nutrients in the cell culture medium in these samples on the following days and may explain their poorer viability at the end.

The presence of bacteria resulted in much slower wound closure in scratch assays from the outset. It is known that bacterial contamination makes cell culture development impossible, as this adversely affects normal cell growth and metabolism. Although contamination was clearly reduced by irradiation, exposure to blue light had negative effects on cell proliferation and wound closure in these contaminated probes.

As we could find little data related the effects of blue light irradiation on keratinocytes, we searched for comparable studies on the effects of blue light on wound healing in general.

While previous studies (26) have reported similar wound closure in fibroblasts for control and treatment groups (wavelength 470 nm; intensity 3, 5, 10, or 55 J/cm2), blue light increased protein synthesis.

In scratch and viability assays using different cell types (fibroblastic, myoblastic, and keratinocytic), Teuschl *et al.* (35) showed that irradiation with blue light (wavelength 470 nm; intensity 50 mW/cm^2) prolonged time to closure (i.e., slower wound healing), with higher apoptosis rate and lower proliferation. Red light, on the other hand, seemed to have opposite effects. Other *in vitro* studies (36) have shown that fibroblast proliferation and migration are slowed by blue light, which may therefore contribute to the treatment of keloids and other fibrotic skin diseases. Although blue light with a 415 nm wavelength is associated with increased reactive oxygen species (ROS) generation (37), pre-treatment of skin fibroblasts with resveratrol prevents effects on fibroblast migration speed (37). Another study (38) showed that blue light at nontoxic fluences reduces proliferation of human keratinocytes and skin-derived endothelial

cells. It was suggested that this may be attributable to differentiation induction, given the increase in differentiation markers (38).

In vivo studies (28,39) have shown that blue light enhances wound healing. Reports that blue light induces angiogenesis may explain these different results *in vivo*; as these positive effects may surpass the negative impact on re-epithelization, wound healing would be improved overall, especially in the case of infected wounds.

In contrast to effects on wound healing, the antimicrobial properties of irradiation with blue light have already been documented in the literature (17-24). However, we did not find a study that showed the logarithmic reduction of bacteria.

We could find no preliminary studies addressing the potential benefits of irradiation with blue light in contaminated or infected wounds *in vitro*. In the present study, we demonstrated that the deleterious effects on bacterial growth also affected other cell types, such as keratinocytes. Many bacteria are known to form biofilms, and we used a model of planktonic inoculum in our study because it is difficult to combine scratch assays with bacterial biofilm, since these normally need some time to grow and a medium change is usually performed after the scratch procedure, so that all samples have the same basic conditions for wound closure and so that cell debris can be fully removed in order to photograph the results. We recommend further studies, for example experiments with different bacterial strains and *in vivo* studies with bacterial biofilms.

Further research is required to clarify the benefits and limitations of light therapy for wound healing in contaminated wounds.

CONCLUSION

Our findings suggest that blue light slows wound healing and re-epithelization *in vitro*. Even in contaminated probes, this negative impact on wound healing overrides any antimicrobial properties. As irradiation with blue light is often linked with heat release, this may explain the improvement in wound healing after phototherapy *in vivo*. Nevertheless, further studies are required to clarify both the potential scope and the limits of this new treatment option. As long as these questions remain unresolved, irradiation with blue light in the context of wound healing should remain subject to critical scrutiny.

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