Biological effects of a new ultraviolet A\textsubscript{1} prototype based on light-emitting diodes on the treatment of localized scleroderma

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Abstract
Ultraviolet A\textsubscript{1} (UVA\textsubscript{1}) phototherapy (spectral range 340-400 nm) is a well-established treatment option for various skin diseases such as localized scleroderma. Recent improvements of conventional UVA\textsubscript{1} light sources (metal-halide or fluorescent lamps) have brought attention to a new light-emitting diode (LED) technology with remarkable advantages in handling and clinical routine. This study provides a preclinical histological and molecular evaluation of an LED-based UVA\textsubscript{1} prototype with a narrower spectral range (360-400 nm) for treating localized scleroderma. Scleroderma mouse models and fibroblasts in vitro were exposed to LED-based UVA\textsubscript{1} phototherapy or to irradiation with a commercially available metal-halide lamp emitting low-dose (20, 40 J/cm\textsuperscript{2}), medium-dose (60 J/cm\textsuperscript{2}) and high-dose (80, 100 J/cm\textsuperscript{2}) UVA\textsubscript{1} light. Both UVA\textsubscript{1} light sources affected inflammatory genes (IL-1\textalpha and IL-6) and growth factors (TGF\beta-1 and TGF\beta-2). Increased collagen type 1 was reduced after UVA\textsubscript{1} phototherapy. Matrix metalloproteinase-1 was more enhanced after a medium dose of LED-based UVA\textsubscript{1} phototherapy than after conventional treatment. In vivo, dermal thickness and the amount of collagen were reduced after both treatment methods. Remarkably, myofibroblasts were more effectively reduced by a medium dose of LED-based UVA\textsubscript{1} phototherapy. The study indicates that LED-based UVA\textsubscript{1} phototherapy yields similar or even better results than conventional treatment. In terms of biosafety and patient comfort, LED-based UVA\textsubscript{1} phototherapy offers clear advantages over conventional treatment because of the use of a narrower and less harmful UVA\textsubscript{1} spectrum, less heat generation and shorter treatment times at the same irradiation intensity. Clinical studies are required to confirm these results in patients with localized scleroderma.

KEYWORDS
extracellular matrix, fibroblasts, LED-based UVA\textsubscript{1}, localized scleroderma, phototherapy
1 | INTRODUCTION

Localized scleroderma (LS) is a distinctive inflammatory disease that leads to sclerosis of the skin and subcutaneous tissues.[1-4] Management of LS is difficult, and no causal therapy is available so far. Topical and systemic immunosuppressants such as corticosteroids are commonly used as anti-fibrotic agents but with considerable side effects and limited efficacy.[5,6] The beneficial effects of different forms of phototherapy in the treatment of LS have been described in many studies.[7-13] The development of UVA1 phototherapy (340-400 nm) has highly improved treatment of LS.[14,15] Because UVA light is less erythemogenic than broadband UVA, it is possible to apply much higher doses of UVA without the risk of sunburn. UVA light penetrates deeper into the skin than UVB and UVA.[16] In the first prospective study on LS, high-dose UVA light (130 J/cm²) was highly effective in reducing skin sclerosis, and some patients even showed complete clearance.[11] Lower UVA1 doses (30 J/cm²) were also highly effective and well-tolerated by patients with LS who showed a significant reduction in skin thickness.[17]

Commercially available UVA light sources for therapeutic use are fluorescent lamps (ie TL10R 100W, Philips) or high-output metal-halide lamps (ie Sellamed 4000W, Sellas Medizinische Geräte GmbH). Low-dose to medium-dose UVA phototherapy is usually conducted by means of fluorescent lamp cubicles, that is full-body UV cabinets, allowing the irradiation of large body areas; in contrast, metal-halide lamps are more often used for high-dose UVA treatments.[14,18,19] Despite the therapeutic success of conventional lamps in the treatment of LS, these light sources should be further developed in terms of biosafety and patient comfort. For patients, conventional therapy involves long irradiation times per therapy session (up to 60 minutes) and treatment durations (up to 40 sessions) as well as possible discomfort due to severe heat from the light sources (up to 40°C). The new light-emitting diode (LED) technology provides remarkable advantages over conventional treatment. The higher power of the LEDs significantly reduces the treatment time per session. Furthermore, LEDs only develop very low waste heat. LED technology also allows the manufacturing of small hand-operated UVA1 devices that require less space. In this way, only skin areas requiring treatment may be targeted. Irradiation of healthy tissue can be avoided when only circumscribed areas need to be treated. The narrower UVA spectrum due to the mixture of different LEDs is also of importance. Patient safety could be improved by eliminating potentially more damaging short-wave UVA radiation (340-360 nm). This study investigated the—so far—unresolved question whether molecular or cellular mechanisms are affected by the different UV spectrum.

2 | METHODS

2.1 | Cell culture

The in vitro study included human dermal fibroblasts from n = 6 different donors (#231340, #7F3943, #7F3950, #2F0621, #9F0438 and #9F0889) purchased from CellSystems® (Biotechnologie Vertrieb GmbH). Fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Fisher Scientific) and supplemented with 10% fetal bovine serum (both from PAN Biotech) and 1% penicillin-streptomycin, as well as 1% L-glutamine (both from Sigma-Aldrich). All cells were cultured in 75 cm² Falcon™ Flasks (Corning Inc), incubated under humid conditions in a 5% CO₂ incubator at 37°C and split 1:3 every 3 days.

2.2 | Animals and treatment groups

The mouse experiments were conducted with 129Sv/Ev male mice (original strain from Robertson Lab of Dunn School Pathology of the University of Oxford, UK). All animals (n = 6 animals per group) were between 6 and 8 weeks old at the start of the study and were divided into experimental groups according to their cage occupation. Mice were maintained under specific pathogen-free and controlled conditions (22°C, 55% humidity and 12 hours day/night rhythm) and had free access to water and chow. They received human care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The backs of the mice were shaved (2 × 2 cm²). The animals were subjected to a well-established bleomycin-induced scleroderma model[20-23] based on the study by Yamamoto et al on BALB/C mice[24] with small modifications adapted for the 129Sv/Ev mouse strain. Mice in the experimental groups (group 2-6) received local subcutaneous injections of 100 µl bleomycin (500 µg/ml; Hexal AG Holzkirchen) five times a week within 6 weeks as well as further injections 1-2 times per week (over the 6 weeks of phototherapy) to maintain the local sclerosis status. Control group 1 received subcutaneous injections of 100 µl of phosphate-buffered saline (PBS) (Sigma-Aldrich) instead of bleomycin (Table S1). To induce a comparable level of local fibrosis that was as homogeneous as possible, the injections were always given by the same person. Groups 3-6 received phototherapeutic treatment for 30 days (5 times per week). The high-dose UVA1 groups (groups 3 and 4) were treated for 3330 minutes (= 100 J/cm²) per session and the medium-dose UVA1 groups (groups 5 and 6) for 2000 minutes (= 60 J/cm²). Groups 3 and 5 were exposed to irradiation with the metal-halide lamp and groups 4 and 6 to irradiation with the LED prototype (Table S1). The mice were treated in their usual cages to avoid any additional stress. After UVA1 therapy, all animals were killed by cervical dislocation, and the bleomycin-treated or PBS-treated skin area was removed and subdivided for histological preparation or molecular biological examination.

2.3 | UVA1 devices, illumination set-up, and treatment of cells and mice

This UVA1 phototherapy study included two UVA1 devices (Figure S1A,B) that differed with regard to the type of lamp and the UVA1...
spectrum (Figure S1C). Besides the conventionally used UVA₁ device (340-400 nm) with an installed metal-halide lamp (exposure area of 25 × 25 cm), the study used a UVA₁ prototype (360-400 nm) with light-emitting diode-based technology (exposure area of 30 × 30 cm with 144 single LEDs) (both from Sellas Medizinische Geräte GmbH). Both UVA₁ light sources were positioned and installed to emit the same irradiation power of 50 mW/cm². During the UVA₁ treatments, the distance between the metal-halide lamp and the animal cage was 2 cm and between the LED-based UVA₁ prototype and the cage 60 cm.

For UVA₁ treatment, 50,000 cells were seeded onto 6-well cell culture plates and irradiated with different doses of UVA₁ light (20 J/cm² - 640 minutes; 40 J/cm² - 13 20 minutes; 60 J/cm² - 20 00 minutes; 100 J/cm² - 33 30 minutes). During UVA₁ irradiation, cells were maintained in 2 ml of phosphate-buffered saline (PBS) (Sigma-Aldrich). Untreated control cells and UVA₁-treated cells simultaneously underwent a change in medium (2 ml DMEM) immediately after irradiation. Cells were incubated for 6 and 24 hours after UVA₁ treatment followed by RNA isolation.

Mice received a medium dose (60 J/cm² - 20 00 minutes) and a high dose (100 J/cm² - 3330 minutes) of UVA₁ irradiation dependent on their assigned experimental group (Table S1).

Both the in vitro and the in vivo studies were conducted with the two UVA₁ light sources (a metal-halide UVA₁ lamp and the LED-based UVA₁ prototype) under the same conditions to allow a direct comparison. The only difference in the set-up was the use of a fan in the experiment with the metal-halide lamp to protect the animals from severe heat.

2.4 | RNA isolation and reverse transcription

Cellular RNA was isolated using the NucleoSpin® RNA Kit (Macherey-Nagel) according to the manufacturer’s instructions. 2 µg of RNA was then transcribed into cDNA by reverse transcriptase reaction using the Super Script™ II Kit (Invitrogen, Thermo Fisher Scientific).

2.5 | Quantitative real-time PCR analysis

Cellular gene expression analysis consisted of quantitative real-time PCR with specific sets of primers (Sigma-Aldrich) and conditions (Table S2) using LightCycler technology (Roche Diagnostics) as described elsewhere.[24] PCR reactions were evaluated by melting curve analysis. β-actin was amplified to ensure cDNA integrity and to normalize expression. Each real-time PCR was repeated at least 3 times.

2.6 | Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants were collected 48 hours after UVA₁ treatment and of untreated control fibroblasts and were analysed by total MMP-1 ELISA, respectively, according to the manufacturer’s instruction. The human total MMP-1 ELISA (DuoSet ELISA Development System) was received from R&D Systems, Wiesbaden-Nordenstadt, Germany, and detects active and pro-Matrix Metalloproteinase-1. Each supernatant was assayed in duplicates, and the entire experiments were performed three times with dermal fibroblasts from n = 6 different donors.

2.7 | MTT assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) test is based on the reduction in tetrazolium salt by mitochondrial enzymes to form coloured formazan as an indicator of vitality.[25] To evaluate the cell vitality of human fibroblasts (n = 6) after UVA₁ treatment, 10⁴ cells/well were seeded onto a 96-well microtitre plate and irradiated with different doses of UVA₁ light (20 J/cm² - 640 minutes; 40 J/cm² - 1320 minutes; 60 J/cm² - 20 00 minutes; 80 J/cm² - 2640 minutes; 100 J/cm² - 3330 minutes) or remained untreated. During UVA₁ irradiation, cells were maintained in 100 µL of PBS. After irradiation, PBS was replaced by 100 µL of DMEM, and the microtitre plate was incubated under humid conditions in a 5% CO₂ incubator at 37°C for 24 hours. 10 µL of MTT reagent (5 mg/mL in PBS, Sigma-Aldrich) was added directly to the medium in the wells of the microtitre plate and incubated for 4 hours. Insoluble formazan was formed in the cells in proportion to the activity of the dehydrogenases. The formazan was finally dissolved in 100 µL 20% SDS (sodium dodecyl sulphate, Sigma-Aldrich) and incubated overnight in the incubator. Absorbance of the dissolved solution was observed with a microtitre plate reader (MWG-Biotech) at 540 nm. Each sample was assayed in duplicates, and the entire experiments were conducted three times.

2.8 | Histochemical analysis

Immunohistochemistry was conducted on formalin-fixed and paraffin-embedded full-skin preparations from all mice (n = 6 mice per group). Skin sections measuring 2 µm were stained with anti-alpha-smooth muscle actin (anti-α-SMA) antibody (EPR5368) (1:1000; Abcam) according to the following protocol: sections were deparaffinized, rehydrated and placed into a steamer in Tris/EDTA buffer (pH 8) for 30 minutes. After cooling down to room temperature, the tissue slides were washed in PBS, incubated with the primary antibody at room temperature for 1 hour, washed again with PBS and incubated with Histofine solution (Nichirei Biosciences INC, Tokyo, Japan) for 30 minutes. Tissue slides were washed again with PBS and incubated with AEC substrate chromogen (ready to use; Dako-Agilent) at room temperature in the dark for 15 minutes. The staining reaction was stopped with H₂O₂, and counterstaining was done with haematoxylalin (Roth).

H&E staining on murine skin sections was done as described previously.[26] Extracellular matrix accumulation in skin sections was
determined by staining with Sirius red/fast green.\textsuperscript{[27]} The intensity of the Sirius red staining of the murine tissue sections was quantified with the image J software (http://www.imagej.softonic.de) and expressed as a percentage of the total area. All tissue slides were scanned with a histo-scanner (PreciPoint, M8), and representative pictures were visualized.

\subsection*{2.9 Analysis of collagen content}

Collagen in mouse tissues was analysed with the QuickZyme collagen assay according to the manufacturer’s instructions (QuickZyme Biosciences).

\subsection*{2.10 Statistical analysis}

Data are expressed as mean values ± standard deviation (SD). Data were analysed with GraphPad Prism 5 software (GraphPad Software Inc). Groups were compared by one-way ANOVA with Tukey’s honestly significant difference (HSD) post hoc test analysis. Differences were considered statistically significant at \( P < .05 \).

\subsection*{2.11 Study approval}

The animal study was conducted according to the NIH Guide for the Care and Use of Laboratory Animals, with the appropriate permission from the Animal Rights Commission of the State of Bavaria, and approved by the Committee on the Ethics of Animal Experiments of the University of Regensburg, Germany (permit number 55.2 DMS-2532-2-461).

\section*{3 RESULTS}

The study purpose was to evaluate molecular and histological effects of an LED-based UVA\textsubscript{1} prototype (spectral range 360-400 nm) on human fibroblasts and dermal fibrosis in a bleomycin-induced mouse model of scleroderma in comparison to conventional UVA treatment with a high-pressure metal-halide lamp (spectral range 340-400 nm). For the in vitro approach, different UVA\textsubscript{1} dose regimens (20, 40, 60, 80 and 100 J/cm\textsuperscript{2}) were evaluated, and for the in vivo approach 2 different treatment regimens (high dose: 100 J/cm\textsuperscript{2} and medium dose: 60 J/cm\textsuperscript{2}).

\subsection*{3.1 UVA\textsubscript{1} phototherapy equipment and emission spectrum}

Figure S1 shows the exposure unit and the emission spectrum of the UVA\textsubscript{1} metal-halide high-pressure lamp (SELLASOL) (Figure S1A, C) and the LED-based UVA\textsubscript{1} prototype (Figure S1B,C).

\subsection*{3.2 Examination of morphology and cell vitality of human fibroblasts after UVA\textsubscript{1} treatment}

To compare the two UVA\textsubscript{1} light sources with respect to cell vitality, cell morphology and cell vitality were determined 24 hours after UVA\textsubscript{1} treatment by means of an MTT test. Human fibroblasts exposed to high UVA\textsubscript{1} doses (80 and 100 J/cm\textsuperscript{2}) appeared swollen, broken and round, and the damage was most critical after treatment with either light source (Figure 1A). Single UVA\textsubscript{1} treatments with up to 60 J/cm\textsuperscript{2} did not change the morphology of the fibroblasts. Cell vitality significantly decreased with increasing UVA\textsubscript{1} doses (Figure 1B). Doses > 60 J/cm\textsuperscript{2} were defined as the irradiation limit for both UVA\textsubscript{1} light sources and were not used in further cell culture experiments.

\subsection*{3.3 Effects of UVA\textsubscript{1} on cytokines, growth factors and collagen metabolism in fibroblasts}

mRNA expression of IL-1α and IL-6, which was initially analysed 6 hours after exposure to UVA\textsubscript{1} was induced after treatment with both the metal-halide lamp and the LED-based prototype in comparison with untreated control. mRNA expression did not seem to be dependent on the UVA\textsubscript{1} dose (Figure 2A,B). Interestingly, induction of IL-1α was significantly higher with the LED-based UVA\textsubscript{1} prototype at the low dose of UVA\textsubscript{1} of 20 J/cm\textsuperscript{2}. Next, the mRNA expression levels of matrix metalloproteinase-1 (MMP-1), collagen type 1 (Col-1), and transforming growth factor ß-1 and transforming growth factor ß-2 (TGFß-1 and TGFß-2) were examined 24 hours after UVA\textsubscript{1} exposure. MMP-1 mRNA expression had increased after UVA\textsubscript{1} treatment with the highest induction at the UVA\textsubscript{1} dose of 60 J/cm\textsuperscript{2} for either light source. Here, the LED-based prototype showed a significant induction advantage (Figure 2C). Investigations of MMP-1 at the mRNA level were supplemented with data showing the human total MMP-1 (human active MMP-1 and pro-MMP-1) in cell culture supernatants collected 48 hours after UVA\textsubscript{1} treatment. The results showed that in the supernatants collected from UVA\textsubscript{1}-treated fibroblasts significantly more total MMP-1 protein can be detected when using a UVA\textsubscript{1} dose of 40 or 60 kJ/cm\textsuperscript{2} compared to the untreated control (Figure S2). In analogy to the mRNA data, a dose of 60 kJ/cm\textsuperscript{2} also showed a significantly increased amount of total MMP-1 protein in the supernatant when using the LED UVA\textsubscript{1} device compared to the metal-halide lamp. The expression of Col-1 was significantly down-regulated at doses ≥40 J/cm\textsuperscript{2}. Lower UVA\textsubscript{1} doses with either light source did not seem to have any reducing effect on the mRNA expression level of Col-1 (Figure 2D). Expression of TGFß-1 was induced after UVA\textsubscript{1} irradiation of normal human fibroblasts compared to untreated cells (Figure 2E). Interestingly, mRNA induction seemed to be dose-dependent for metal-halide UVA\textsubscript{1} treatment and not dose-dependent for LED-based UVA\textsubscript{1} treatment. In contrast, TGFß-2 was down-regulated with the strongest effect at the dose of 60 J/cm\textsuperscript{2} for either light source (Figure 2F). In summary, these results indicate that both UVA\textsubscript{1} light sources affect identical biological mechanisms in fibroblasts with similar or even better effects of the new LED-based light
source with a UVA\textsubscript{1} spectrum (340-400 nm) that is 20 nm narrower than that of the conventional metal-halide lamp (460-400 nm).

### 3.4 Evaluation of dermal thickness, collagen content and the number of α-SMA-positive myofibroblasts after UVA\textsubscript{1} treatment

The biological effects of the two UVA\textsubscript{1} light sources were compared by means of a well-established bleomycin-induced murine scleroderma model in a preclinical in vivo approach.\textsuperscript{[23]} After 30 subcutaneous bleomycin injections, the mice had developed dermal sclerosis and were divided into different phototherapeutic treatment groups (Table S1). UVA\textsubscript{1}-treated groups (groups 3-6) received 30 UVA\textsubscript{1} irradiation sessions and 10 additional bleomycin injections to maintain the status of sclerosis. The mean dermal thickness of the bleomycin control group 2, which contains 30 bleomycin injections and 10 additional bleomycin injections to maintain the status of sclerosis was 550.81 µm (Std. D. ± 102.8) in comparison with 186.51 µm (SD ± 34.8) in the group of healthy mice (group 1). Dermal thickness was significantly reduced after high-dose (100 J/cm\textsuperscript{2}) UVA\textsubscript{1} treatment with the metal-halide lamp
(group3: 426.10 µm; SD ± 58.6) and the LED-based UVA₁ prototype (group 4:413.10 µm; SD ± 31.2) in comparison with group 2 (Figure 3A). Dermal thickness was also reduced after medium-dose UVA₁ treatment regimens, but the reduction was not significant with either light source in comparison with the untreated group 2 (group 5:446.20 µm; SD ± 80.70; group 6:441.30 µm; SD ± 56.4). The collagen content in the affected skin after UVA₁ treatment was histologically analysed by means of Sirius red/fast green staining (Figure 4B) and image analysis (Figure 3B) and quantified using a hydroxyproline assay (Figure 3C). The amount of collagen was reduced after each UVA₁ treatment regimen; however, compared to the bleomycin-only group (group 2), the reduction was only significant after high-dose treatment with either light source (Figure 3C). Finally, the number of α-SMA–positive myofibroblasts was determined by a dermato-histopathologist. Myofibroblasts were significantly reduced after high-dose UVA₁ treatment with the metal-halide lamp and medium-dose UVA₁ treatment with the LED-based UVA₁ prototype (Figure 3D). The obviously stronger α-SMA staining in groups 3-6 (Figure 4C) was due to the blood vessels that were also α-SMA positively stained and whose synthesis is known to increase after UVA₁ irradiation[28]. In summary, these in vivo results indicate that both UVA₁ light sources are effective.

**FIGURE 2** Gene expression marker analyses in human fibroblasts after UVA₁ treatment. Different fibroblasts (n = 6 different donors) were exposed to UVA₁ (metal-halide UVA₁ or LED-based UVA₁) using different treatment regimens (20, 40 and 60 J/cm²) or remain untreated (ctrl.; 0 J/cm²). RNA was isolated 6 or 24 h after treatment as indicated in the text, and different sclerosis-related molecular markers were analysed. A, IL-1α, (B) IL-6, (C) MMP-1, (D) Col-1, (E) TGFβ-1 and (F) TGFβ-2 (*P < .05, ANOVA with Tukey’s multiple comparison test).
in reducing sclerosis in localized scleroderma with minor differences according to the dose of UVA\textsubscript{1} irradiation applied.

4 | DISCUSSION

The aim of this study was to evaluate the impact of a new LED-based UVA\textsubscript{1} prototype with a spectral range of 360 to 400 nm on the treatment of LS. Normal human fibroblasts and a dermal fibrosis mouse model were used for analysis. In terms of biosafety and patient comfort, LED-based UVA\textsubscript{1} light technology offers clear advantages over conventional metal-halide lamps with a broader spectral range of 340 to 400 nm. Next to the narrower and less harmful UVA\textsubscript{1} spectrum, the LED-based UVA\textsubscript{1} prototype has the advantage of less heat development and shorter treatment times at the same irradiation intensity.\cite{29} However, the molecular and histological effects are also decisive when comparing different UVA\textsubscript{1} light sources. Therefore, we analysed the effects on cell morphology, cell vitality, gene expression of sclerosis-relevant marker molecules, dermal thickness, collagen content and the status of myofibroblast activation in vivo.

First of all, we focused on the morphology and vitality of normal human fibroblasts. Different UVA\textsubscript{1} doses (20, 40, 60, 80 and 100 J/cm\textsuperscript{2}) were examined, and morphological changes (swollen, broken and round) were observed at doses of 80 J/cm\textsuperscript{2} and higher. No morphological differences were found between the conventional lamp and the LED-based prototype. Cell vitality decreased dose-dependently and was significantly reduced after doses of 80 J/cm\textsuperscript{2} and higher with either light source. Hereby, the narrower UVA\textsubscript{1} spectrum of the LED-based UVA\textsubscript{1} prototype did not seem to affect cell vitality. Up to date, only a few murine studies are available using a narrowband UVA\textsubscript{1} light source for the treatment of scleroderma.\cite{30,31} Karpec and coauthors described the safety and efficacy of 365 nm LED-based UVA\textsubscript{1} phototherapy in bleomycin-induced scleroderma in

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**FIGURE 3** Evaluation of dermal thickness, collagen content and the number of α-SMA–positive myofibroblasts in a bleomycin-induced scleroderma model after UVA\textsubscript{1} treatment. A, Dermal thickness was determined in µm in the 6 mice groups. Representative images of mice (n = 6 mice per group) were taken, and 5 measurements per image were used for the calculation (\( ^* \times P < .05 \), ANOVA with Tukey's multiple comparison test). B, Determination of the fibrotic region by quantifying the Sirius red-positive area by image analysis (\( ^* P < .05 \), ANOVA with Tukey's multiple comparison test). C, Protein quantification of total collagen in the dermis within the 6 mice groups (n = 6 mice per group) (\( ^* P < .05 \), ANOVA with Tukey's multiple comparison test). D, Quantification of α-SMA–positive fibroblasts (myofibroblasts) (average of three hpf/animal; n = 6 animals per group) (\( ^* * P < .05 \), ANOVA with Tukey's multiple comparison test). SMA, smooth muscle actin; hpf, high-power field

**FIGURE 4** Histological overview of the bleomycin-induced scleroderma model after UVA\textsubscript{1} treatment. Representative images of (A) H&E, (B) Sirius red/fast green and (C) α-SMA–stained sections of local skin fibrosis in different treatment groups (group 1: healthy control; group 2: bleomycin (Bleo) control; group 3: metal-halide UVA\textsubscript{1} (100 J/cm\textsuperscript{2}); group 4: LED UVA\textsubscript{1} (100 J/cm\textsuperscript{2}); group 5: metal-halide UVA\textsubscript{1} (60 J/cm\textsuperscript{2}); and group 6: LED-based UVA\textsubscript{1} (60 J/cm\textsuperscript{2}). α-SMA–stained vessel structures are strongly increased after UVA\textsubscript{1} treatment and are marked with dotted arrows in the enlarged sections. Full arrows in the enlarged sections indicate α-SMA positively stained fibroblasts. Scale bars: 500 µm
mice, but the optimum UVA\textsubscript{1} spectrum for scleroderma treatment has not yet been determined and needs to be examined further.

To determine possible differences between metal-halide lamps and the LED-based UVA\textsubscript{1} prototype on the molecular level, we analysed the gene expression of UVA\textsubscript{1}-affected biomolecules involved in scleroderma. Increased MMP-1 expression is one of the most important indicators for successful UVA\textsubscript{1} treatment and significantly improves the clinical condition of LS\textsuperscript{11} MMP-1 is specifically responsible for the breakdown of collagen. Collagen type I (Col-1) is the predominant collagen component of the skin and plays a central role in LS development. Its excessive production by fibroblasts leads to the typical phenotype of dermal fibrosis with accompanying skin hardening due to pathological collagen excess in the dermis. The two biomarkers MMP-1 and Col-1 interact with each other and maintain hardening due to pathological collagen excess in the dermis. The two biomarkers MMP-1 and Col-1 interact with each other and maintain the balance of collagen metabolism in healthy fibroblasts. UVA\textsubscript{1}-induced MMP-1 induction in fibroblasts and the reduction in Col-1 has been described in many studies\textsuperscript{32-36}. In the present study, we also observed significant MMP-1 induction at the dose of 60 J/cm\textsuperscript{2} with either light source and a higher activation rate in fibroblasts irradiated with narrowband LED-based UVA\textsubscript{1}. This higher induction capacity of LED-based UVA\textsubscript{1} for MMP-1 maybe a clear advantage of the LED-based UVA\textsubscript{1} light source that potentially results in a better clinical response to treatment. So far, we do not know the mechanism responsible for the higher induction capacity for MMP-1 when using the LED-based UVA\textsubscript{1} lamp. However, comparison of both spectra shows that the LED emission spectrum shows three distinct maxima at about 367, 375 and 385 nm. When neglecting the small peaks, the metal-halide lamp shows five maxima, whereas three maxima can be found in the spectral range of the LED emission spectrum at about 366, 377 and 384 nm. Thus, the maxima in the comparable wavelength show almost the same wavelengths. Besides the small part in the visible spectrum, the major difference of both spectra can be assigned to the short wavelength part of the UVA spectrum. Thus, at equal radiant exposure, the LED-based UVA\textsubscript{1} lamp provides more photons in the short wavelength range from about 360 to 400 nm. A set of molecules in cells like flavins and vitamin A show absorption maximum in the range of 370-380 nm\textsuperscript{37,38}. Part of these molecules efficiently generate singlet oxygen which in turn may induce MMP-1\textsuperscript{39,40}. This hypothesis is purely speculative and must be investigated in further studies.

In addition to MMP-1 induction, our study also showed reduction in Col-1. Although collagen balance is of particular importance in sclerotic diseases, collagen degradation by UVA\textsubscript{1} treatment plays a relatively minor but not less important role than the induction of MMP-1. In percentage terms, UVA\textsubscript{1} irradiation has a significantly stronger effect on MMP-1 activation than on the reduction in Col-1\textsuperscript{32} as confirmed by our study.

In addition to the intervention in the collagen balance of fibroblasts, another important mechanism of UVA\textsubscript{1} irradiation is its immunomodulatory effect. This effect particularly concerns pro-inflammatory cytokines such as IL-1\textalpha and IL-6 that signal mediate functions inside and outside a cell. Stimuli of cytokine production in the cells are different stress factors such as exposure to UV light. The synthesis of pro-inflammatory cytokines forms a mutually stimulating network, in which IL-1\textalpha plays a special role. IL-1\textalpha may influence the collagen metabolism and is a major stimulus for the synthesis of other profibrotic regulatory proteins such as IL-6 or IL-8\textsuperscript{41,42}. Kreuter and colleagues described significantly down-regulated mRNA expression of IL-6 and IL-8 after UVA\textsubscript{1} treatment in patients with LS\textsuperscript{43}. Vielhaber et al. controversially discussed in their study that increased UVA\textsubscript{1}-induced MMP-1 production is connected with the upregulation of the cytokine IL-1\textalpha and the resulting increase in IL-6 synthesis\textsuperscript{44}. Increased IL-1\textalpha and IL-6 gene expression after UVA\textsubscript{1} irradiation of fibroblasts was also found by Wlaschek et al\textsuperscript{36,40}. Any comparison of study results should distinguish between normal fibroblasts and LS-derived fibroblasts. Our study involved normal fibroblasts. Similar to the results of Wlaschek et al\textsuperscript{36,40}, we observed a significant induction of IL-1\textalpha and IL-6 expression for both UVA\textsubscript{1} light sources. In addition, the growth factors of the TGF\beta family are considered to be further important indicators for fibrosis and play an essential role in scleroderma. In the dermis, TGF\beta stimulates the proliferation of dermal fibroblasts, which in turn secrete increased amounts of ECM components such as Col-1 and have a reducing effect on MMP-1 expression\textsuperscript{45-48}. In addition, TGF\beta may suppress the production of IL-1, TNF\alpha and IL-8, which in turn shows the interplay between pro- and anti-inflammatory functions in cytokine-mediated inflammatory reactions\textsuperscript{49}. The two most important isoforms of TGF\beta are TGF\beta-1 and TGF\beta-2, both crucial factors in the pathogenesis of fibrosis\textsuperscript{47,50-53}. In activated scleroderma fibroblasts, the growth factor TGF\beta-1 is excessively secreted\textsuperscript{45}. In addition, mRNA gene expression of TGF\beta in lesional skin areas significantly decreases after UVA\textsubscript{1} therapy in patients with LS\textsuperscript{45}. Other in vivo studies using normal human skin samples yielded contradictory results. Quan et al. described the significant upregulation of TGF\beta-1 combined with a simultaneous decrease in TGF\beta-2 after exposure to UV irradiation\textsuperscript{54}. In experiments with skin fibroblasts, Yin et al also observed the significant induction of TGF\beta-1 after UVA\textsubscript{1} treatment\textsuperscript{48}. The results of Quan et al and Yin et al\textsuperscript{48,54} are also reflected in our study. 24 hours after UVA\textsubscript{1} exposure, TGF\beta-1 expression was increased simultaneously with reduced TGF\beta-2 expression in normal fibroblasts.

Effective UVA\textsubscript{1} treatment of scleroderma in vivo decreases dermal thickness primarily due to the degradation and reduction in collagen\textsuperscript{32}. In our in vivo experiment, all groups except control group 1 received bleomycin injections and developed the typical picture of the fibrosis mouse model described by Yamamoto et al\textsuperscript{20-23}. The bleomycin control group 2 clearly showed thicker dermis than groups 3, 4, 5 and 6 that were additionally treated with UVA\textsubscript{1}. Also clearly visible were the fat cells displaced by dermal fibrosis that resulted in complete absence of these cells due to highly pronounced fibrosis, especially in control group 2 of this study. Here, fat cells are replaced by fibrotic tissue through adipocyte-myofibroblast transformation\textsuperscript{55,56}. Slight regeneration of fatty tissue was observed in the experimental groups treated with UVA\textsubscript{1}. Increased myofibroblast activity and associated profibrotic processes significantly contributing to the development of fibrosis\textsuperscript{21,57} could be observed in the \textalpha-SMA stains of
groups 2-6 treated with bleomycin. Quantification of α-SMA-positive fibroblasts by a dermato-histopathologist showed a decrease in activated myofibroblasts after UVA<sub>1</sub> treatment. Myofibroblasts were significantly reduced compared to the untreated control group 2 after high-dose treatment with the metal-halide lamp (group 3). Interestingly, similar significant effects could be already achieved with a medium dose of the LED-based UVA<sub>1</sub> prototype (group 6). The induction of neangiogenesis occurring as wound healing process in UVA<sub>1</sub>-treated mice could also be observed by means of α-SMA staining, as Trompezinski et al. showed in an experimental approach.<sup>[28]</sup> These α-SMA-stained vascular structures dominate the histological picture of the UVA<sub>1</sub> treated groups and were of course excluded in the quantification of α-SMA-positive myofibroblasts.

Compared to already published results, our histological analysis showed that 30 irradiations (cumulative dose of 3000 J/cm<sup>2</sup>) with a high UVA<sub>1</sub> dose (100 J/cm<sup>2</sup>) with either light source significantly reduced the dermal diameter by about 25%; the reduction achieved with medium-dose UVA<sub>1</sub> treatment (60 J/cm<sup>2</sup>) (cumulative dose of 1800 J/cm<sup>2</sup>) was about 20%. These results indicate that both light sources are effective in treating sclerosis and have almost the same effect on reducing dermal thickness. Karpec and colleagues described in their animal study that even a cumulative dose of UVA<sub>1</sub> with a narrowband (365 ± 5 nm) LED-based UVA<sub>1</sub> light source that was 5 times lower than that of conventional broadband UVA<sub>1</sub> lamps had almost the same effect on decreasing dermal thickness (skin thickness was reduced by 34% after irradiation with a cumulative dose of 600 J/cm<sup>2</sup> and by 36% using 3000 J/cm<sup>2</sup>).<sup>[22]</sup> In our similar mouse model, reduction in the dermal diameter in the 129Sv/Ev mouse strain was lower than that achieved by Karpec using two different devices for UVA<sub>1</sub> irradiation. This difference may be due to the different animal strains (129Sv/Ev versus DBA/2), differences in the performance of the bleomycin-induced sclerosis model (maintenance of the sclerosis status versus no further bleomycin treatment during the period of UVA<sub>1</sub> therapy) or the different spectral ranges of the LED source (360-400 nm vs 365 ± 5 nm).

Nevertheless, this study indicates that the two tested UVA<sub>1</sub> light sources are effective in reducing skin sclerosis with similar or even better effects of the new LED light source on the histological and molecular level. In terms of biosafety and patient comfort, the LED-based UVA<sub>1</sub> light source offers clear advantages such as an arrower UVA<sub>1</sub> spectrum, less heat generation and shorter treatment times. Further clinical studies are required to confirm these results in patients with LS or related sclerotic skin diseases such as eosinophilic fasciitis, lichen sclerosus et atrophicus or chronic sclerodermiform graft-versus-host (GvHD) disease, in which UVA<sub>1</sub> phototherapy is also a treatment option.

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Conflict of Interest

The authors have no conflict of interest to declare.

Author Contribution

SA designed the experiments and carried out the animal study together with CL. CL carried out the in vitro experiments together with PU. SA and CL wrote the paper. PU performed molecular biological tests and prepared the histological preparations. WB organized the study and together with MB supported the study with photobiological expertise. SK directed the study, performed the histological evaluations and participated in the data analysis and discussion with all other authors. All authors read and approved the final manuscript.

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References

[1] R. Buense, M. Bouer, I. A. G. Duarte. An Bras Dermatol. 2012, 87, 63.
[2] M. F. Careta, R. Romiti. An Bras Dermatol. 2015, 90, 62.
[3] O. Distler, A. Cozio. Semin. Immunopathol. 2016, 38, 87.
[4] R. Laxer, F. Zulian. Curr. Opin. Rheumatol. 2006, 18, 606.
[5] T. Herzinger, M. Berneburg, K. Goreschi, H. Gollnick, E. Holzle, H. Honigsmann, P. Lehmann, T. Peters, M. Rocken, K. Scharffetter-Kochanek, T. Schwarz, J. Simon, A. Tanew, M. Weichenthal, J. Dtsch. Dermatol. Ges. 2016, 14, 853.
[6] R. Knobler, P. Mainzadeh, H. Hunzelmann, A. Kreuter, A. Cozio, L. Mouthon, M. Cutolo, F. Rongioletto, C. P. Denton. Econ. Dermatol. 2001, 17, 149.
[7] C. J. Gruss, G. von Kobyletzki, S. C. Behrens-Williams, J. Linnering, T. Reuther, M. Kerscher, P. Altmeyer. Photodermatol. Photoimmunol. Photomed. 2001, 17, 149.
[8] M. Kerscher, T. Dirschka, M. Volkenandt, A. Bree Aleson, I. Földvari, C. Frances, A. Jaili, U. Just, V. Kahari, S. Karpadi, K. Kofoed, D. Krasowska, M. Løsowski, A. Orteu, J. Painielli, A. Parodi, A. Petit, P. Quarilino, A. Ranki, J. M. Sanchez Schmidt, J. Senescal, A. Skrok, M. Sterichinger, C. Sunderkotter, A. Taieb, A. Tanew, P. Wolf. M. Worm, N. J. Wute, J. Eur. Acad. Dermatol. Vener. 2017, 31, 1401.
[9] J. W. Steger, J. H. Matthews. J. Am. Acad. Dermatol. 2009, 60, 786.
[10] J. Steger, M. Berneburg, S. Humke, M. Klammer, M. Grewe, S. Grether-Beck, R. Boederker, T. Diepggenn, K. Dierks, G. Goerz, T. Ruzicka, J. Krutman. J. Am. Acad. Dermatol. 1997, 36, 938.
[11] J. W. Steger, J. H. Matthews. J. Am. Acad. Dermatol. 1999, 40, 787.
[12] R. C. W. Su, L. Y. Chong, K. K. Lo, H. Stege, J. Krutman, J. Am. Acad. Dermatol. 1998, 39, 517.
[13] T. Gambichler, L. Schmitz, Front. Med. 2018, 5, 237.
[14] U. Keyal, A. K. Bhatta, X. L. Wang, J. Am. Transl Res. 2017, 9, 4280.
[15] T. Gambichler, S. Terras, A. Kreuter. Clin. Dermatol. 2013, 31, 438.
[16] O. Su, N. Onsun, H. K. Onay, Y. Erdemoglu, D. B. Ozkaya, F. Cebeci, A. Somay, Int. J. Dermatol. 2011, 50, 1006.
[17] N. R. York, H. T. Jacob, Int. J. Dermatol. 2010, 49, 623.
[18] S. Zandi, S. Kalia, H. Lui. 2012:17. http://www.skintherapyletter.com/cutaneous-t-cell-lymphomas/uva1-phototherapy/
[19] T. Yamamoto, Arch. Dermatol. Res. 2006, 297, 333.
[20] T. Yamamoto, K. Nishioka, Clin. Immunol. 2002, 102, 77.
[21] T. Yamamoto, K. Nishioka, Exp. Dermatol. 2005, 14, 81.
Additional supporting information may be found online in the Supporting Information section.

Figure S1. UVA$_2$-phototherapy equipment.

Figure S2. Total MMP-1 protein in the supernatants of UVA$_2$-treated fibroblasts.

Table S1. Timeline overview of the mouse study. Bleomycin (Bleo)-injections for 30 d (5 times per week for 6 wk; except for healthy control group 1 treated with PBS). 30 UVA$_2$-irradiation sessions (5 times per week for 6 wk; except for healthy control group 1 and Bleo control group 2) were followed by 10 additional Bleo injections (1-2 times per week for 6 wk; except for healthy control group 1 treated with PBS) to maintain the sclerosis status.

Table S2. Primers and conditions. Quantitative real-time PCR was conducted with specific sets of primers and conditions as indicated in this table. Ann, annealing temperature; melt, melting temperature.

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