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Light Emitting Diode-Generated Blue Light Modulates Fibrosis Characteristics: Fibroblast Proliferation, Migration Speed, and Reactive Oxygen Species Generation

Andrew Mamalis[1,2], **Manveer Garcha**[1], and **Jared Jagdeo**[1,2,3]

[1]Department of Dermatology, University of California Davis, Sacramento, CA, USA

[2]Dermatology Service, Sacramento VA Medical Center, Mather, CA, USA

[3]SUNY Downstate Medical Center, Department of Dermatology, Brooklyn, New York, USA

Abstract

Background and Objective—Blue light is part of the visible light spectrum that does not generate harmful DNA adducts associated with skin cancer and photoaging, and may represent a safer therapeutic modality for treatment of keloid scars and other fibrotic skin diseases. Our laboratory previously demonstrated that light-emitting diode (LED) red and infrared light inhibits proliferation of skin fibroblasts. Moreover, different wavelengths of light can produce different biological effects. Furthermore, the effects of LED blue light (LED-BL) on human skin fibroblasts are not well characterized. This study investigated the effects of LED-BL on human skin fibroblast proliferation, viability, migration speed, and reactive oxygen-species (ROS) generation.

Methods and Materials—Irradiation of adult human skin fibroblasts using commercially-available LED-BL panels was performed in vitro, and modulation of proliferation and viability was quantified using the trypan blue dye exclusion assay, migratory speed was assessed using time-lapse video microscopy, and intracellular ROS generation was measured using the dihydrorhodamine flow cytometry assay. Statistical differences between groups were determined by ANOVA and Student s t-test.

Results—Human skin fibroblasts treated with LED-BL fluences of 5, 30, 45, and 80 J/cm^2 demonstrated statistically significant dose-dependent decreases in relative proliferation of 8.4%, 29.1%, 33.8%, 51.7%, and 55.1%, respectively, compared to temperature and environment matched bench control plates, respectively. LED-BL fluences of 5, 30, 45 and 80 J/cm^2 decreased fibroblast migration speed to 95 ± 7.0% ($p = 0.64$), 81.3 ± 5.5% ($p = 0.021$), 48.5 ± 2.7% ($p < 0.0001$), and 32.3 ± 1.9% ($p < 0.0001$), respectively, relative to matched controls. LED fluences of 5, 10, 30, and 80 J/cm^2 resulted in statistically significant increases in reactive oxygen species of 110.4%, 116.6%, 127.5%, and 130%, respectively, relative to bench controls.

Conclusion—At the fluences studied, LED-BL can inhibit adult human skin dermal fibroblast proliferation and migration speed, and is associated with increased reactive oxygen species generation in a dose-dependent manner without altering viability. LED-BL has the potential to

Corresponding author: Jared Jagdeo, MD, MS, jrjagdeo@gmail.com, Telephone: (917) 837-9796, Fax: (916) 451-7245.

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contribute to the treatment of keloids and other fibrotic skin diseases and is worthy of further translational and clinical investigation.

INTRODUCTION

Light in the ultraviolet (UV) spectrum (300–400 nm) is used to treat various skin diseases, however, UV light causes DNA adducts that have been linked to skin cancers and premature photoaging [1,2]. Visible light in the 400–760 nm range is presumably not associated with harmful DNA adducts, and may represent a safer alternative to UV phototherapy, however, the biological effects, underlying mechanisms, and clinical uses of different wavelengths of visible light are not well characterized [3]. Non-coherent light-emitting diode generated 415 +/− 15 nm light is technically violet light, but is commonly referred to as blue light and is part of the visible light spectrum. Currently, light emitting diode blue light (LED-BL) phototherapy is clinically used in dermatology primarily for photorejuvenation and acne, and likely has potential for treatment of other skin diseases, such as skin fibrosis [4–6].

LED phototherapy is mechanistically based upon the photobiomodulatory effects caused by light in the visible spectrum. Specifically, LED-BL phototherapy is hypothesized to function through either direct generation of reactive oxygen species (ROS) or through photostimulation of the flavin group attached to complex I (nicotinamide adenine dinucleotide (NADH)-dehydrogenase) of the mitochondrial electron transport chain [7,8]. Blue light has been demonstrated to cause alterations in fibroblast proliferation and antioxidant capacity, TGF-beta signaling, and myofibroblast differentiation [9,10]. In addition, LED phototherapy has been shown to lead to alterations in cytokines, growth factors, and inflammatory mediators [7,11,12].

Skin fibrosis is a progressive reaction to chronic injury or inflammation. The hallmark of skin fibrosis is an excessive deposition of extracellular matrix (ECM) components, such as collagen, that eventually result in thickening and stiffness of the skin [13–16]. Skin fibrosis is the distinguishing feature of many chronic skin diseases, including systemic sclerosis, graft versus host disease (GVHD), hypertrophic scars, keloids, nephrogenic systemic fibrosis, porphyria cutanea tarda, restrictive dermopathy and other conditions. These fibrotic diseases are associated with increased morbidity and mortality, increased incidence of depression and other psychiatric comorbidities, and decreased quality-of-life [17–24]. Despite the significant clinical and social burdens associated with skin fibrosis, there are few FDA-approved anti-fibrotic drugs [25]; thus, research in this area is crucial in addressing this deficiency.

We hypothesized that LED-BL may be capable of modulating key cellular functions in human adult fibroblast cells that are associated with skin fibrosis including proliferation, migration speed, and reactive oxygen species. We chose to study the effects of LED-BL doses from 5–80 J/cm^2 because these doses demonstrated a dose-dependent inhibition of normal fibroblast proliferation in pilot experiments. We believe that LED-BL phototherapy has the potential to be used as a non-invasive treatment for skin fibrosis. Due to the safety, portability, and cost-effectiveness of LEDs, LED-BL phototherapy holds potential benefits over other modalities used to treat skin fibrosis such as surgery, intralesional steroids and

ablative and non-ablative laser treatments that are costly and associated with significant side effects. Furthermore, LED-BL could potentially be used in combination as an adjunctive therapy with other devices and drugs used to treat skin fibrosis.

The effects of LED-BL on human skin fibroblasts are not well characterized. This study investigated the effects of LED-BL on human skin fibroblast proliferation, and viability in vitro. This research builds upon our previously reported findings that LED red light and LED infrared light can decrease normal human skin fibroblast proliferation in vitro [26,27]. Herein, we report the effects of LED-BL on normal human skin fibroblast proliferation, viability, migration speed, and reactive oxygen-species (ROS) generation.

METHODS

Cell Culture

Monolayers of primary human skin fibroblasts (AG13145, Coriell Institute) were cultured in Dulbecco modified Eagle medium (Gibco/Invitrogen, Carlsbad, CA) with 10% bovine calf serum and 1% antibiotic-antimycotic mixture. The cell cultures were incubated at 37°C with 5% carbon dioxide. Fibroblasts were plated at 2×10^4 cells per 35-mm dish. All experiments were performed on cell cultures passage 12 or less.

Light-emitting Diode Irradiation

Cells were irradiated 24 hours after plating, using an LED-BL array (415 ±15 nm, Omnilux Clear-U, Photo Therapeutics, Carlsbad, CA) at a power density of 350 W/m^2 at room temperature. Media temperatures measured using a digital thermometer (HH506RA, Omega Engineering, Stamford, CT) throughout irradiation remained constant at 32–34°C. By remaining within physiologic temperatures, heat stress was not induced. Each experimental plate receiving LED-BL treatment was randomly matched with a bench control plate (BCP) to ensure that the measured effect was a result of LED-BL treatment and not due to ambient light or environment. BCPs were derived from the same stock of cell suspension, taken out of the incubator at the same time as their matched treatment pairs, protected from the LED light source, and placed on a digital warming block (Multi-Blok 2001, Thermo-Scientific, Waltham, MA) with a negative feedback temperature control system set at 32 °C and BCP media temperatures remained 32–34°C.

Cell Counts and Viability with Trypan Blue

Following treatment with LED-BL, treated plates and BCPs were then returned to the incubator and incubated for 48-hours. Following incubation, cell counts were taken that included all cells in the sample, including media change, trypsinization products, and washes. Cells counts were calculated by sampling a known volume (10 μL) and measuring the cell concentration using a hemocytometer and trypan blue. Counting with trypan blue dye allows the microscopist to distinguish live from necrotic dead cells during cell counts because the dye is impermeable to the intact membranes of live cells. Viability can then be calculated based upon the ratio of live (total minus dead) to total cells. Mean percentage proliferation and viability (live cells/total [live + dead] cells) relative to non-irradiated

controls are reported as means and standard errors of the mean. Cell count and viability experiments were repeated three times to verify data reproducibility and accuracy.

Time-Lapse Video Cell Migration Microscopy

Time-lapse video migration experiments were performed as described with modifications [28]. For migration experiments, primary human fibroblast cells were plated on 35 mm^2 culture dishes with CO_2-independent medium (Invitrogen) and incubated for 24 hours. Immediately following irradiation, time-lapse images of the cell migration were captured every 30 minutes for 4 hour in an environmental chamber monitored under microscopy (ECLIPSE TE2000-E, Nikon Instruments, Tokyo, Japan) with the temperature maintained at 37 °C. The time-lapse videos were generated using Volocity Image Software (PerkinElmer) and were analyzed using Openlab Software (PerkinElmer) to measure cell migration speed. “Migration speed” is the average speed in μm per minute that the cells travel in a 4-hour period. The migration speed of 50 cells was measured from each group. Statistical analysis of data was performed using the paired two-tailed Student s t-test, significance level was set at $p < 0.05$. Migration experiments were repeated three times to verify data reproducibility and accuracy.

Dihydrorhodamine Flow Cytometry Assay

Cells were assessed for intracellular ROS generation as previously described [29]. Briefly, 2×10^4 per dish were intravitally stained with dihydrorhodamine for 30 minutes, washed with PBS, and then irradiated as described above. The positive control samples were exposed to 1.2 mM H_2O_2 (Sigma-Aldrich) for 30 minutes. Following irradiation or H_2O_2 exposure, cells were washed with PBS, collected with trypsin, and processed for flow cytometry analysis. The level of intracellular ROS was evaluated by flow cytometry analysis using a Guava easyCyte HT system at 525 nm. Median fluorescent intensity was measured for each group and statistical analysis of the data was performed using ANOVA and the paired two-tailed Student s test, significance level was set at $p < 0.05$. Dihydrorhodamine ROS flow cytometry experiments were repeated twice to verify data reproducibility and accuracy.

Statistical Analysis

Statistical analysis with analysis of variance (ANOVA) was performing using using GraphPad Prism version 6.00 for OSX (GraphPad Software, San Diego, CA, USA) to compare treatment arms and the Student s t-test to compare each treatment arm with the paired bench control arm.

Results

Our experimental results demonstrate that treatment of primary adult human skin fibroblasts using LED-BL (415 nm) statistically significantly inhibits fibroblast proliferation in a dose-dependent manner without causing significant effects on viability at fluences of 10, 15, 30, or 80 J/cm^2 (Figures 1 and 2). Light-emitting-diode (LED) generated blue light (LED-BL) (415 nm) significantly decreases the relative proliferation rate of normal human skin fibroblasts at fluences of 0 J/cm^2 (0) = 100 ± 1.7%, 5 J/cm^2 (1:55) = 91.6 ± 3.7% ($p = 0.75$), 10 J/cm^2 (3:50) = 70.9 ± 2.9% ($p = 0.001$), 15 J/cm^2 (5:45) = 66.2 ± 2.1% ($p = 0.00013$), 30

J/cm^2 (11:30) = 48.3 ± 1.8% (p = 0.000005), 80 J/cm^2 (30:40) = 44.9 ± 3.1% (p = 0.000006), compared to matched bench control plates (Figure 1). No statistically significant decreases in cell viability were noted compared to matched bench control plates (Figure 2).

LED-BL irradiation with fluences of 5, 30, 45 and 80 J/cm^2 decreased fibroblast migration speed to 95 ± 7.0% (p = 0.64), 81.3 ± 5.5% (p = 0.021), 48.5 ± 2.7% (p < 0.0001), and 32.3 ± 1.9% (p < 0.0001), respectively, relative to matched bench control (Figure 3).

Irradiation with LED-BL resulted in significantly increased generation of intracellular reactive oxygen species as measured by dihydrorhodamine. Fluences of 5, 10, 30, and 80 J/cm2 resulted in statistically significant increases in median fluorescent intensity of 110.4% (9404 ± 232, p=0.0487), 116.6% (9930 ± 394 p=0.035), 127.5% (10864 ± 205, p = 0.0014), and 130% (11077 ± 201, p < 0.0001), respectively, relative to bench control (8519 ± 214) (Figure 4).

Discussion

This study confirms the findings of previous blue light photobiomodulation research and adds novel findings pertaining to fibroblast modulation and mechanism associated with LED-BL in vitro [10,30]. Our research is distinct from previously published studies as we utilized a widely used commercially available LED-BL array and therefore our findings may more likely be clinically translatable and used as a foundation for future clinical studies. We evaluated the photobiomodulatory effects of LED-BL using commercially available human skin dermal fibroblasts in contrast to previously evaluated chinese hamster lung fibroblasts [30] and primary human dermal fibroblasts [10]. Similarly to other researchers, we assessed the photobiomodulatory effects of LED-BL on proliferation, viability, and ROS with similar but distinct methodologies and range of LED-BL doses [10,30]. Additionally, we used time-lapse video microscopy to study the effects of LED-BL on single cell migration, which has not been previously reported.

Our data demonstrate that LED-BL can decrease the proliferation of adult fibroblasts in vitro in a dose-dependent manner (Figure 1). LED-BL statistically significantly inhibits fibroblast proliferation in a dose-dependent manner without causing significant effects on viability at fluences of 10, 15, 30, and 80 J/cm^2. Treatment with these fluences resulted in statistically significant decreases in cell number without a significant decrease in viability as compared to BCPs (Figure 2). These alterations in final cell count may have resulted from modulation of cell cycle, increases in autophagy, and/or apoptosis. It is possible that blue light, generated by LEDs or other light sources, has biologically relevant features similar to UVA light such as ROS generation and immunomodulatory effects due to the proximity of UVA and blue light on the electromagnetic spectrum.

LED-BL irradiation with fluences of 5, 30, 45 and 80 J/cm^2 decreased fibroblast migration speed compared to matched bench control in a dose-dependent manner. This is significant because increased migration speed is seen in tissue fibrosis and increased cellular migration is believed to play a role in the pathogenesis of skin fibrosis [31,32]. Specifically, the increased migration speed of fibroblasts may contribute to increased local recruitment of

fibroblasts to the affected skin and recurrence or maintenance of fibrotic conditions [31,32]. Therefore, using LED-BL to inhibit fibroblasts migration may promote skin health and prevent promotion and perpetuation of fibrosis. Additional in vitro-in vivo correlation studies are needed as it is possible that long term photoaging, post-inflammatory hyperpigmentation, increased MMP-1/procollagen type I ratio are possible undesirable effects that could happen with constant and prolonged use of blue LEDs.

Furthermore, our data demonstrates that irradiation with LED-BL resulted in significantly increased generation of intracellular reactive oxygen species in a dose-dependent manner as measured by dihydrorhodamine relative to bench control (Figure 4). Given that the increased ROS generation due to LED-BL is also dose dependent, the dose-dependent decreases in migration speed and proliferation may be related to increases in ROS. Therefore, ROS generation is a potential mediator of the observed cellular effects of LED-BL. Further study is warranted to explore if neutralizing LED-BL-generated ROS can reverse or prevent the LED-BL effects on proliferation, migration, and other measures.

We recently reported similar findings using LED-generated infrared and red light to treat normal human skin fibroblasts [26,27]. To our knowledge, this is the first report of the inhibitory effect of LED-BL on human skin fibroblast migration in vitro. We believe that our findings serve as “proof of principle” and may establish the foundation for the translation of these “bench” findings to clinical studies to determine the anti-fibrotic effects of LED-BL for the treatment of keloids and other cutaneous scarring conditions. A limitation of blue light is that it does not penetrate past 2 mm into the dermis and is not likely a good therapeutic option for treating skin conditions that involve the deep reticular dermis.

Attention to bias is necessary when attempting to demonstrate light-induced effects on human skin fibroblasts. Specifically, the observed effects may be attributed to variations in proliferation patterns between the experimental and control plates. The effects may also be attributed to various environmental factors other than the light energy that the LED device generated. We controlled for environmental conditions by using bench controls, monitored to confirm matching temperatures, and repeated all experiments three times to account for variation in cellular proliferation patterns. Further studies are needed to translate these findings to clinical use.

LED-BL phototherapy may represent a safe and cost-effective modality to improve patient outcomes with keloids or other fibrotic skin disorders and therefore further study is warranted. Further in vitro studies are needed to elucidate the mechanism underlying LED-BL phototherapy and to investigate its effects on key pathogenic processes related to skin fibrosis, such as collagen production and the MMP-1/Procollagen I ratio.

These data may serve as the in vitro foundation for the investigation of LED-BL phototherapy for the treatment of skin fibrosis such as keloids or other forms of scarring. We hypothesize that our findings will translate to clinical therapy of keloid and other forms of fibrotic disease. Future studies are aimed at investigating the effects of LED-BL on collagen production, TGF-beta signaling, and investigating the precise role of ROS generation in eliciting the LED-BL related cellular effects. Future research will focus on establishing

dosing regimens, elucidation of mechanism of action through interrogation of the TGF-Beta pathway and other molecular signaling pathways linked to skin fibrosis, and translation of these findings to clinical studies to improve the lives of patients with skin fibrosis.

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Abbreviations

ECM	extracellular matrix
LED	Light emitting diode
LED-BL	Blue light emitting diode
MMP-1	matrix metalloproteinase 1
NADH	Nicotinamide adenine dinucleotide
TGF-Beta	Transforming growth factor beta
ROS	Reactive oxygen species
UV	Ultraviolet light
UVA	Ultraviolet A

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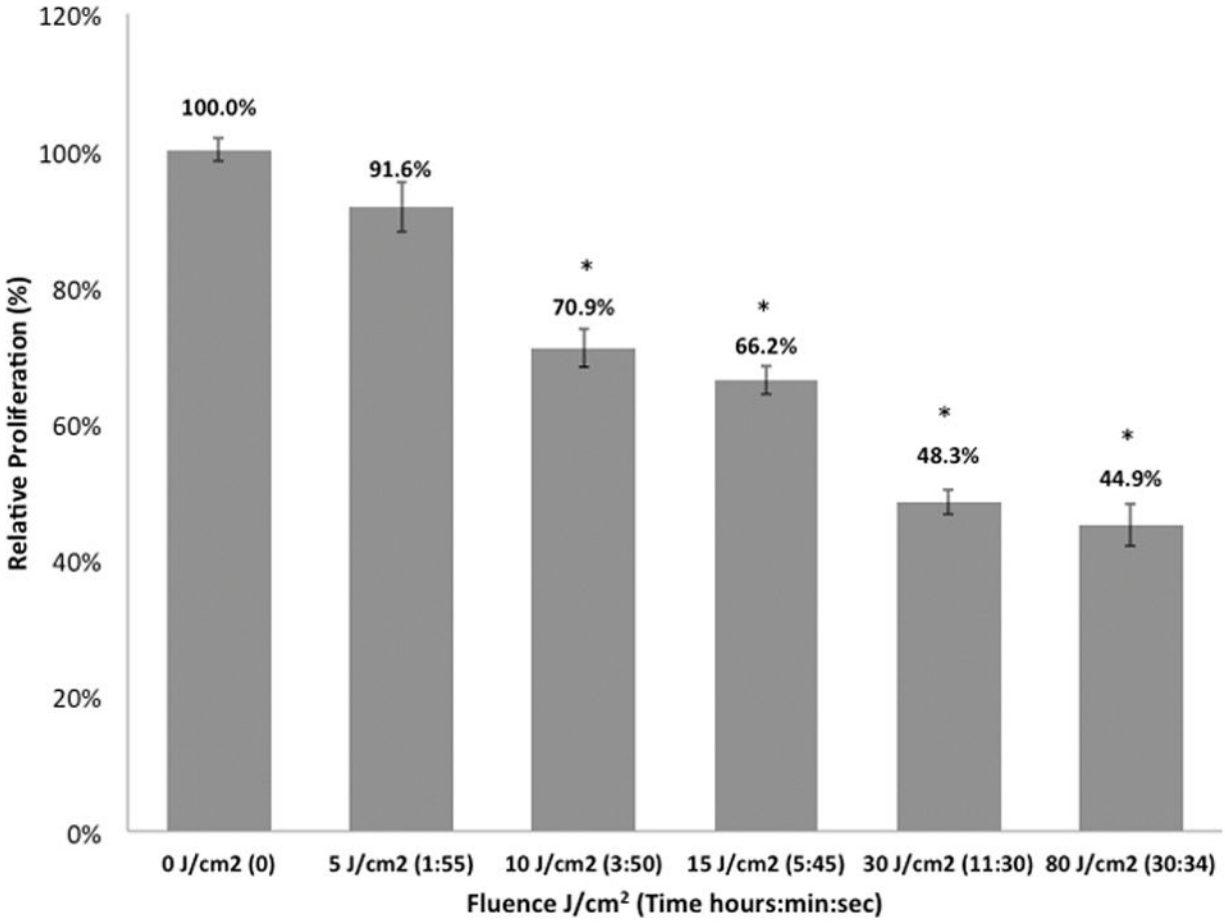

Figure 1. Light-emitting diode generated blue light (LED-BL) decreases fibroblast relative proliferation in a dose-dependent manner

LED-BL significantly decreases the relative proliferation determined by cell counts of normal human skin fibroblasts at fluences of 0 J/cm2 (0) = 100 ± 1.7%, 5 J/cm2 (1:55) = 91.6 ± 3.7% ($p = 0.75$), 10 J/cm2 (3:50) = 70.9 ± 2.9% ($p = 0.001$), 15 J/cm2 (5:45) = 66.2 ± 2.1% ($p = 0.00013$), 30 J/cm2 (11:30) = 48.3 ± 1.8% ($p = 0.000005$), 80 J/cm2 (30:40) = 44.9 ± 3.1% ($p = 0.000006$), compared to matched bench control plates. Assessed using Trypan blue assay. Proliferation is presented as percentage of control. Error bars represent standard error of the mean. (* represents $p < .01$).

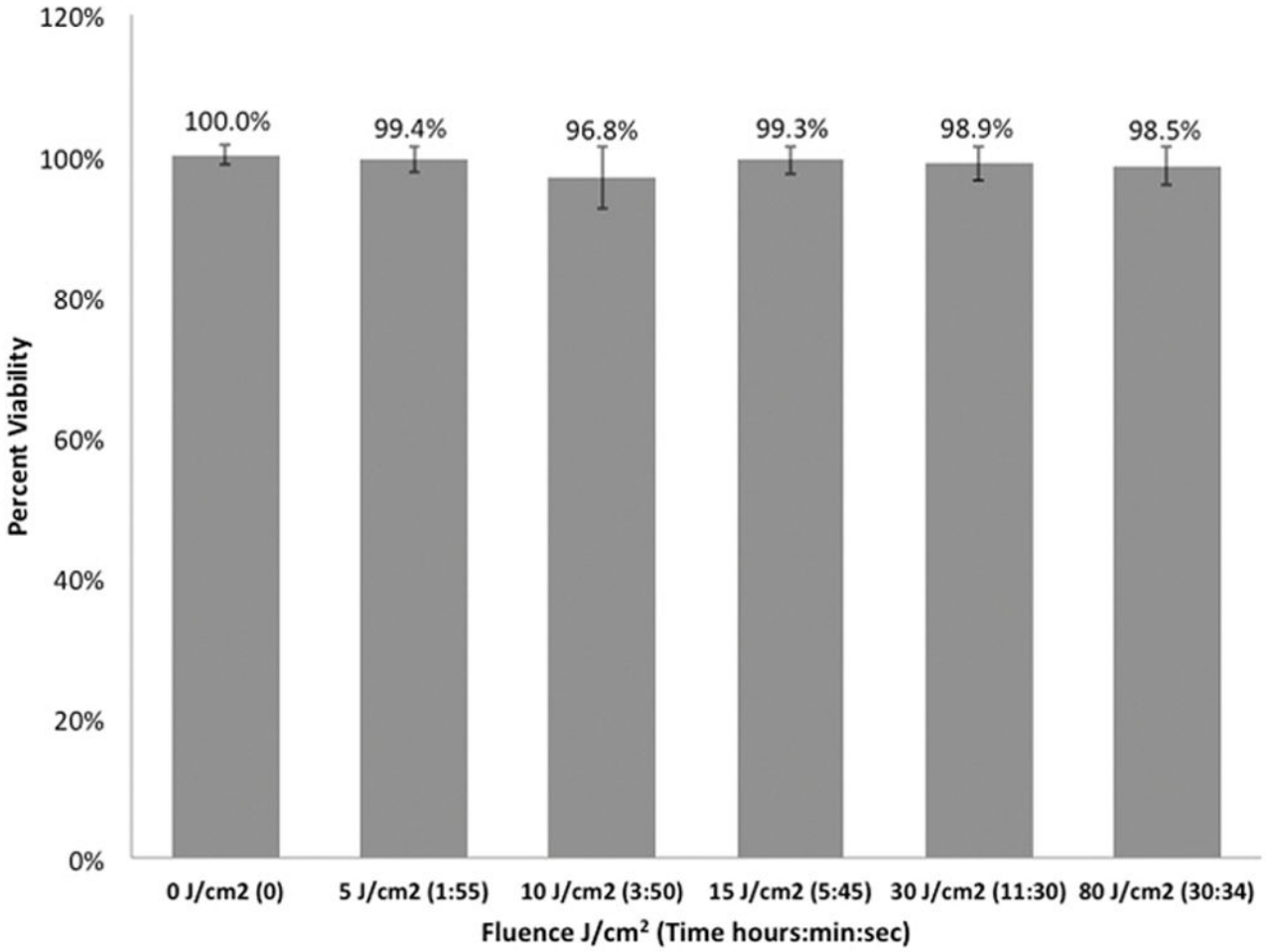

Figure 2. Light-emitting diode generated blue light (LED-BL) does not significantly alter fibroblast viability

In adult human fibroblasts treated with LED-BL, cellular viability is maintained in vitro at fluences of: 0, 5, 10, 15, 30, and 80 J/cm2. 48-hours following irradiation, cells were assessed by Trypan Blue Assay. Relative viability is presented as percent of the control. No statistically significant differences were found between these groups when comparing viability. Error bars represent standard error of the mean. (* represents $p < .01$).

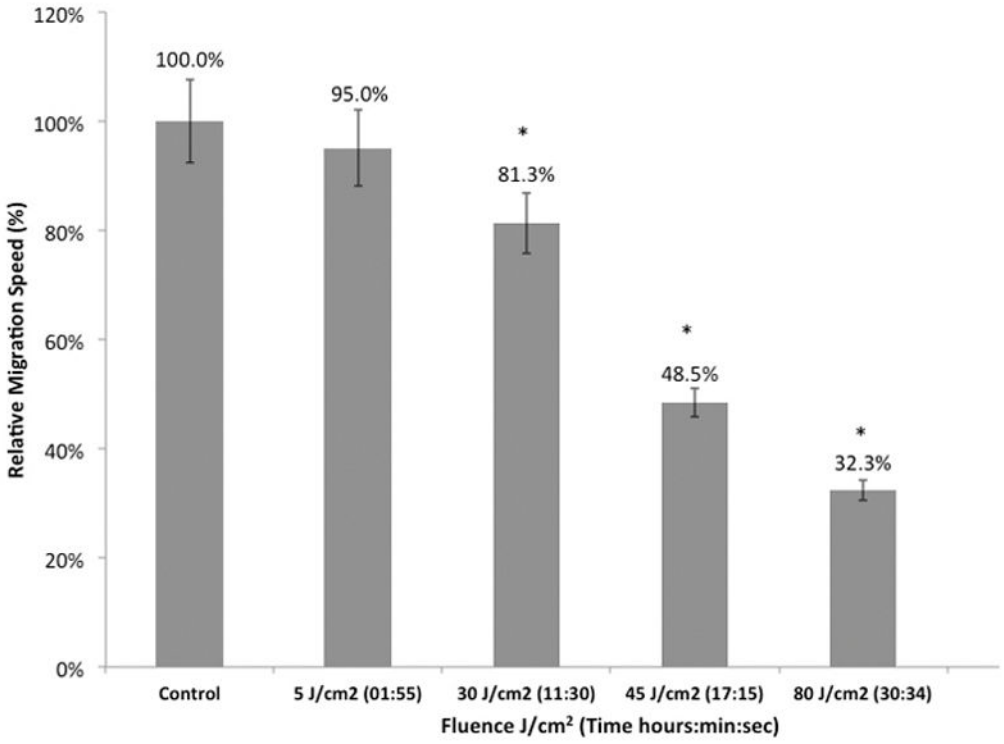

Figure 3. Light-emitting diode generated blue light (LED-BL) decreases fibroblast migration speed relative to control in a dose-dependent manner

LED-BL irradiation with fluences of 5, 30, 45, and 80 J/cm^2 decreased fibroblast migration speed to 95 ± 7.0% ($p = 0.64$), 81.3 ± 5.5% ($p = 0.021$), 48.5 ± 2.7% ($p < 0.0001$), and 32.3 ± 1.9% ($p < 0.0001$), respectively, relative to matched bench control. Error bars represent standard error of the mean. (* represents $p < .01$).

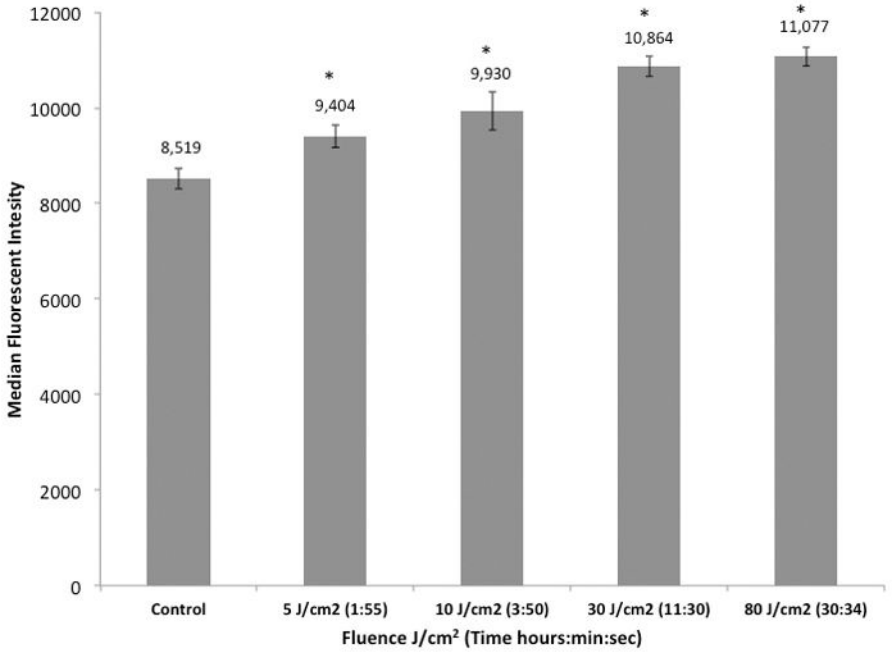

Figure 4. Light-emitting diode generated blue light (LED-BL) increases intracellular reactive oxygen species (ROS) levels in a dose-dependent manner

Irradiation of normal human fibroblasts with LED-BL at fluences of 5, 10, 15, 30, and 80 J/cm2 resulted in significantly increased generation of endogenous reactive oxygen species as measured by dihydrorhodamine. Fluences of 5, 10, 30, and 80 J/cm2 resulted in statistically significant increases in median fluorescent intensity of 110.4% (9404 ± 232, p=0.0487), 116.6% (9930 ± 394 p=0.035), 127.5% (10863 ± 205, p = 0.0014), and 130% (11077 ± 201, p < 0.0001), respectively, relative to bench control (8519 ± 214). Error bars represent standard error of the mean. (* represents p < .01).