

Low-Level Red LED Light Inhibits Hyperkeratinization and Inflammation Induced by Unsaturated Fatty Acid in an *In Vitro* Model Mimicking Acne

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Background and Objective: *Acne vulgaris* is a chronic inflammatory disease of the pilosebaceous units (PSU), associated with increased sebum production, abnormal follicular keratinization (hyperkeratinization), follicular overgrowth of *Propionibacterium acnes* (*P. acnes*), and increased inflammatory mediator release. Light therapy has attracted medical interests as a safe alternative treatment for acne. Both blue and red light therapies at high doses $>10\text{ J/cm}^2$ have demonstrated marked effects on inflammatory acne lesions. However, few studies have investigated the effects of lower doses of light. The aim of this study is to investigate the biological effects of lower doses of red light at $0.2\text{--}1.2\text{ J/cm}^2$ for acne using an *in vitro* model previously developed to mimic the inflammation and hyperkeratinization observed clinically in acne.

Materials and Methods: Human epidermal equivalents were topically exposed to an unsaturated fatty acid, oleic acid (OA), followed by red light-emitting diode (LED) light treatments (light-plus-OA treatments). Endpoints evaluated included the proinflammatory cytokine IL-1 α , epidermal barrier integrity, as measured by transepithelial electrical resistance (TEER), and stratum corneum (SC) thickness to monitor hyperkeratinization.

Results: OA-induced IL-1 α release was significantly ($P < 0.05$) reduced following red LED light at 0.2, 0.5, and 1.2 J/cm^2 , from $266 \pm 11\text{ pg/ml}$ of no-light-plus-OA-treated (OA treatment without light) controls to 216 ± 9 , 231 ± 8 , and $212 \pm 7\text{ pg/ml}$, respectively. Histological examination showed that SC thickening following OA treatment was reduced from 43% of total epidermis for no-light-plus-OA treatment to 37% and 38% of total epidermis following 0.5 and 1.1 J/cm^2 red light plus OA treatment, respectively ($P < 0.05$). Moreover, 1.1 J/cm^2 red-light-plus-OA treatment improved OA-induced TEER changes from 29% of baseline for no-light-plus-OA treatment, to 36% of baseline.

Conclusion: Low level red LED light therapy could provide beneficial effects of anti-inflammation, normalizing pilosebaceous hyperkeratinization, and improving barrier impairment in *Acne vulgaris*. Lasers Surg. Med. © 2017 Wiley Periodicals, Inc.

Key words: red light-emitting diode (LED) light; inflammation; oleic acid; hyperkeratinization; barrier; acne photobiomodulation therapy

INTRODUCTION

Acne vulgaris is a chronic inflammatory skin disorder of the pilosebaceous unit [1,2]. Pathogenic factors of acne include increased sebum production, abnormal follicular keratinization, follicular overgrowth of *Propionibacterium acnes* (*P. acnes*), and increased inflammatory mediator release [3]. Human sebum is composed of triglycerides, fatty acids, cholesterol, squalene, and wax esters. Higher sebum secretion and larger transepithelial water loss (TEWL) are associated with mild to moderate acne [4,5]. *P. acnes* in the hair follicle canal and on the skin surface can produce digestive enzymes, including lipase, resulting in free fatty acid release from triglycerides in human sebum, which may lead to skin irritation [6–8]. Topically applied OA induced calcium influx and abnormal keratinocyte differentiation in hairless mice [9], ultrastructural changes on rabbit ears like those seen in human comedones [10], and impaired barrier associated with an increased interleukin-1 α (IL-1 α) and an increased SC thickening in human epidermal equivalents [11]. Inflammatory events occurring during early acne lesion development include interleukin-1 α (IL-1 α) bioactivity found in open comedones [12], as well as in papules of known duration less than 6 hours [13].

Acne therapy can be administered based on acne type and severity. Mild acne can be treated with topical retinoids, azelaic acid, salicylic acid, or benzoyl peroxide.

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and have disclosed the following: WHL, AF, CB, RP, and MDS were employees of Johnson & Johnson Consumer Inc. when these experiments were conducted.

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For moderate acne, an oral antibiotic is often added. For severe cases of acne, oral retinoids are prescribed and hormonal therapy might be used adjunctively. The treatment for acne can be done either topically or systemically. Topical treatments include benzoyl peroxide (BPO), azelaic acid, and retinoids as well as combinations of adapalene/BPO and clindamycin/BPO. The most common systemic treatments include isotretinoin, oral antibiotics, and hormonal agents [14]. However, a combination treatment targeting more than one of the mechanisms of acne pathogenesis yields more successful results. The combination of a topical retinoid with an antimicrobial therapy is currently recommended as the standard of care for the management of acne [15–17]. Furthermore, adjunctive and/or emerging treatments for acne include topical dapsone, taurine bromamine, resveratrol, antimicrobial peptides, chemical peels, high energy light therapy, as well as complementary and alternative medications [17].

Light-based therapies are among the oldest therapeutic modalities used to treat disease conditions. Currently, light-based therapy for mild to moderate inflammatory acne includes a variety of high energy light technologies including intense pulsed light (IPL), lasers, and photodynamic therapy (PDT) [18,19]. It has been found that these types of therapy improve inflammatory acne, that is, blue and blue-red light treatments have the greatest effect on mild to moderate inflammatory acne lesions with fewer side effects, and enhance patient satisfaction [17,19,20]. The mechanisms of action for light-based therapy for acne have been postulated to be that light-based therapies decrease *P. acnes* levels in pilosebaceous units and reduce sebaceous gland size and function [21]. For example, blue light is absorbed by porphyrins produced naturally by *P. acnes*. Subsequently, photo-excited porphyrins lead to generation of intrabacterial reactive free radicals and singlet oxygen that exert bactericidal effects on *P. acnes* [22,23]. Whereas, red light can penetrate deeper into the skin and target sebaceous glands to down-regulate sebaceous lipid production [24]. Moreover, 0.054 J/cm² blue light was shown to decrease the interferon- γ and TNF- α -induced IL-1 α and intercellular adhesion molecule 1 (ICAM-1) in keratinocytes, *in vitro* [25], and 3.6 J/cm² red light inhibited calcium-dependent phospholipase A2 (cPLA2), secreted phospholipase A2 (sPLA2), and cyclooxygenase (COX) expressions in arachidonic acid (AA)-treated human gingival fibroblasts and prevented prostaglandin E2 (PGE2) release [26]. Denda and Fuziwara [27] studied visible light effects on skin barrier after tape stripping hairless mice and they found that blue light (430–510 nm) delayed barrier recovery, whereas red light (550–670 nm) accelerated it, compared with a dark control. Lee et al. [28] showed that red LED light (630 nm) significantly attenuated cathelicidin (LL-37), Toll-like receptor 2 (TLR-2), and kallikreins (KLKs) mRNA expressions, and suppressed protease activity in normal human epidermal keratinocytes (NHEKs). In an acne clinical study using blue (50.96 J/cm²) and red (68.32 J/cm²) LED light treatments, Kwon et al. [29]

showed that both inflammatory and noninflammatory acne lesions had decreased significantly, by 77% and 54%, respectively, in the treatment group following 12 weeks of treatment. They demonstrated a concomitant decrease of IL-1 α , IL-8, matrix metalloproteinase 9 (MMP-9), TLR-2, nuclear factor- κ B (NF- κ B), insulin-like growth factor-1 receptor (IGF-1R), and sterol response element binding protein (SREBP)-1 by immunohistochemical staining on acne lesional biopsies.

Since few studies have investigated the mechanism of low level light therapy for acne, the purpose of the current study was to examine the effect of low-level red LED light for acne *in vitro* utilizing an *in vitro* model [11] whereby a topically applied OA on human epidermal equivalents (HEEs) induced proinflammatory cytokine IL-1 α release and induced SC abnormal keratinization *in vitro*, mimicking symptoms present in inflammatory acne lesions *in vivo*.

MATERIALS AND METHODS

Red LED Light Board Setup

A LED light board *in vitro* apparatus (Fig. 1) was designed to irradiate a Falcon[®] transparent wall 6-well plate (ThermoFisher Scientific, Hudson, NH) and to provide a mean of delivering specific doses of light to specimens placed into a 6-well plate in a repeatable and reliable fashion. The average height of each red LED light (peak wavelength 637 nm) was 18 mm above the bottom of each well. The power was set at 0.499–0.583 mW/cm² for the red LED light board. Light intensity was measured using a light sensor (818-ST2/DB, Newport Corporation, Irvine, CA) and Newport Power Meter Model 1918C (Newport Corporation). For dose calculations (J/cm²), the power density measured using the light sensor (mW/cm²) was multiplied by the irradiation time (seconds).

Human Epidermal Equivalents (HEEs)

Human epidermal equivalents (HEE) in 9 mm inserts were purchased from MatTek Company (Ashland, MA). Upon receiving, human epidermal equivalents were incubated in hydrocortisone-free, pheno-red-free MatTek assay medium overnight. The HEE tissues that were exposed to different light treatment doses were kept in separate plates. The HEE tissues that were exposed to light treatment were placed in the center of their corresponding wells during the light exposure. The surface of each HEE tissue is only \sim 0.64 cm², whereas, the surface of each well of the 6-well plate used is \sim 9.5 cm², and each LED light is centered in each well and uniformly distributed over the plate, thereby covering the entire surface of each tissue placed in the center of each well, with minimal cross-talk in between wells. Five percent oleic acid in propylene glycol/ethanol (3/7, Sigma–Aldrich, St. Louis, MO) was applied on top of HEEs followed by red light treatment once per day for 2 days. The total doses of red LED light used were 0.2, 0.5, 1.1, and 1.2 J/cm². For each study, epidermal barrier was assessed by trans-epithelial electrical resistance (TEER) measurement of HEE at the day 0 before the light treatment and 24 hours after the

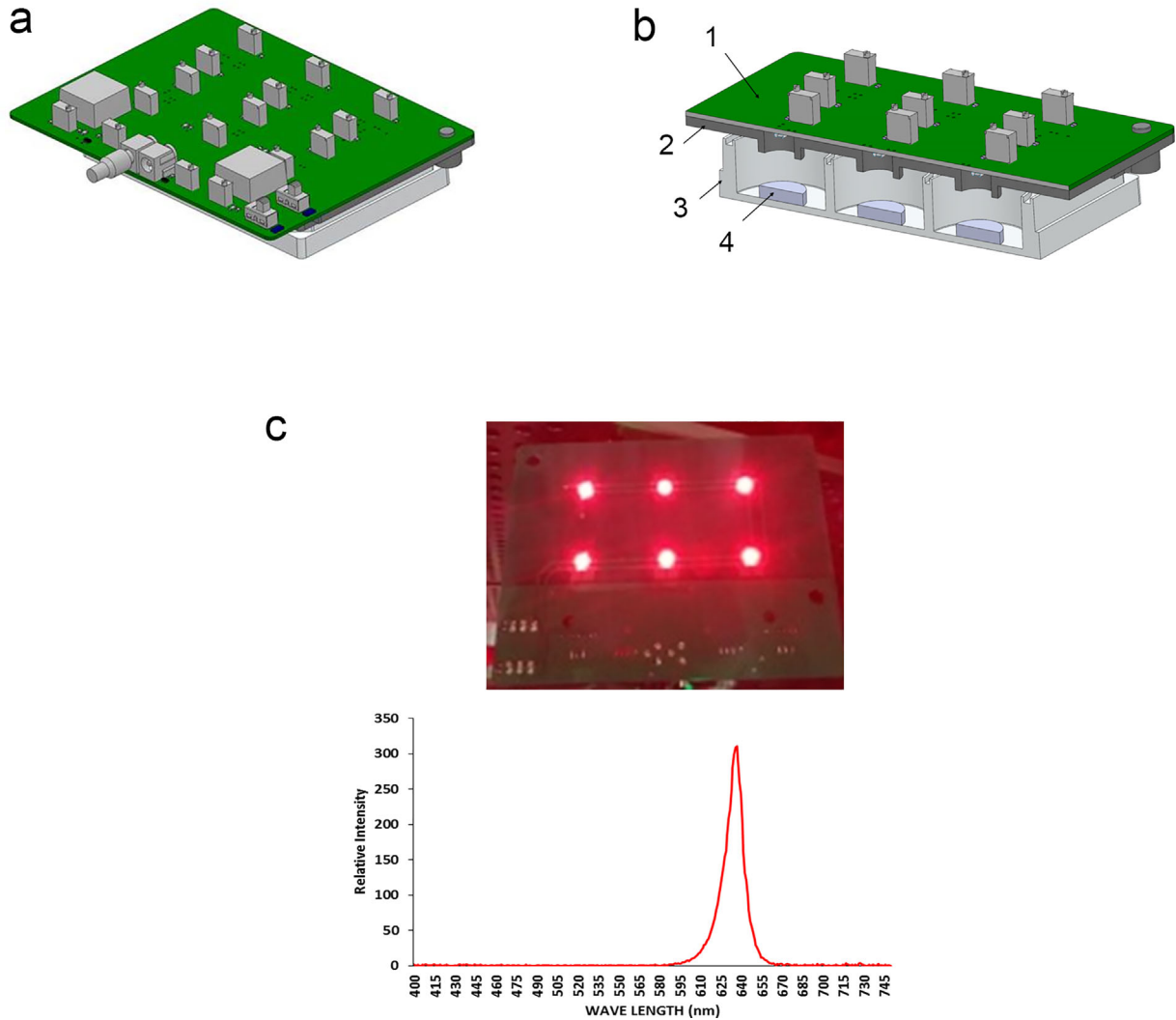


Fig. 1. Experimental setup of the *in vitro* irradiation system. (a) Top view of the red LED light board. (b) Side view of the red LED light board (1), plate adaptor (2), a 6-well plate (3), and specimen (4). (c) A photograph of the bottom view of a red light board showing the red LED lights were turned on and the emission spectrum of the red LED light.

second light irradiation in each study, as described below. The media were collected 24 hours after the second light irradiation for IL-1 α release, measured by Milliplex MAP kit (EDM Millipore, Billerica, MA) following manufacturer's instruction. The HEEs were harvested 24 hours after the second light irradiation for histological examination. At least three independent experiments were performed.

Epidermal Barrier Assessment by Trans-Epithelial Electric Resistance (TEER) Measurement of HEE

Trans-epithelial electric resistance of HEEs was measured using the epithelial ohmmeter Millicell ERS (EDM Millipore) at day 0 and day 2. HEEs were placed in 6-well plates containing 1 ml of culture medium and overlaid with 400 μ l of 1x PBS (phosphate buffered saline) for the time

required to measure TEER. Two electrodes were positioned with one in the outside of the well submerged in medium and the other above the equivalent submerged in 1x PBS. The electrical resistance readings were expressed in kilo-ohm (k Ω). The TEER values of the HEEs were normalized by the readings of the HEEs at day 0 and expressed as % of baseline.

Histological Analysis

HEE samples were fixed at the end of treatment in 10% neutral buffered formalin solution (Thermo Fisher Scientific, Hudson, NH), dehydrated and embedded in paraffin. Tissue sections (5 μ m thick) were cut perpendicular to the filter and were stained with hematoxylin–eosin. Three pictures per section were taken for each HEE. The thickness of the stratum corneum and live epidermis

were measured using ImagePro Plus (Media Cybernetics Inc., Rockville, MD) and normalized by total thickness of epidermis and expressed as % total epidermis.

Statistics

Statistical analyses were performed using two-tailed two-sample unequal variance student *t*-test (Microsoft Office Excel 2007; Microsoft, Redmond, WA). Data were expressed as mean \pm standard deviation. Differences were considered statically significant if $P < 0.1$ (\dagger), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)

RESULTS

Oleic Acid Induced Abnormal Keratinization of Stratum Cornea (SC), Barrier Defect, and the Release of IL-1 α from Human Epidermal Equivalents

Oleic acid (OA, C18:1, n-9) was used in the *in vitro* studies to induce inflammatory responses mimicking that of acne lesions *in vivo*. HEEs with vehicle treatment had 224 ± 15 pg/ml of IL-1 α protein detected in medium, $37 \pm 2\%$ of baseline in TEER and $31 \pm 1\%$ of total epidermis

in SC thickness. HEEs with OA treatment showed significant induction of IL-1 α protein release from 224 ± 15 to 317 ± 17 pg/ml in culture media ($P < 0.01$, Fig. 2a), and of barrier impairments as reflected by a decrease in TEER from $37 \pm 2\%$ to $29 \pm 2\%$ of baseline ($P < 0.05$, Fig. 2b). HEEs treated with OA also showed an increase of their SC thickness as compared to that of vehicle-treated HEEs (Fig. 2c). The quantitative analysis of the thickness of SC of HEEs showed a significant increase in SC thickening in OA-treated HEEs, from $31 \pm 1\%$ to $49 \pm 2\%$ of total epidermis ($P < 0.001$, Fig. 2d).

Red LED Light Inhibited OA-Induced Inflammation by Reducing IL-1 α Release

To evaluate the red LED light effect on OA-induced inflammation, 0.2, 0.5, and 1.2 J/cm^2 of red LED light were used to treat HEEs in the presence of OA. Figure 3 showed that IL-1 α protein detected in vehicle-treated HEEs was 205 ± 8 pg/ml and IL-1 α release induced by no-light-plus-OA-treated HEEs was 267 ± 11 pg/ml. For light-plus-OA treatments, 0.2, 0.5, and 1.2 J/cm^2 of red LED light

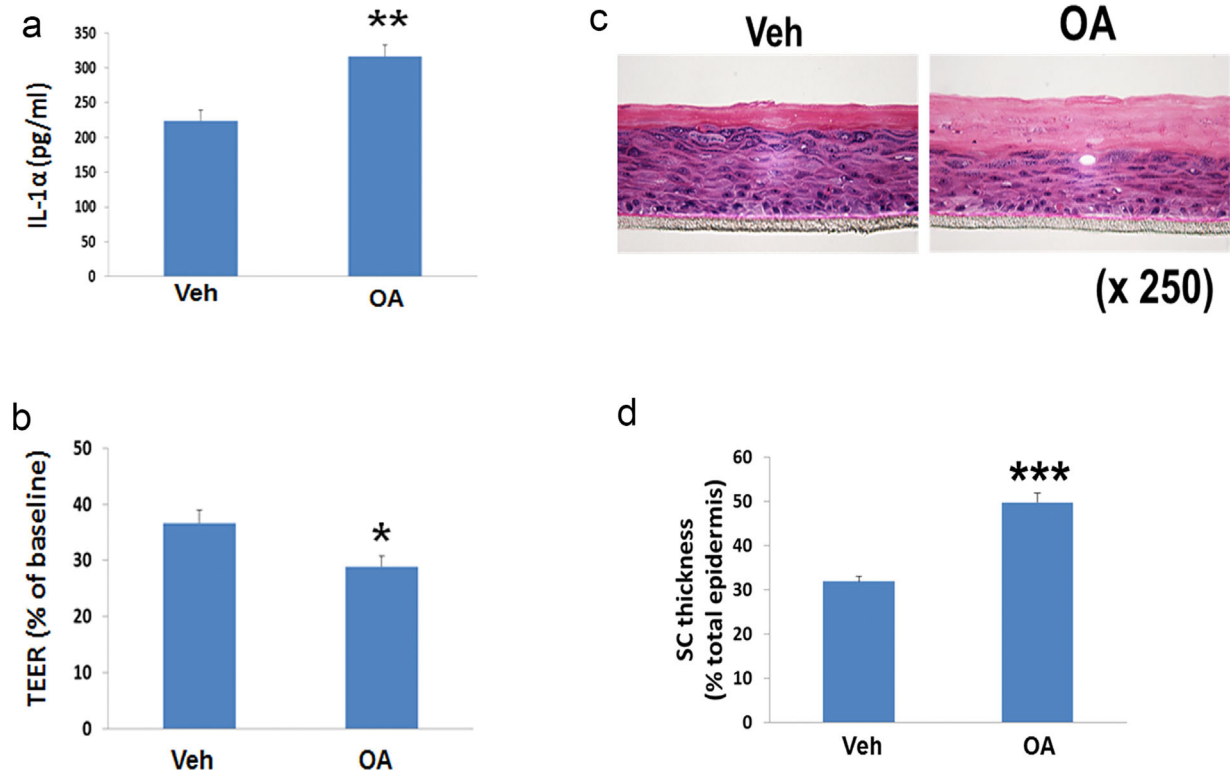


Fig. 2. Oleic acid induced IL-1 α release, barrier defect, and stratum cornea thickening in human epidermal equivalents. Topical 5% oleic acid treatment caused IL-1 α release (a) and trans-epithelial electrical resistance (TEER) decreased (b) as compared to that of vehicle treatment. (c) Histology of human epidermal equivalents treated with vehicle or oleic acid. (d) Quantitative analysis of stratum cornea and epidermis in vehicle-, and oleic acid-treated human epidermal equivalents. Oleic acid caused an increase in the thickening of stratum cornea as compared to that of vehicle treatment. Original magnification, 250 \times . Veh, vehicle; OA, oleic acid. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

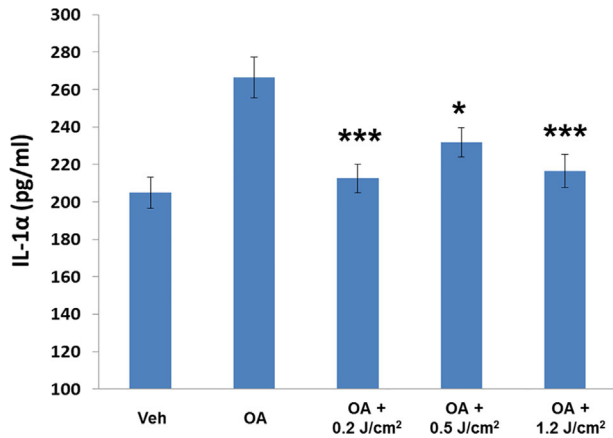


Fig. 3. Red light demonstrated anti-inflammatory property by decreasing oleic-acid-induced IL-1 α release in human epidermal equivalents. Oleic-acid-induced IL-1 α releases were reduced when treated red light at 0.2, 0.5, and 1.2 J/cm² as compared to OA treatment. * $P < 0.05$; *** $P < 0.001$.

treatment significantly reduced OA-induced-IL-1 α release in HEEs to 213 \pm 8, 232 \pm 8, 216 \pm 9 pg/ml, respectively, as compared to that of no-light-plus-OA-treated HEEs ($P < 0.05$ for 0.5 J/cm², $P < 0.001$ for 0.2 and 1.2 J/cm²).

Red LED Light Inhibited OA-Induced Hyperkeratinization by Reducing Stratum Corneum (SC) Thickening

To evaluate the red LED light effect on OA-induced hyperkeratinization, two doses (0.5 and 1.1 J/cm²) of red LED light were used to treat HEEs in the presence of OA. SC thickening of OA-treated HEEs was 43 \pm 1 of total epidermis (Fig. 4a and c). For light-plus-OA treatments, both doses of red LED light caused decrease of SC thickening in OA-treated HEEs as compared to that of no-light-plus-OA-treated HEEs (Fig. 4a). The quantitative analysis of the thickness of SC of red-LED-light-plus-OA-treated HEEs showed that both 0.5 and 1.1 J/cm² of red light irradiation led to a significant decrease in OA-induced-SC thickening from 43 \pm 5% to 37 \pm 1% and 38 \pm 1% of total epidermis, respectively ($P < 0.01$, Fig. 4b).

Red LED Light Improved OA-Induced Barrier Defect

To evaluate the red LED light effect on OA-induced barrier defect, 1.1 J/cm² of red LED light was used to treat HEEs in the presence of OA. No-light-plus-OA treatment caused a decrease of TEER from 100% of baseline to 29 \pm 2% of baseline, but in case of light-plus-OA treatment, 1.1 J/cm² of red LED light improved OA-induced barrier defect by increasing TEER from 29 \pm 2% of baseline to 36 \pm 3% of baseline ($P < 0.1$, Fig. 5).

DISCUSSION

Light therapy is used widely as a dermatological treatment for acne and acne scars. Medical devices using low level light therapy have also been reported to

reduce acne lesions and improve global assessments of acne [30,31]; however, few studies have investigated the mechanism of action of these low-level doses of light. The present study was conducted to determine whether low-level red light modulates the inflammatory process in acne. The data presented here show that low level red LED light treatments significantly reduce inflammatory mediator expression, inhibit excess keratinization and help reduce barrier damage, which are important contributing factors in development of acne.

The pathogenesis of acne is multifactorial, involving increased sebum production, abnormal follicular keratinization, *P. acnes* overgrowth within the hair follicles, and inflammatory mediator release [3]. An *in vitro* model was developed by using topical OA to induce an increase of IL-1 α release and SC thickening in human epidermal equivalents [11]. The current study utilized this human epidermal equivalent acne model to assess the effect of low-level red light on biomarkers associated with acne. It was shown that red light at 0.2–1.2 J/cm² inhibited OA-induced IL-1 α release (Fig. 3), demonstrating that the low level red light exerts anti-inflammatory activity via causing a reduction in IL-1 α levels. Low-level red light also mitigated unsaturated fatty acid induced stratum corneum thickening (Fig. 4.), which is thought to be mediated through the anti-inflammatory property of red light since IL-1 α has been shown to be the culprit in inducing hyperkeratinization *in vitro* [32–34].

In addition to the anti-inflammatory activity, our initial testing suggested low-level red LED light may also reduce barrier defects induced by OA treatment (Fig. 5); however, further studies are required to confirm this finding. Results published by others have shown that similar treatments (550–670 nm) accelerated the recovery of skin barrier after tape stripping [27], and may be beneficial in acne treatment. Indeed, it was recently suggested that acne subjects may also have an impaired skin barrier. The barrier functions of both inter- and intrafollicular epithelial linings are important to consider as they may be involved in the excessive keratinization associated with acne and with impaired physiological properties of the SC [35]. Yamamoto et al. [36] described that male patients, age 14–26, with mild to moderate acne exhibited markedly higher sebum secretion, larger trans-epidermal water loss (TEWL), and markedly decreased SC conductance (i.e., lower SC hydration), suggesting a deficient intercellular lipid membrane which correlates with impairment of the SC permeability barrier. Moreover, more epidermal and sebaceous lipids as well as more sebaceous free fatty acids were detected in the faces of acne subjects than those detected in the faces of healthy subjects [11]. In addition, total sebum lipids and TEWL were higher in acne subjects than that of normal subjects [5], further suggesting that high sebum may lead to barrier impairments in acne subjects.

There exist a number of devices reported to either treat acne or mitigate the post-lesional hyperpigmentation associated with acne including ablative and non-ablative lasers, radiofrequency, and low level light treatment.

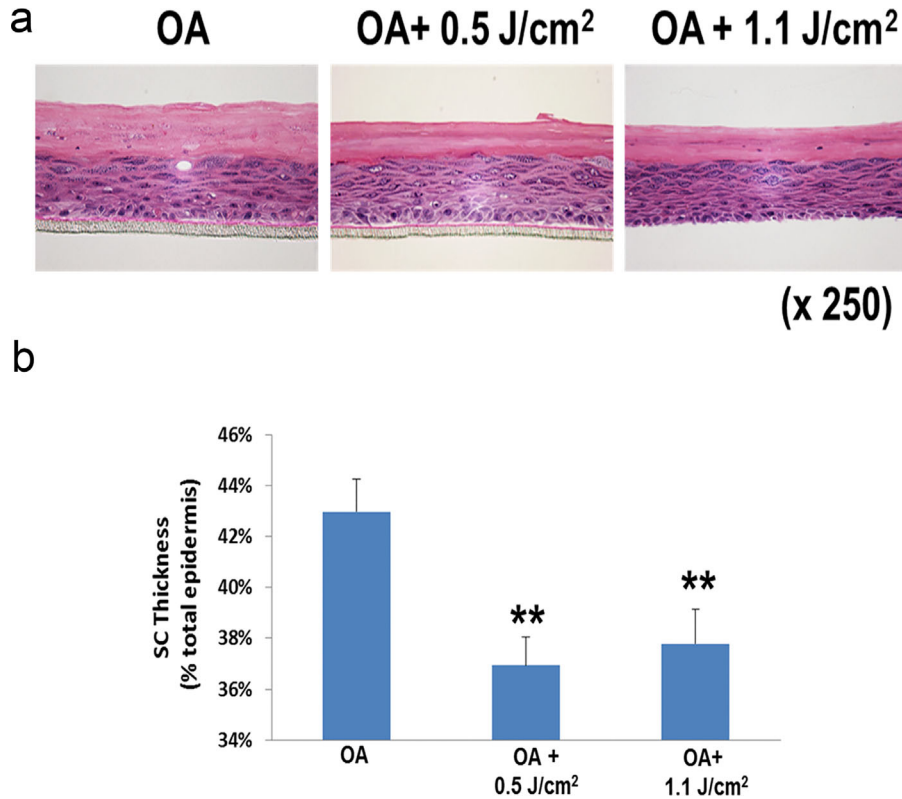


Fig. 4. Red light reduced oleic-acid-induced stratum corneum thickening in human epidermal equivalents. (a) Histology of human epidermal equivalents treated with oleic acid or with red light at 0.5 and 1.1 J/cm² in the presence of oleic acid. (b) Quantitative analysis of stratum corneum and epidermis in oleic acid-treated and red-light-plus-oleic-acid-treated human epidermal equivalents. Red light treated at 0.5 and 1.1 J/cm² caused a decrease of oleic-acid-induced thickening of stratum corneum. Original magnification, 250 \times . ** $P < 0.01$.

Ablative lasers, such as CO₂ and Erbium, work by vaporizing the outer layers of the skin causing the skin to undergo a wound healing process which results in the clinical improvement. In contrast, non-ablative lasers work by heating up the targeted tissue without actually destroying the skin and thus are less invasive than ablative treatments [37]. Both ablative and non-ablative lasers have been used in the treatment of acne, particular acne scars [38]. In addition to the post-acne scarring benefits, non-ablative laser treatments may also be effective in reducing erythema and post acne inflammation [39]. Non-ablative treatments using radio frequency (RF) have been reported to be effective for the treatment of mild acne. RF works by passing a electric current into the skin at specific depth to produce a micro thermal injury in the dermis which causes a wound healing response that results in the improved appearance of acne scars or by targeting sebaceous glands RF maybe an effective treatment of acne [40]. LED light therapy is another form of non-ablative treatment which has been reported to reduce the number of acne lesions in mild to moderate acne [19,20]. Multiple light therapy devices have been developed for acne treatment *in vivo*, using red light over a broad range of treatment parameters, mostly at relatively

high dosimetries. For example, Goldberg and Russell [41] showed a reduction in clinical acne lesion count in as little as 4 weeks of treatment, and 80% reduction in lesion count 8 weeks after the final treatment, by using a red-light regimen delivering 80 mW/cm² with an energy of 96 J/cm² per week for 4 weeks, at a total red light energy of 384 J/cm² delivered. In addition, Kwon et al. [29] showed significant reductions in inflammatory and non-inflammatory acne lesion counts with a relatively lower energy red light treatment regimen at 8.1 mW/cm² for 2.5 minutes per treatment (1.2 J/cm² per treatment), treated twice daily for 4 weeks, delivering a total of 68 J/cm² over the study. Using an *in vitro* model of acne, we demonstrated that low-level red light delivering 0.2–1.2 J/cm² showed anti-inflammatory benefit associated with improvements of tissue keratinization and barrier defects induced by OA treatment. Our study provided *in vitro* evidence that low-level red light could be used for acne treatment clinically. Nestor et al. [30] showed that treatment with a lower intensity of red light (0.156 mW/cm² for 15 minutes a day, or 0.14 J/cm² per day) for a total red light energy dose of 11.8 J/cm² over 12 weeks of treatment resulted in 24% reduction of acne lesions and was found to be safe and effective therapy for mild-to-moderate acne. Miller

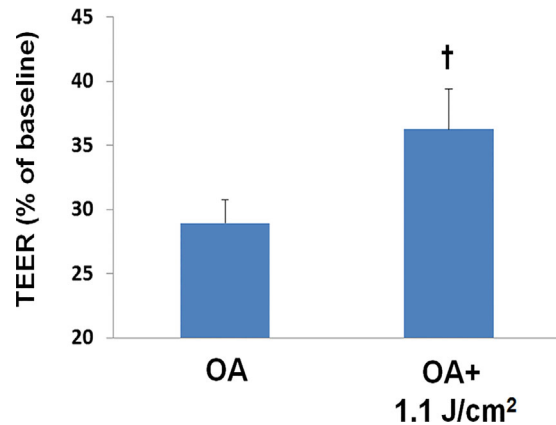


Fig. 5. Red light improved oleic-acid-induced barrier defect in human epidermal equivalents as measured by trans-epithelial electrical resistance (TEER). Oleic acid damaged the skin barrier by reducing TEER from 100% of baseline to ~30% of baseline whereas red light treatment at 1.1 J/cm² improved the skin barrier by increasing TEER to 36% of baseline. †*P* < 0.1.

et al. [31] reported that treatment with a low level red light (0.5 mW/cm² for 10 minutes a day, or 0.3 J/cm² per day) for a total red light energy dose of 25.2 J/cm² over 12 weeks produced significant reductions in inflammatory and non-inflammatory acne lesion counts and investigator global assessment. These two clinical results clearly demonstrate a broad effective range of light dosimetry for low-level light therapy of acne which are comparable to the levels used in our studies. Taken together, the data presented here suggest that low-level light therapy using red LED light treatments can inhibit inflammation and normalize pilosebaceous duct hyperkeratinization, which is an important factor in development of acne, and medical devices using low-level red LED light therapy may provide effective treatment for acne.

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