Evaluation of Fluorescent Light Energy for the Treatment of Acute Second-degree Burns

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ABSTRACT

Introduction:
The use of photobiomodulation has been proposed to improve wound healing for the last two decades. Recent development in photobiomodulation has led to the development of a novel biophotonic platform that utilizes fluorescent light energy (FLE) within the visible spectrum of light for healing of skin inflammation and wounds.

Materials and Methods:
In this article, FLE was used in preliminary analysis on 18 case studies of acute second-degree burns and in a pilot study using an *ex vivo* human skin model. Efficacy of FLE on wound healing and tissue remodeling was evaluated by monitoring improvements in the treated tissues, assessing pain for the patients, and by performing human genome microarray analysis of FLE-treated human skin samples.

Results:
Healing was reported for all 18 patients treated with FLE for acute second-degree burns without reported adverse effects or development of infections. Furthermore, preliminary *ex vivo* skin model data suggest that FLE impacts different cellular pathways including essential immune-modulatory mechanisms.

Conclusions:
The results presented in this article are encouraging and suggest that FLE balances different stages of wound healing, which opens the door to initiating randomized controlled clinical trials for establishing the efficacy of FLE treatment in different phases of wound healing of second-degree burns.

INTRODUCTION

Wound healing consists of three phases: (1) hemostasis and initiation of inflammation, (2) wound closure through proliferation and re-epithelialization, and (3) maturation via extracellular matrix deposition and remodeling.[1] A balanced wound healing process is vital to ensure wound closure and tissue regeneration without excessive inflammation and scar formation. Immune cells are important in regulation of the wound healing process through removal of invading bacteria, regulating inflammation, and facilitating extracellular matrix deposition and remodeling.[1,2] First, myeloid cells reside in the skin where they patrol the largest barrier of the body and are thus among the first immune cells at the sites of inflammation. They clean up the tissue by removing invading bacteria as well as dead cells and damaged tissues. Second, activated phagocytes relay activation to the later responding and persistent adaptive defense (T cells), through presentation of antigen on major histocompatibility complex (MHC) molecules. Third, immune cells regulate angiogenesis and deposition of ECM through secretion of factors such as fibroblast growth factor (FGF), vascular endothelial growth factor, and transforming growth factor beta (TGF-β) important for activity of fibroblasts and keratinocytes.[2,3]

Imbalanced wound healing leads to excessive inflammation and formation of keloid or hypertrophic scars in the injured tissue.[1] Second-degree (or partial-thickness) burns affect both the outer epidermal as well as the deeper dermal layer of the skin, and hence require longer healing time than superficial epidermal burns (first-degree burns). Because of longer healing time, second-degree burns are common causes of persistent bridle type and hypertrophic scars.[4] Treatment for second-degree burns requires painful dressings and for deep burns hospitalization for skin grafting resulting in prolonged wearing of compression garments or other specific

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treatments, generating significant healthcare costs and having a profound effect on patients' quality of life (QoL).[4,5]

Despite leading progress in the treatment of burns, the strategies implemented have not yet significantly improved burn healing in a clinical setting. The prognosis for the scarring of burns is difficult to establish during the initial healing phase and the possibility of predicting development of a pathological scar remains uncertain. Burns cause severe inflammation and the risk of infection is significant in deep second-degree lesions, associated with escalated inflammation, prolonged healing time, and increased risk of improper healing and substantial scar formation.[6] Progression of burn injury through deeper tissues (conversion) is a critical aspect of prehospital burn trauma care as it often contributes to greater depth and surface area of the injured tissue. This larger, deeper wound has multiple local and systemic consequences that increase complications and morbidity. Halting burn conversion is a critical aspect of medical intervention in burn treatment.[5] Silver sulfadiazine (SSD) is the most prescribed antibacterial agent for topical treatment of burns; however, the efficacy of SSD treatment is still debated.[7] New therapies are needed to accelerate a balanced healing process.[6]

Photobiomodulation (PBM) therapy has proven effects on a variety of inflammatory skin conditions and healing of different types of acute and chronic wounds.[8-11] Studies have described that different wavelengths can affect different stages of inflammation and wound healing.[12,13] A study investigating PBM therapy in healing of second-degree burns in a rat model showed that 660 nm light was highly efficient in the early inflammatory and acute phase by enhancing crust formation that support cellular migration, whereas 780 nm light was more efficient in the later and regenerating phase of wound healing observed by fewer fibroblasts, less granulation tissue, and enhanced collagen deposition at the end of the experimental period, overall supporting formation of newly formed normal dermal tissue.[14]

A specialized type of PBM therapy utilizes a biophotonic platform that consists of a gel containing chromophores, which are activated by a light-emitting diode (LED) lamp. This combination creates a spectral output of fluorescent light energy (FLE) in a broad spectrum of wavelengths ranging from 400 to 700 nm.[15] Several reports have described a positive effect of FLE on wound healing, as well as on skin rejuvenation and inflammatory skin conditions such as acne and rosacea.[15-23] Cellular mechanisms for FLE include lowered secretion of pro-inflammatory cytokines (IL-6 and TNF-α), promotion of angiogenesis, and stimulation of collagen from primary fibroblasts.[15,2,23] Efficacy of FLE therapy was recently shown in human clinical studies on chronic wounds such as venous leg ulcers, diabetic foot ulcers, and pressure ulcers.[24,25] Furthermore, FLE was found to promote wound healing *in vivo* in a canine deep pyoderma model.[23] Studies on the impact of PBM on cellular activity have shown that mitochondrial activity is affected by PBM.[8] Research into the molecular actions of FLE are ongoing, but so far suggest that mitochondrial biogenesis is involved. FLE was found to normalize mitochondrial morphology and increase expression of genes essential for mitochondrial ATP production in inflamed human dermal fibroblasts *in vitro*,[26] and alter mitochondrial morphology while enhancing wound healing *in vivo*.[23]

We have evaluated the effect of FLE therapy in prospective observational trials (Investigator Initiated Trials [IITs]) on the initial treatment of superficial and deep second-degree burns in 18 patients divided in 2 IITs. Moreover, we have included preliminary data from an *ex vivo* human skin model to give perspective on the cellular mode of action behind promotion of wound healing and tissue regeneration mediated by FLE.

MATERIALS AND METHODS

Ethics Statement

Both studies conducted were case series in IITs following the CE-marked approved intended use of the FLE platform; hence, no approval from ethics committees was needed. Informed written consents were obtained from all participants. The human *ex vivo* pilot study was performed under written informed consent according to the Helsinki guidelines.

Patients Included in IITs

Ten patients with thermal second-degree burns were included in the IIT in Montpellier University hospital (France) (IIT-1). Criteria for inclusion of patients in IIT-1 are listed in Table A1. Eight patients with second-degree burns (four superficial and four deep) were included in IIT-2 in Padova University Hospital (Italy).

FLE System

The biophotonic platform consists of a chromophore-containing-gel and a multi-LED lamp delivering blue light with wavelengths ranging from 440 to 460 nm and a power density between 55 and 129 mW/cm^2. The gel is prepared immediately before use by mixing chromophores into a neutral hydro-carrier gel, as previously described.[23,25]

FLE Treatment

The gel was applied to the wound (area to be treated) in a 2 mm thick layer, and illuminated by the multi-LED lamp at a distance of 5 to 9 cm for 5 minutes, as previously described.[16,20]

IIT-1: Patients were treated with FLE twice per week for 2 to 3 weeks, in combination with usual dressings made for burns and with the application of SSD. Before FLE treatment, the wound was cleaned with sterile saline, gel was applied, and illumination by the multi-LED lamp was performed at a distance of 9 cm for 2×5 minutes. After FLE treatment, the gel was removed by sterile saline wash. SSD was then applied to all burns. FLE treatment was performed one to five times. IIT-2: Patients were treated with FLE two to three times per

week, in combination with usual dressings made for burns. Before FLE treatment, the wound was cleaned with sterile saline, gel was applied, and illumination by the multi-LED lamp was performed at a distance of 5 to 9 cm for 5 minutes, with a minimum of 2 days interval between treatments. After FLE treatment, the gel was removed by sterile saline wash. FLE treatment was performed two to eight times.

Pain Assessment

Pain assessment was performed for every patient during treatment (IIT-1) or before initiation of the trial and after FLE treatments were terminated (IIT-2), according to the Visual Analog Scale (VAS).[27] The pain was assessed with the numerical scale ranging from 0 (no pain) to 10 (severe pain).

Clinical Assessment of Wound Healing

In both IITs, wounds were photographed at treatment initiation and follow-up control, and at several time points during the treatment period.

Ex vivo Human Skin Model

Human skin sample was obtained from a routine face-lift procedure after informed consent from the patient. Organ cultures of full-thickness scalp skin were prepared by 4-mm biopsy punches cultured at the air-liquid interface in serum-free William's E medium (Biochrom, Cambridge, UK) supplemented with 100 IU/mL penicillin (Sigma, St Louis, MO, USA), 10 μg/mL streptomycin (Sigma), 10 μg/mL insulin (Sigma), 10 ng/mL hydrocortisone (Sigma), and 2 mmol/L L-glutamine (Invitrogen, Paisley, UK). The cultures were maintained at 37 °C, 5% CO_2.

Punches (samples) were divided into three groups (1) untreated control (prepared on day 0), (2) LED (blue light alone), and (3) FLE (gel and blue light) with two punches per group and illuminated by the multi-LED lamp at a distance of 5 cm for 9 minutes. Untreated control samples (group 1) were prepared immediately, while samples from groups 2 and 3 were prepared 24 hours after treatment. The model was established by and purchased from the Monasterium Laboratory Skin & Hair Research Solutions GmbH (Münster, Germany).

Immunohistochemistry and Immunofluorescence

At the indicated time points, half of each punch was embedded into cryomatrix (Thermo Fisher Scientific, Schwerte, Germany), frozen in liquid nitrogen, and stored at −80 °C for later analysis. For histological analysis, 6 μm thick sections were prepared from tissue in the center of the punch. Hematoxylin and eosin stain was done to demarcate nuclei and acidophilic structures (collagen and elastin) to evaluate tissue integrity. Antibody used was anti-collagen I (5D8-G9, Abcam, Cambridge, UK), rabbit anti-mouse Alexa-Flour 488 (Invitrogen), and nuclei stain was done with standard 4′, 6-diamidino-2-phenylindole. Omission of the primary antibody served as negative control. Images were obtained with a digital microscope (Keyence, Neu-Isenburg, Germany).

Quantification of Collagen I

Quantification of collagen I in the dermis was done by quantitative immunohistomorphometry. Relative intensity of collagen I immunoreactivity was assessed in defined dermal tissue reference areas (visual fields) using ImageJ (NIH, Bethesda, MD). Data are presented as arbitrary units of collagen I immunoreactivity from 8 to 16 visual fields from 2 individual punches from each group.

Affymetrix GeneChip Human Genome Array

At the indicated time points, half of each punch was stored in 500 μL RNA later, overnight at 4 °C. RNA was extracted by RNeasy Mini Kit (Qiagen, Hilden, Germany), samples were sent on ice to Immunology, Frederiksberg, Copenhagen University, and stored at −80 °C until analysis. Samples were analyzed by the Center for Genomic Medicine (Copenhagen University Hospital, Denmark) using a Human Gene 2.0 ST Array (includes lincRNA probes) microRNA expression Arrays (Affymetrix, Santa Clara, CA, USA). Data were analyzed using Expression Console Software 1.4 (Affymetrix) and Transcriptome Analysis Console Version 4.0.1.36 (Thermo Fisher Scientific).

RESULTS

The use of FLE treatment was proven to be nonirritating to the skin and safe for use on wounds according to previous *in vitro* assays, *in vivo* studies conducted in different animal models, as well as in previous clinical trials and case studies.[15-25,28]

In this article, we tested FLE treatment on 18 patients with second-degree thermal burns ranging from superficial to deep burns in 2 independent preliminary studies (IIT-1 and IIT-2). We found that in all cases complete healing was observed without the need for skin grafting, and no adverse events related to the FLE treatment were reported. No patient developed an infection.

The healing time reported in IIT-1 ranged from 7 days for some partial thickness burns extending to a maximum of 21 days for full-thickness burns. Patients started FLE treatment between day 1 and 8 (average: day 2.9) after their injury and underwent between 1 and 5 treatments. Patients terminated their FLE treatment between day 7 and 21, and follow-up was carried out until day 30 for all burns except one at day 50 (Table A2). Assessment of wound healing in both IIT-1 and IIT-2 was monitored throughout the studies by clinical assessment of wound healing and of clinical signs of infection, and pictures before each FLE treatment, exemplified by seven cases in Figs. 1 and 2. Steady improvement through wound closure, reduced inflammation, and tissue healing was observed throughout the treatment and follow-up period (Figs. 1 and 2). Furthermore, the clinical assessment of

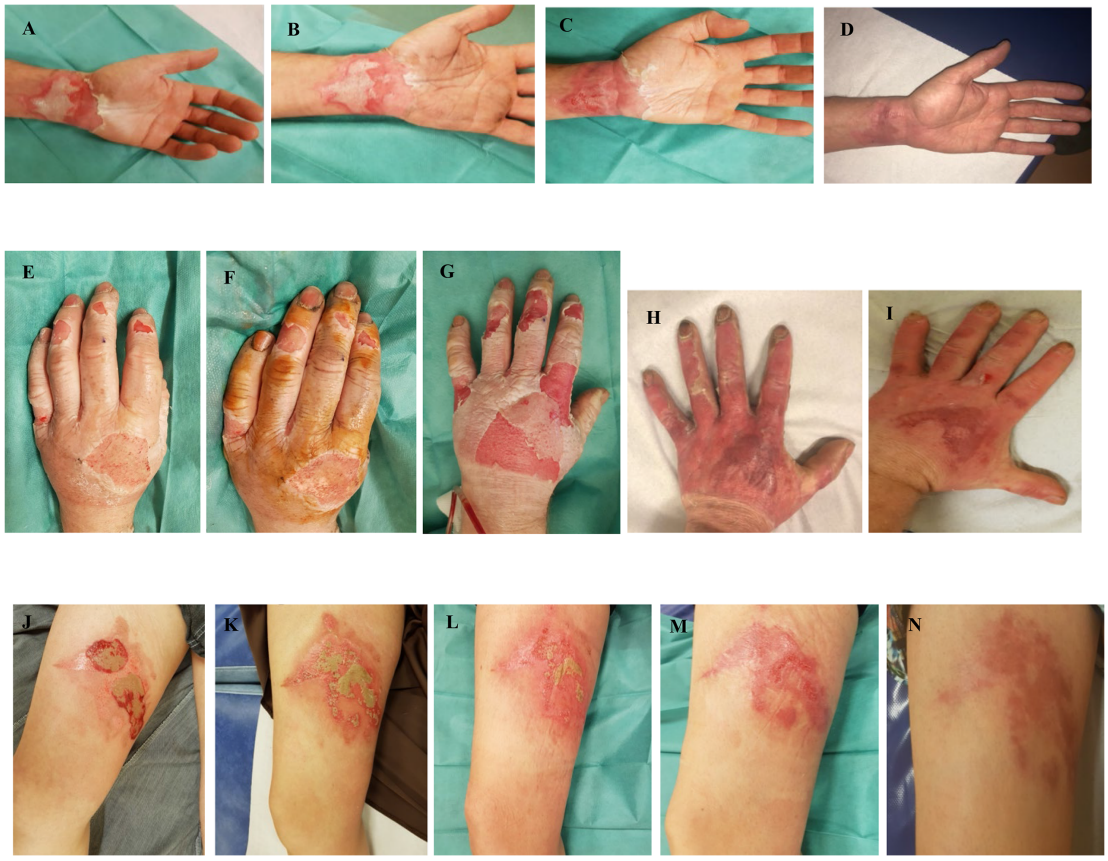

FIGURE 1. Wounds from deep second-degree burns of three cases in Investigator Initiated Trial 1. **Case 1,** burn of the wrist. **(A)** Day 8, before fluorescent light energy (FLE) treatment was initiated. **(B)** Day 12, before the second FLE treatment. **(C)** Day 19, after FLE treatment was terminated. **(D)** Day 30, follow-up. **Case 2,** burn of the back of the hand. **(E)** Day 1, before FLE treatment. **(F)** Day 1, immediate appearance after the first FLE treatment. **(G)** Appearance at Day 3 **(H)** Day 18, complete healing after four FLE treatments. **(I)** Day 30, follow-up. **Case 3,** burn of the anterior thigh. **(J)** Day 1, before FLE treatment was initiated. **(K)** Day 5, appearance before second FLE treatment. **(L)** Day 9, before the third FLE treatment. **(M)** Appearance at Day 16. **(N)** Day 30, appearance at follow-up.

the burns at follow-up visits, although insufficient to predict the occurrence of a hypertrophic scar, showed no evidence of a pathological early scar, even for deep burns since this risk often associates with the degree of inflammation.[29]

In addition, in IIT-1 pain assessment was carried out during FLE treatment. At the first treatment, the VAS intensity for the pain score ranged from 2 to 8 for 10 patients. No correlation was observed between the VAS intensity and the depth of the burn. The pain assessment showed that the gel application and illumination by the LED lamp was well tolerated by patients, although five patients reported some pain at the beginning of the treatment. In the IIT-2 study, pain assessment was done at the beginning and at the end of the treatment period with FLE. The VAS intensity ranged from 2 to 9 before FLE and dropped to 0 for all FLE treated patients. In summary, healing was achieved without the need of surgery in all 18 patients included in IIT-1 and IIT-2 within 1 to 3 weeks leaving the efficacy profile of FLE treatment for second-degree burns in this study to can be considered satisfactory. Data are summarized in Tables A2 and A3.

These results of both IITs suggest that FLE therapy supports healing in the early stages of the wound healing and tissue remodeling process. To understand how FLE regulates these processes, we performed a pilot study using an *ex vivo* human skin model to screen for target molecules, cells, and pathways directly affected by FLE treatment. Interestingly, preliminary data in Fig. 3 show increased dermal collagen I after FLE treatment (Fig. 3Aand B), without affecting the integrity of the skin barrier or inducing inflammation (Fig. 3C). In line with previous reports, we found that also several immune factors were affected by FLE treatment.[15,30] Specifically, these data indicate that several MHC class II molecules are downregulated by FLE treatment, while MHC class I molecules are largely unaffected (Fig. 3D). MHC molecules are vital for presenting antigen to T cells and thereby relay an activating signal to the adaptive immune response to continue inflammation.[31] Finally, we saw that expression of FGF2 and anti-inflammatory cytokines TGF-β1 and TGF-β3 were induced by FLE treatment (Fig. 3E).

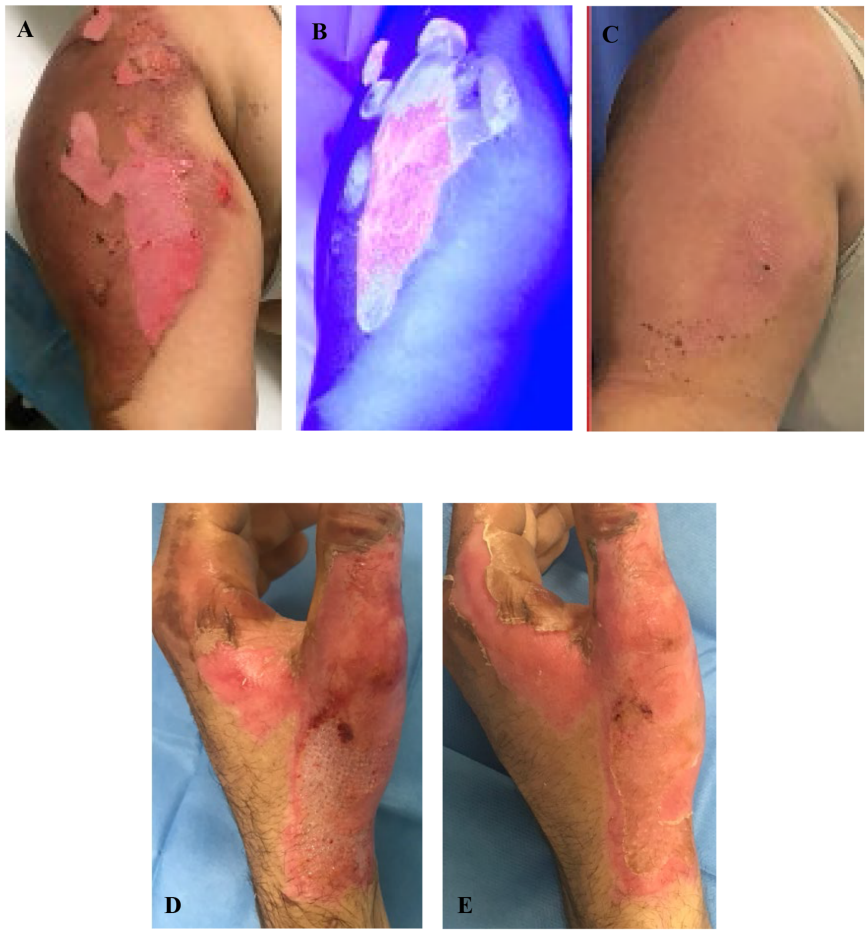

FIGURE 2. Wounds from superficial and deep second-burns of two cases included in Investigator Initiated Trial 2. **Case 1a,** burn of the upper arm. **(A)** Day 1, appearance before fluorescent light energy (FLE) treatment was initiated. **(B)** Picture taken during FLE treatment. **(C)** Day 10, appearance after 3 FLE treatments. **Case 2a**, burn of the hand. **(D)** Day 1, appearance before FLE treatment was initiated. (E) Day 5, appearance after termination of FLE treatment.

Although preliminary, these data shed light on the mechanisms underlying the observed clinical effects of FLE treatment on various inflammatory conditions and at different stages of wound healing and remodeling, indicating that FLE balances essential pathways in inflammation and healing.

DISCUSSION

The impact of wavelengths ranging from the visible to the infrared spectrums on healing is evident. In particular, the use of visible light in PBM therapy has been shown to reduce inflammation and stimulate wound healing in several studies using animal models as well as in clinical trials.[10-12]

The preliminary observational studies presented in this article were conducted to assess the effect of FLE treatment, primarily on the acute phase of the healing process in second-degree burns in humans. Findings indicate that the combined actions of blue LED light and a biophotonic gel, generating FLE was beneficial in treatment of second-degree burns (both superficial and deep). Healing was obtained for all 18 observed patients with acute second-degree burn wounds and no patient developed an infection. These findings are in line with a recent study showing that FLE accelerates wound healing after skin grafting in a dermal mouse model (Ding et al., "Fluorescent light energy (FLE) accelerates wound closure and re-epithelialization in a skin graft mouse model" manuscript under review in Journal of Tissue Science and Engineering). In addition, FLE treatment has previously been reported to reduce inflammation and improve overall skin texture,[21] which will likely aid in reducing the risk and appearance of scar tissue from burn wounds. This is in line with previous findings in which the effect of FLE treatment was assessed on mature scars from burn wounds on the lowers limbs. Throughout the study period, the scar tissue became softer, pliable, and less itching and painful resulting in increased QoL scores for the patients (Bassetto et al., Fluorescent Light Energy: a new tool for the treatment of hypertrophic scars? Plast Aesthetic Res 2020; In Press).

The advantage of FLE is the noninvasive, easily applicable, and efficient nature of this therapy. Main disadvantages are associated with the biweekly sessions according to the intended use and followed in these IITs, and to pain reported by some patients in IIT1. Furthermore, some patients reported pain in the beginning of the treatment. Both issues are currently being evaluated and are central for optimizing protocols

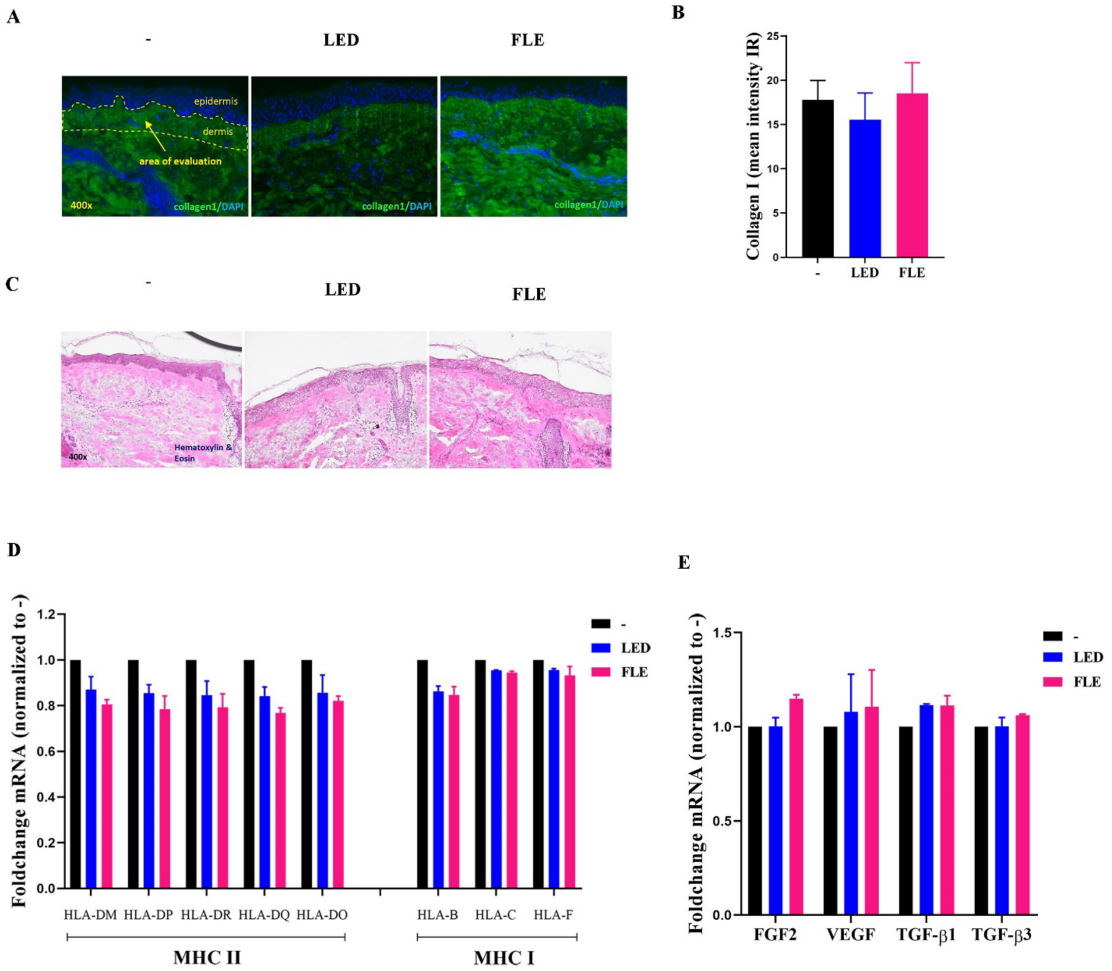

FIGURE 3. Pilot study on *ex vivo* human skin model. *Ex vivo* human skin punches was treated with light-emitting diode (LED), fluorescent light energy (FLE) or prepared directly after excision without treatment (-). **(A)** Staining for Collagen I (green) and DAPI (blue) in dermal tissue **(B)** Quantification of Collagen I in dermal tissue. **(C)** Hematoxylin and eosin staining of tissue. **(D-E)** Transcriptional regulation of MHC class I and class II **(D)**, as well as FGF2, VEGF (collected VEGF-A/B/C), TGF-β1, and TGF-β3 **(E)** were analyzed in total RNA (from full thickness skin samples) 24 hours after treatment.

in future studies. It is possible that the use of desensitizing creams (e.g., containing lidocaine) or targeted optimization of the gel can reduce the sensation of heat and pain associated with FLE treatment.

Interestingly, although data are preliminary, a human *ex vivo* skin study suggested that FLE treatment promotes fibroblast activation and dermal collagen production without inducing inflammation. Furthermore, these data suggested a specific down-modulation of MHC class II molecules. This could indicate that FLE potentially hampers activation of the adaptive immune response initially in the wound healing process, thus preventing development of chronic inflammation and wounds. These suggestive *ex vivo* findings needs repetition for validation along with further in-depth research into the cellular and molecular mechanisms regulated by FLE is ongoing to fully understand the impact of this therapy. Since FLE affects different cell types in the skin leading to a balanced healing process, we hypothesize that a common cellular organelle/molecule is target for the energy delivered by FLE. Cytochrome c oxidase is part of the mitochondrial respiratory chain that can respond to PBM via absorption of delivered photons resulting in enhanced mitochondrial ATP production.[32] Based on the indicated impact of FLE on mitochondrial activity, we speculate that cytochrome c oxidase is an important acceptor of FLE although this needs further experimental validation. Insight into the mode of action of FLE is important to optimize both components of the treatment (the gel and the LED source) and will provide knowledge important to better target protocols that are applicable and benefits both wound care units and the patients.

In line with repetition of *ex vivo* findings to support the observations that FLE is noninvasive and function by targeting pathways in different stages of regeneration, more comprehensive randomized and controlled clinical studies are needed to establish the beneficial effects of FLE treatment on healing of second-degree burn wounds observed and reported in this article.

Management of second-degree burns is a challenge in wound care. Diagnosis is vital for directing treatment, especially in the acute phase of the wound healing process. This

is challenging in burn patients and often result in complicated wound healing leading to prolonged treatment, scarring and pain in affected tissues, - factors that significantly impact the patients QoL.

FLE therapy is easily applicable, safe, and noninvasive, targeting and balancing several phases of wound healing and tissue regeneration. Based on preliminary data presented here in line with previous reported results,[15-17,19,20,22-25,28] we suggest that FLE should be considered for wound healing treatment for burn wounds, possibly as an alternative to surgery or in combination with surgical interventions such as skin grafting.

CONCLUSION

These preliminary data suggest that the FLE treatment has potential in the management of superficial and deep second-degree burns. Larger clinical trials and randomized controlled studies are needed to back and confirm these results.

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SUPPLEMENTARY MATERIAL

Supplementary material is available at *Military Medicine* online.

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CONFLICT OF INTEREST

M. Mellergaard, L. Hebert, and M. Nielsen are employees of Klox Technologies Ltd. S. Fauverghe is a former employee of Klox Technologies Ltd. F. Bassetto and L. Téot are medical consultants for Klox Technologies Inc. The other authors have no conflicts of interest relevant to the content of this manuscript.

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