Portable Multicolor LED-Based System for the Photobiomodulation Therapy on Wound Healing Process *In Vitro*

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Research Article

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Abstract

Photobiomodulation is a practical and non-invasive treatment that triggers cell proliferation, cell differentiation, wound healing, new tissue formation, inflammation and pain reduction with low-level light therapy. Light-emitting diodes (LEDs) are energy-saving, affordable and safe alternatives to laser devices which are recently preferred in photobiomodulation. Although the wavelengths between 600–700 nm are most preferred ones, there is a lack of practical optical systems which study this mechanism *in vitro* with different wavelengths simultaneously. In this study, a portable and remotely controlled multicolor LED-based system was designed and tested on the wound healing process of human keratinocytes by irradiating the cells homogenously with 3 different wavelengths (460–475 nm as blue, 515–535 nm as green, and 585–595 nm as orange) on different experimental groups at the same time. Its proliferative and wound healing effect was evaluated with cell viability (MTT) analysis and cell migration (scratch) assay, respectively. It was observed that orange-LEDs were designated as the most triggering wavelength in terms of cell proliferation. Also, it was revealed with this device that different wavelengths can reach the intended accelerated wound healing process, so this optical system will be an advantageous design for future practical photobiomodulation studies *in vitro*.

Introduction

Photons are the smallest unit of light used in modern medicine to diagnose and cure diseases since the 1800s. The physical nature of light and its mutual interaction with matter enabled its usage for therapeutic purposes [1]. The interaction mechanisms between light and matter reveal important information related to the characteristics of matter and tissue as well [2]. Thus, light with improved optical instruments has become a revolutionary breakthrough in the field of medicine and biomedical sciences [3].

Among various application areas, light can be used to destroy pathogens or cancer cells [4–6] and also promote cell proliferation and differentiation [7, 8]. Photobiomodulation is specifically used to stimulate or inhibit cellular processes by changing cellular biochemical activities with the absorption of photon energy by certain cellular components at non-thermal and low-level irradiances [9]. Filtered lamps and light-emitting diodes (LEDs) are commonly used in this mechanism as incoherent light sources. Besides, laser devices are commonly preferred as coherent light sources for this purpose [10]. The visible (400–700 nm) and near-infrared region (700–1000 nm) are the most widely used light spectrum in photobiomodulation [11]. The primary cellular responses to photobiomodulation are cell proliferation, enhancing tissue repair and regeneration, decreasing inflammation and pain, relaxation of nerves and muscles, and inhibition of tissue degradation via light-induced biochemical pathways [12–14]. The energy and power densities of light sources should be kept at low doses not to induce any photothermal effects such as cutting, ablation, and coagulation of the tissues thermally [15].

Different LED-based light sources and laser devices have become advantageous optical instruments to trigger photobiomodulation mechanisms [10, 13]. Although the biostimulation effect of different

wavelengths emitted from these light sources is considerably known, it is still necessary to design a more efficient system to illuminate the cells for the investigation of the underlying mechanism of photobiomodulation. The main problem in these applications is that there is an energy loss during irradiation with the existing light systems [3, 10, 16, 17]. In this study, we aimed to design and produce a portable LED-based photobiomodulation system that fits 12-well cell culture plates and works as a cover for those plates. Its LED-array system was built up with 3 pieces of 460–475 nm blue, 3 pieces of 515–535 nm green, and 3 pieces of 585–595 nm orange-LED light sources. A single LED corresponds to a single well of the plate and 3 wells were left blank without any LED for the control group. Each LED was placed in the middle-top of each well for homogenous illumination. The purpose of this study with this system was to make clear which wavelength is more efficient to induce cell proliferation and wound healing processes on human keratinocyte cells after triple light treatments with 24-hour intervals.

Results

Wound Healing Assay After Triple Light Treatment

During triple light treatment on keratinocytes, the cells were observed by microscopy and their microscopic images were captured every 24 hours for the control, blue, green, and orange-LED groups. Figure 1 shows the results of the light applications at 1 J/cm^2. These images represented that the wounds in the blue, green, and orange-LED groups were closed to a large extent at around the 24th hour, while total closure was accomplished at the 48th hour. However, the wounds of the control group did not heal and were not closed in this period (Fig. 1).

Figure 1.

Figure 2 shows the results of the light applications at 3 J/cm^2. These images demonstrated that the wounds of the green LEDs healed completely at the 48th hour and the wounds of the orange-LEDs healed completely at the 72nd hour. However, the light applications with blue-LEDs did not close the scratches at this energy density in three days. Similarly, the wounds of the control group were not closed, either (Fig. 2).

Figure 2.

Figure 3 shows the results of the light applications at 5 J/cm^2. These images represented that the light applications of the orange and green-LEDs closed the wounds completely at the 48th and 72nd hour, respectively. The applications with blue-LEDs closed the wounds to a greater extent nearly at the 72nd hour. However, the wounds of the control groups did not heal in three days (Fig. 3).

Figure 3.

Cell Viability Analysis After Triple Light Treatment

Cell viability analysis was performed with MTT analysis at the end of a three-day follow-up process. Triple light treatments on human keratinocyte cells induced maximum cell proliferation after the application with green-LEDs at 1 J/cm^2 and orange-LEDs at 3 J/cm^2 and 5 J/cm^2 as depicted in Fig. 4. The amount of cell proliferation in a green-LED group at 1 J/cm^2 energy density was 196% as a percentage. The amounts of the cell proliferation in orange-LED groups at 3 and 5 J/cm^2 energy densities were 150% and 214%, respectively. The orange-LED group after 3 J/cm^2 application and blue, green, and orange-LED groups after 5 J/cm^2 application had the highest significance level (***) when they were compared with the control groups (Fig. 4).

Figure 4.

Discussion

Although the proliferative effects at the wavelengths of the red and near-infrared region were proven in many studies [18–20], it is thought that there is a need for a design of a more efficient optical system to obtain consistent outcomes. In addition to this problem, the majority of the existing studies still evaluates the possible effect of the wavelengths in the range of 600 to 700 nm [21], but only the minor researches show the promise of other wavelengths in the visible spectrum, such as the wavelengths in the range of 400 to 600 nm [16, 17, 22].

Throughout the improvement of the Portable Multicolor LED-Based System for photobiomodulation therapy, we have paid attention to produce a system that is portable, cost-effective, small-sized, lightweight, durable, user-friendly, self-supporting, and easy to clean. We have designed this optical system with 9 LEDs (3-pieces of blue (460–475 nm), 3-pieces of green (515–535 nm), and 3-pieces of orange-LED light sources (585–595 nm)) and each of them corresponded to a single well of the 12-well plate to illuminate the single well homogenously with a single LED. The three wells of the culture plate were left blank without any LED to be used as the control group in the same experiment.

In this system, power LED light sources and 15° LED lenses were used together to provide a more homogenous illumination in the wells, due to the non-coherent structure of LED light and the location of LEDs which was the middle-top of each well. Here, LED lenses played a crucial role in reducing the spread of light to ensure homogenous illumination as in the work of Hwang et al [23]. Furthermore, black insulating materials that fit the border of a well were produced from the PLA filament to inhibit the transmission of the light beam from a well to the adjacent ones. To obtain maximum performance from the LEDs, transistors, LED drivers, and aluminum coolers were used and the LEDs were connected in series. With the help of the transistors, the LED drivers, and the aluminum cooler, the internal environment of the device was kept cooler during its performance and LEDs worked efficiently without losing their performance. Furthermore, a 12V 1.5A adaptor was used to activate 3-pieces metal-oxide-semiconductor field-effect transistors (MOSFETs) and LED Drivers, and to lead the 9-pieces of LEDs by Arduino Uno. Thus, the current and the voltages of the LED were kept constant without any loss during its operation

and the output powers that were obtained from a single LED for each color were enough and suitable to induce photobiomodulation.

Until recently, it has been believed that the laser devices are more suitable light sources to induce photobiomodulation [9], because of their specific characteristics which are being collimated, coherent, and monochromatic [10]. The LEDs are energy-saving, affordable, and safe alternatives to lasers with having a smaller range of wavelengths. They have great promise in photobiomodulation, too. Generally, red-LEDs have been handled in clinics and scientific researches [22], but the information for the use of blue, green, and orange-LEDs are limited. Even so, it is known that low energy blue, green, and orange LEDs can promote several biological effects on the tissue and cellular levels [16, 17, 22].

Chabert *et al.* worked with an LED system that emitted the wavelengths of 590 nm yellow light, 630 nm red light, and a combination of them as an orange light at 4, 8, and 12 J/cm^2 energy densities on the cell viability and the morphology of the normal human keratinocyte and fibroblast cells. They irradiated the cells twice with a 48 h interval. The best outcome regarding the cell morphology of the fibroblasts was obtained with 12 J/cm^2 energy density. For both fibroblasts and keratinocytes, orange LED irradiation induced better improvements in the cell shape, cell viability, and protein expression such as Collagen 1. Thus, it was stated that orange light can be a promising wavelength to combat skin aging [17]. Similarly in our study, the highest proliferative effect on keratinocyte cells obtained with the triple orange-LED application at the energy densities of 3 J/cm^2 and 5 J/cm^2 with the rates of 150.73% and 214.53%, respectively.

Wang *et al.* examined the effects of 420 nm blue-LED array, 540 nm green filtered lamp, 660 nm red diode laser, and 810 nm NIR diode laser at the fluence of 3 J/cm^2 for five times in every two days on the osteogenic differentiation of human adipose-derived stem cells (hASCs) for three weeks. Blue and green light irradiations at 3 J/cm^2 provided an increment in the intracellular calcium concentration, and others did not induce dramatic increases. Thereupon, they stated that 420 nm blue and 540 nm green lights were more effective on the osteogenic differentiation with respect to 660 nm red and 810 nm NIR light [16]. In our study, we did not examine the cell differentiation, but we have evaluated the wound healing and cell proliferation issues on keratinocytes. We have observed that triple green light application had a great impact, similar to the study of Wang *et al.*, on the cell viability of keratinocytes with a proliferation rate of 196%.

Zhu *et al.* examined the effect of blue light irradiation on the osteogenic differentiation and the cell proliferation of human gingival mesenchymal stem cells (hGMSCs) via blue-LEDs at 1, 2, 4, and 6 J/cm^2 energy densities. They showed that there was a significant increase in osteogenic differentiation and it was proved with real-time PCR analysis of the specific genes, especially after the 2, 4, and 6 J/cm^2 light applications. Besides, it was found that blue-light irradiation inhibited cell proliferation in the same study. It was thought that these stem cells took the path for cell differentiation. Thus, there was not any significant increase in cell viability [22]. On the contrary, triple blue light application in our study revealed

successful outcomes in cell proliferation of keratinocytes at 5 J/cm^2 energy density, with a rate of nearly 150%.

In the experimental part of this study with the portable multicolor LED-based photobiomodulation system, it was observed that the triple light treatment accelerated the wound healing process significantly and increased the cell viability of the keratinocytes with each wavelength at the different energy densities. Any of these applications created considerable differences when compared with the control group. The highest cell proliferation rates were achieved with the green-LED treatment at 1 J/cm^2 and the orange-LED treatment at 5 J/cm^2. At the beginning of this study, we hypothesized that the Portable Multicolor LED-Based System will be an important design for the examination of different wavelengths in the photobiomodulation process. Now, the obtained results confirmed this hypothesis with the positive outcomes of the green and orange-LED light applications on the accelerated wound healing of keratinocytes.

Conclusion

The Portable Multicolor LED-Based Photobiomodulation System with its specific design that is properly compatible with the cell culture plates was very useful to study photobiomodulation process with different wavelengths at different energy doses and application modes, simultaneously together with the control group. Thus, it may provide plenty of information for the uncertainties and the inconsistent outcomes related to the low-level light applications. For future studies and applications, the design of the Portable Multicolor LED-Based System can be improved and adapted according to the needs of the applications on patients to be handled easily by healthcare professionals to treat some problems such as chronic wounds or skin aging, especially in dermatology clinics.

Materials And Method

Design and Manufacture of the Portable Multicolor LED-Based System

In this study, 9-pieces LED light sources which have 3 different wavelength ranges (3 pieces of 460–475 nm blue, 3 pieces of 515–535 nm green, and 3 pieces of 585–595 nm orange-LEDs) were used to produce a portable device to illuminate a 12-well plate. The mechanical design of this system had 2 main parts, which were the 3D design of the device which was performed in Computer-Aided Design (CAD) modeling of SolidWorks 2016, and the production of this system by the 3D printing technology. Polylactic acid (PLA) filament was used to produce the case of the system by a 3D Printer (Ultimaker 2 + Connect, Utrecht, Netherlands).

Arduino Uno R3 was used for the design of the electrical parts of the system. HC-06 Bluetooth-Serial Module Card was used to control the LEDs of the device at any time remotely for the establishment of an IoT connection between the device and a smartphone. 3-pieces of BUZ80A N-channel MOSFETs were

used to switch loads (LEDs) that would consume high power by the way of Arduino Uno and LED Drivers. Three LED drivers were used to operate 9 Power LEDs connected in series. 3-pieces 460–475 nm blue, 3-pieces 515–535 nm green, and 3-pieces 585–595 nm orange Power LEDs were placed in the lid of a 12-well plate to provide a more homogenous illumination inside the wells directly on the cells. The operating voltages were 3–4 Volts for blue and green-LEDs and 2–3 Volts for orange-LEDs. The output power of each LED was measured with a powermeter (Thorlabs, USA). It was 267 mW for blue LEDs, 188 mW for green LEDs, and 157 mW for orange LEDs. Heat sinks were used to transfer the excess heat from the LEDs to an aluminum cooler, while 15-degree LED lenses were used to enhance the light intensity and emit the light to the bottom of the wells of the plates efficiently. The aluminum cooler was used to receive the excess heat from heat sinks, while the fan was used to cool down all the environment in the device. 12V 1.5A adaptor was used to actuate the system and an LED was used on the outer side of the system to show that it was working.

12V 1.5A adaptor activated the 3-pieces of MOSFET transistors and LED drivers, and lead the 9-pieces of LEDs by Arduino Uno. Additionally, the same color of LEDs was connected in series, so currents were the same but voltages were different. Following the electrical design of the device, software parts of the device were planned to make the system functional, user-friendly, and self-supporting. At first, the Arduino code of the device was written and loaded into the Arduino Uno. Afterward, system control was planned and created by the MIT App Inventor which is an open-source web application sustained by the Massachusetts Institute of Technology (MIT). Then, the template for the system control in the MIT App Inventor was loaded to a smartphone as an Android application package format (Apk). Besides, cylindrical and rectangular shape black insulation barriers were produced with PLA filaments by 3D printing technology. The cylindrical shape barriers totally fit into the wells of the cell culture plate and the rectangular shape insulation barrier fits the bottom of the well plate. They prevented any light diffusion through the wells.

Finally, it was used to induce the photobiomodulation mechanism on human keratinocyte cells by remotely controlling this optical system. Figure 5 shows the steps of the mechanical, electrical, and software design of the system.

Figure 5.

Cell Culture

The human skin keratinocyte cell line (HS2) was used to test the possible biostimulation effects of this system. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, Germany) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, USA), 1% Penicillin (Gibco, USA), and 1% L-Glutamine (Gibco, USA). They were incubated in a humid environment containing 95% air, 5% CO_2 at 37°C until they formed a single confluent layer. The medium was refreshed every 2 days. When they reached 80% confluency, they were washed with Phosphate Buffered Saline (PBS, BioShop, Canada). Then trypsin-EDTA solution (Sigma Aldrich, Germany) was used to detach and transfer the cells from the flasks

to the 12-well plates. $5x10^5$ cells were seeded in each well of the plates and incubated overnight before the applications.

Experimental Procedure for Light Applications

Before each application, all the equipment of the photobiomodulation system was sterilized by UV light in a biological safety cabinet to prevent any contamination. All the insulation barriers were at first incubated in 70% alcohol and then sterilized by UV light before they were placed inner side of the wells and the bottom of the well plate in order to prevent any scattered light between the wells. Thereby, the photobiomodulation system and the environment of the cells were sterilized before the applications. 4 different experimental groups were formed: Control, Blue-LED, Green-LED, and Orange-LED groups, and the applications of all of these groups experimented were performed simultaneously with 3 samples in each group with this photobiomodulation system as shown in Fig. 5. Three different energy densities (1, 3, and 5 J/cm^2) were used to irradiate the cells in each group. The output powers for each color of the LEDs were different from each other. So the exposure time for each color was different to reach the same amount of energy densities. All the light parameters used in these experiments were explained in Table 1. To induce photobiomodulation, the cells received three light applications at the same energy dose with a 24-hour time interval and they were followed up for three days.

Table 1.

Wound Healing Assay

Wound healing assay, which is also known as scratch assay, was performed to analyze the wound healing and cell migration process of the cells after light applications. After the cells were seeded in the 12-well plates and incubated overnight, the growth medium was drawn and the cells were washed with PBS. The middle part of the cell layer in each well was scratched with the tip of a small pipet tip. Then the cells in every 12 wells were irradiated with the photobiomodulation system at 1 J/cm^2, 3 J/cm^2, and 5 J/cm^2 of energy densities three times with a 24-hour interval. After the light applications, the cells were observed by light microscopy (Olympus CKX41, Olympus Co. Ltd., Tokyo, Japan) until the wounds were closed in experimental groups. The microscopic images of the wounds were taken every 24 hours at 10X magnification.

MTT Assay

When the wound healing assay was completed, the cell viability in each group was analyzed by MTT Assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Analysis, Sigma Aldrich, Germany). The growth medium of the cells was removed and newly prepared sterile 5mg/mL MTT solution in 10% PBS and 90% serum-free culture media were added to each well. The cells were incubated in 95% air and 5% CO_2 environment at 37°C for 2 hours. After incubation, the MTT solution was removed, the cells were washed with PBS, and dimethyl sulphoxide (DMSO, Merck, Germany) was added to the wells. The cell culture plates were covered with aluminum foils to prevent any light access to the well plates and they were incubated for 10 minutes. Finally, the absorbance values of each well were measured by a

microplate reader (BioTek, Winooski, VT, USA) at a wavelength of 570 nm. To calculate the viable cell number, a calibration curve was formed and viable cell numbers were calculated by using this equation of the calibration curve.

Statistical Analysis

Initially, all data were analyzed with the help of a one-way Analysis of Variance (ANOVA). Then Student's t-test was performed to compare the data of the control and the light treatment groups. The *p*-value which was less than 0.05 was considered as statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001). Besides, the standard errors were represented as error bars in all figures.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

N. T. and U. B. designed the study. N. T., U. B., and Z. B. Y. Ç. conducted the experiments. U. B. performed the data and statistical analysis. N. T. and U. B. contributed to writing, reading, and editing the manuscript. All authors approved the final manuscript.

ADDITIONAL INFORMATION

The authors declare no competing interests.

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Tables

Table 1. Light parameters used in the triple light applications (energy densities, output powers, and exposure durations corresponding to the different color LEDs of the system).

Energy Density (J/cm^2)	Blue Light (267 mW)	Green Light (188 mW)	Orange Light (157 mW)
1 J/cm^2	57 s	81 s	97 s
3 J/cm^2	171 s	243 s	291 s
5 J/cm^2	285 s	405 s	485 s

Figures

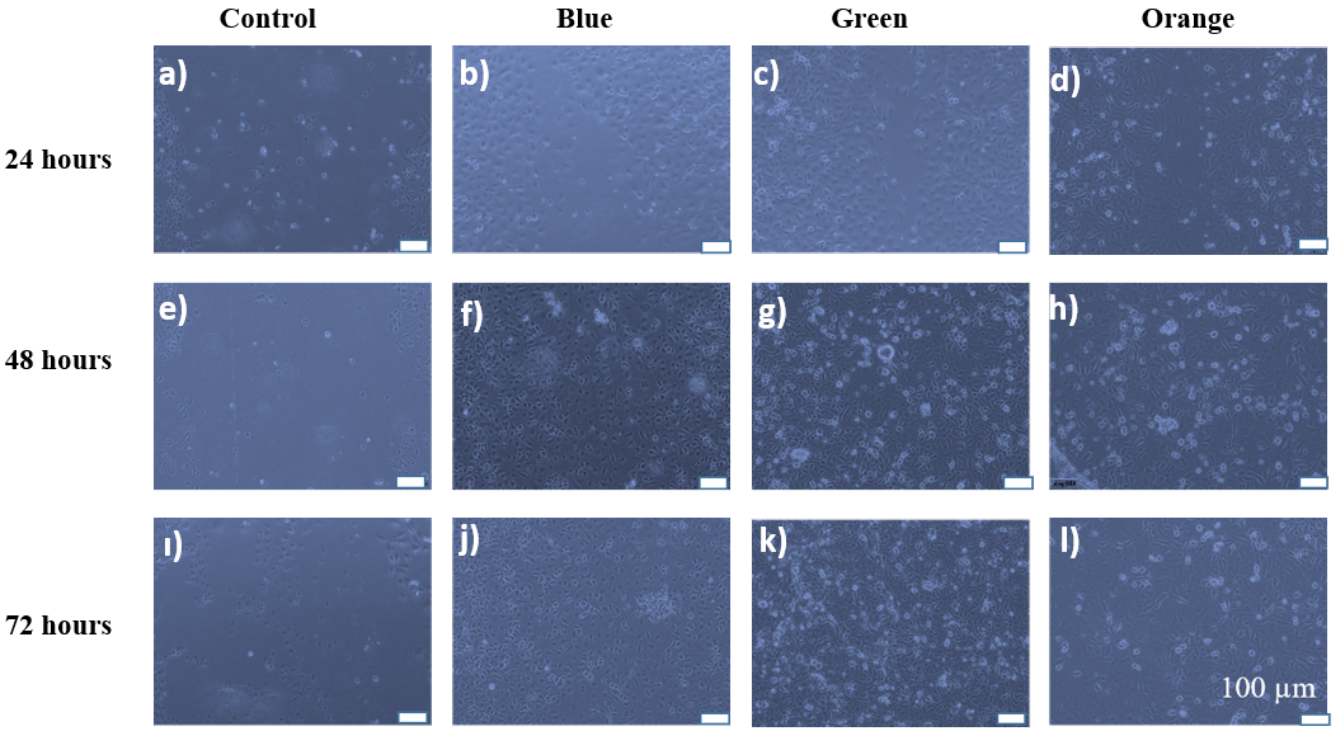

Figure 1

Microscopic images of keratinocytes in wound healing assay after the triple light treatment at 1 J/cm2 for the groups of a) Control at the 24-hour, b) Blue light at the 24-hour, c) Green light at the 24-hour, d) Orange light at the 24-hour, e) Control at the 48-hour, f) Blue light at the 48-hour, g) Green light at the 48-hour, h) Orange light at the 48-hour, i) Control at the 72-hour, j) Blue light at the 72-hour, k) Green light at the 72-hour, l) Orange light at the 72-hour. Scale bar represents 100 µm.

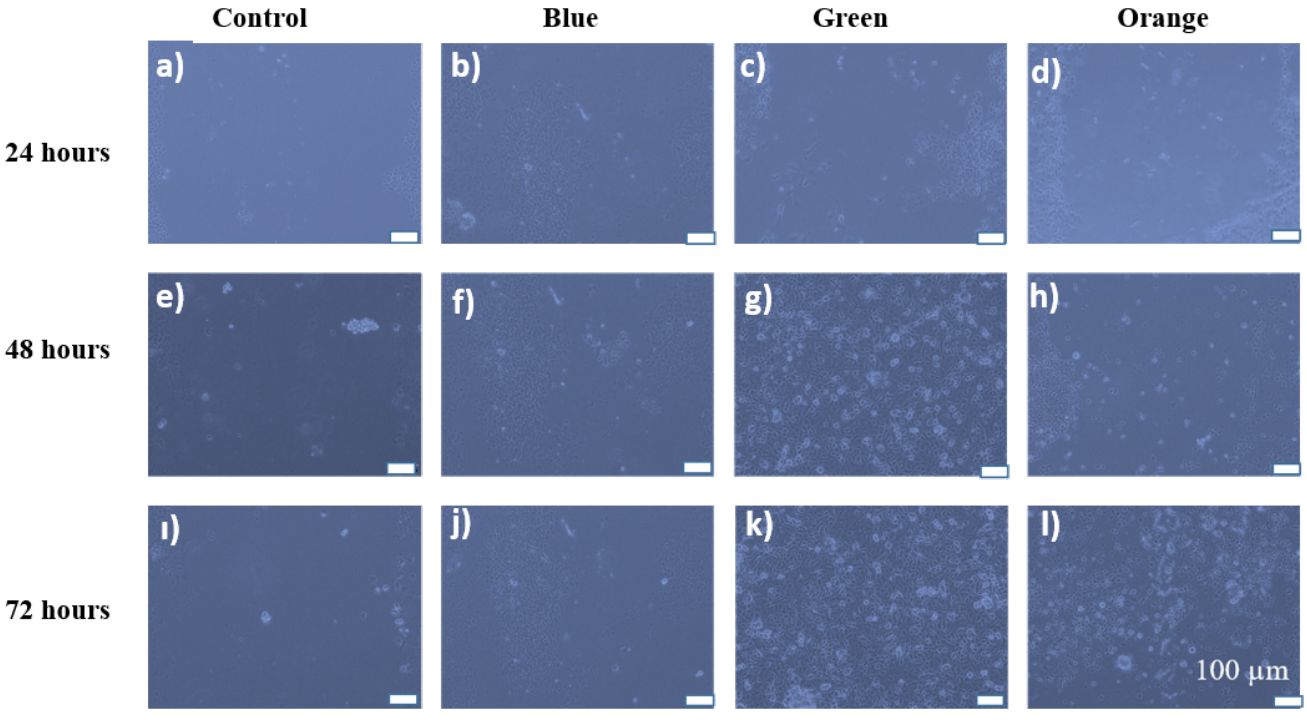

Figure 2

Microscopic images of keratinocytes in wound healing assay after the triple light treatment at 3 J/cm2 for the groups of a) Control at the 24-hour, b) Blue light at the 24-hour, c) Green light at the 24-hour, d) Orange light at the 24-hour, e) Control at the 48-hour, f) Blue light at the 48-hour, g) Green light at the 48-hour, h) Orange light at the 48-hour, i) Control at the 72-hour, j) Blue light at the 72-hour, k) Green light at the 72-hour, l) Orange light at the 72-hour. Scale bar represents 100 µm.

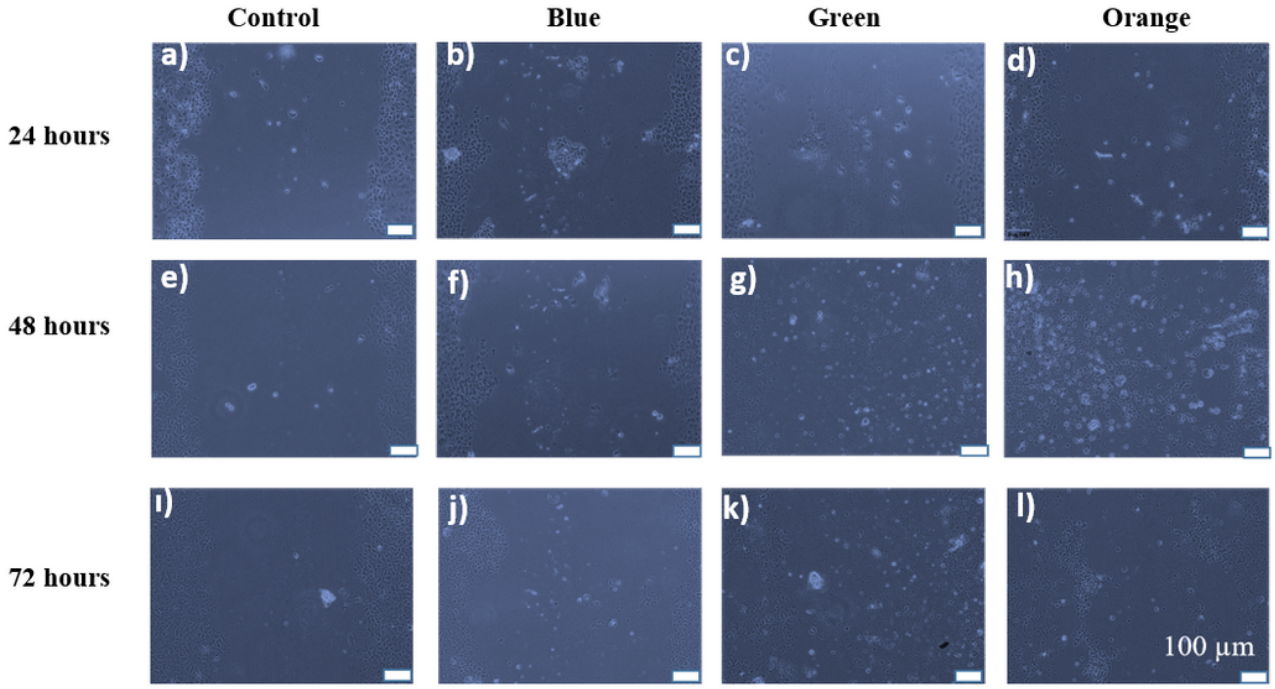

Figure 3

Microscopic images of keratinocytes in wound healing assay after the triple light treatment at 5 J/cm2 for the groups of a) Control at the 24-hour, b) Blue light at the 24-hour, c) Green light at the 24-hour, d) Orange light at the 24-hour, e) Control at the 48-hour, f) Blue light at the 48-hour, g) Green light at the 48-hour, h) Orange light at the 48-hour, i) Control at the 72-hour, j) Blue light at the 72-hour, k) Green light at the 72-hour, l) Orange light at the 72-hour. Scale bar represents 100 µm.

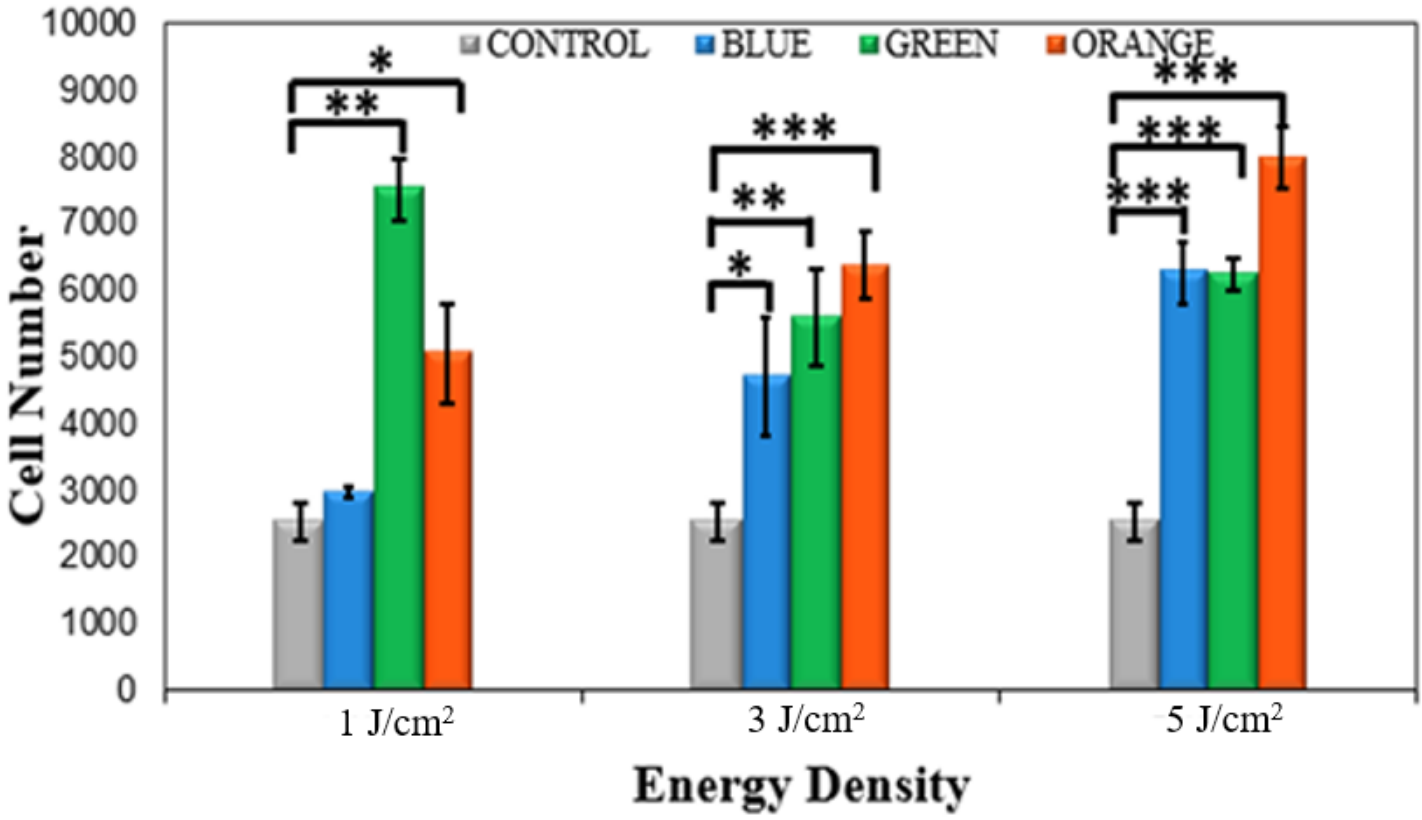

Figure 4

The number of viable cells in Control, Blue, Green, and Orange-LED groups determined by MTT assay after triple light treatment at 1, 3, and 5 J/cm2 energy densities. Data were analyzed by one-way ANOVA and the comparisons between groups were performed by Student's t-test. * indicates p<0.05, ** indicates p<0.01, and *** indicates p<0.001.

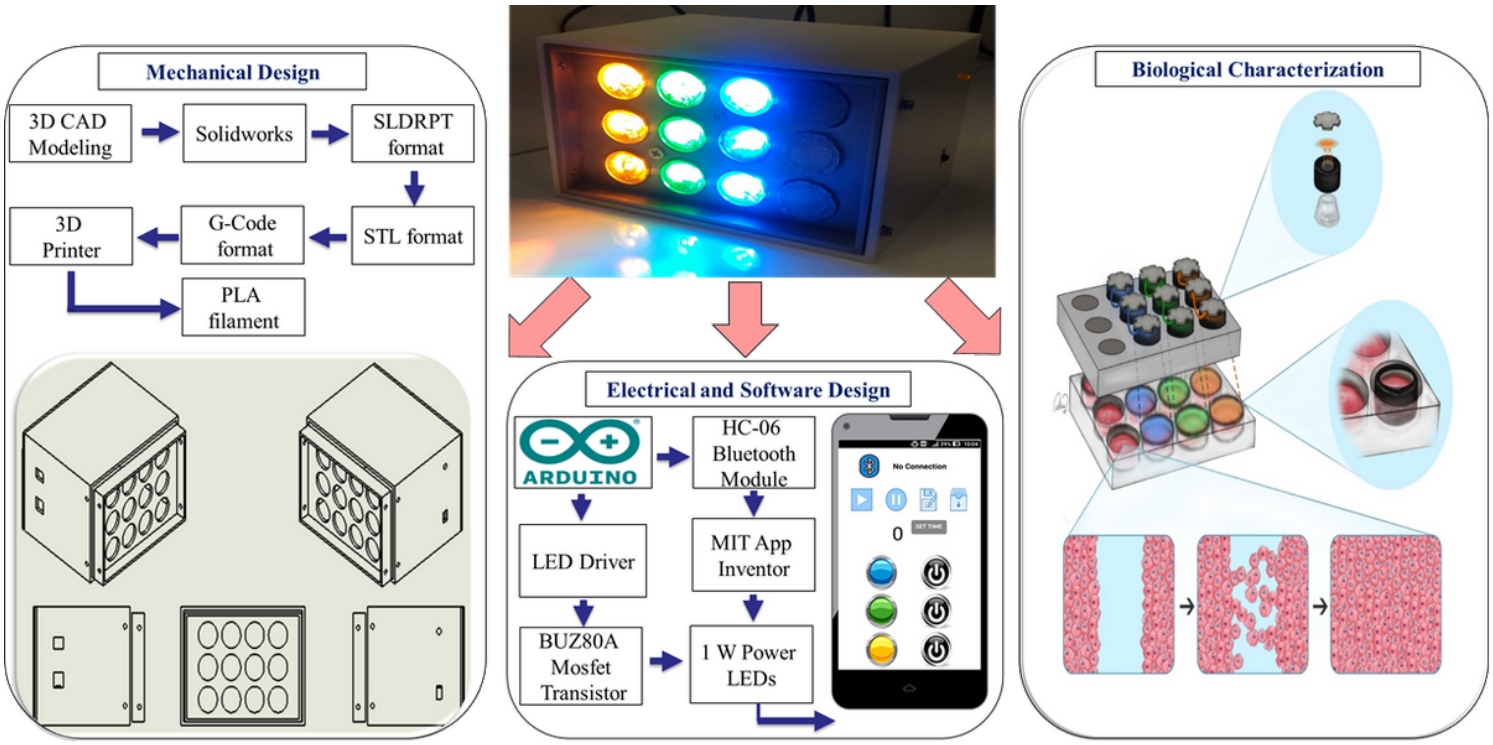

Figure 5

The flowchart for the design and manufacture of the Portable Multicolor LED-Based Photobiomodulation System.