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Effect of Pulsed-810 nm Laser Photobiomodulation on Dermal Wound Healing and Oxidative Stress in Immunosuppressed Rats

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ABSTRACT

Under immunosuppression, the sequential overlapping wound repair phases get hampered due to dysregulated or persistent inflammation leading to non-healing chronic wounds formation. The present study investigates the effect of low-power 810 nm diode laser (70 mW mean output power; 40 mW/cm^2 average irradiance; 24 J/cm^2 total fluence; 10 Hz pulse frequency; duty cycle 50 per cent; 10 min. illumination time once daily for seven days) photobiomodulation (PBM) on dermal penetration ability, wound healing and oxidative stress in hydrocortisone-induced immunosuppressed rats. The results of the penetration ability of 810 nm laser irradiation to the depth of the sub-dermal region revealed that the transmitted power of laser at 10 Hz pulsed-mode was better and easier than continuous-mode. The present findings clearly delineated that PBM with 810 nm laser at 10 Hz significantly augmented healing and reduced oxidative stress as evidenced by decreased free radicals, nitric oxide (NO) levels, enhanced superoxide dismutase (SOD) enzyme activity and wound area contraction facilitating the cellular redox homeostasis and promoting the tissue repair process. In conclusion, PBM with NIR 810 nm laser at pulsed-mode 10 Hz frequency showed better penetration and accelerated dermal wound healing in immunosuppressed rats.

Keywords: Dermal wound healing; Immunosuppressed; Inflammation; Oxidative stress; Pulsed 810 nm photobiomodulation; Reactive oxygen species

1. INTRODUCTION

Photobiomodulation therapy (PBMT) is an emerging non-invasive, non-thermal, drug-less, biophysical modality for enhanced wound healing, neuronal repair, mitigation of pain, inflammation, and restores cellular functions[1]. The cellular mechanism of PBM has been attributed to the absorption of photons by light-absorbing components (chromophores) of the cellular respiratory chain[2]. The light absorption at optimum radiant exposure by cellular chromophores elicits photophysical and photochemical events, which induces downstream molecular signaling cascades. The photons absorbed by endogenous intracellular photoacceptor (principally mitochondrial complex-IV, cytochrome c oxidase enzyme; CCO) leading to the enhancement of mitochondrial respiration, ATP production, calcium influx, alteration in cellular redox state that induces phototransduction reactions at various biological scales by regulating numerous transcription factors, retrograde-mitochondrial and intracellular signaling pathways associated with cell proliferation, migration, adhesion, inflammation, pain, apoptosis, tissue repair and regeneration[3-4].

The absorption and scattering of light in tissue are predominantly wavelength-dependent and it has been found that near-infrared (NIR) light spectrum (800-1100 nm) has less scattering property with greater tissue penetration and absorption at cellular chromophores. This property of NIR light facilitates the photoexcitation of target molecules with minimal losses of photon energy[3]. Pulsed-wave (PW) light has been found to be more effective than continuous-wave (CW) light. PW laser follows a quenching period (pulse-on/ off) and thereby, generates no measurable temperature changes in the irradiated area hence reduce thermal damage by tissue heating. On the contrary, CW causes an increase in the temperature of the intervening and target tissues or organs. Further, PW improves the ability of the laser to penetrate deep tissues achieving adequate energy at the target tissues[5-6].

The normal progression of wound healing includes the well-orchestrated overlapping phases of hemostasis, inflammation, new tissue formation and remodeling within a stipulated time-frame. It involves intricate interactions among different cell types, growth factors, structural proteins, extracellular matrix and proteinases. The impaired wound healing could be due to myriad pathological factors[1]. Chronic non-healing wounds fail to progress through a tightly regulated process of repair. In immunosuppressed subjects, the sequential repair phases get hampered due to dysregulated or persistent inflammation and oxidative damage[5]. Oxidative stress due to overproduction of free radicals viz. reactive oxygen species (ROS), reactive nitrogen species (RNS) and incompetent cellular antioxidant defense system at the wound bed of immunosuppressed subject could leads to a consequence of prolonged inflammation, free radical-induced damage, septicemia, reduced angiogenesis,

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inhibited fibroblast formation and delayed granulation tissue formation[6].

The factors, which are responsible for delayed wound healing are metabolic syndromes (diabetes), autoimmune diseases, burns, genetic disorders, ischemia, vascular disorders, pressure ulcers, immune deficiency, malnutrition and chemotherapy[7]. The abnormal wound healing causes a major deal of psycho-physiological discomfort, which may incur a financial burden on the healthcare system. Currently, different strategies are available for the management of poorly healing wound employing novel modalities like bioactive-dressing, stem cell therapy and pharmacotherapeutic agent interventions and biophysical modalities using non-thermal light-based therapy (PBMT), pulse electromagnetic field and bioelectric stimulation[8].

PBMT has marked potential to regulate different wound healing processes like growth factor, cytokines production, neovascularisation cellular proliferation, differentiation, migration, adhesion and tissue remodeling[3]. In dermatology, the beneficial healing effects of PBMT have been evaluated on wrinkles, acne scar, psoriasis, diabetic and burns injuries[1]. The optimal regime of PBM is important for any particular application and dose higher or lower than the optimal value may have negative or no therapeutic effect[3]. Hence, the present study investigates the effects of pulsed NIR 810 nm diode laser irradiation using optimised optical parameters on dermal penetration ability, wound healing and oxidative stress in hydrocortisone-induced immunosuppressed experimental rats. The present findings showed that PBM with 810 nm laser at pulsed-mode 10 Hz frequency augments cutaneous healing by enhancing the repair process and reducing oxidative stress in immunosuppressed rats.

2. MATERIALS AND METHODS

2.1 Experimental Animals

The male Sprague-Dawley rats weighing 180 ± 20 g were used in the present experiment. Animals were maintained under the standard light: dark cycle (12 h: 12 h), temperature (25 ± 1 °C) and housed in the separate sterile cage, provided with free access to rodent diet and water *ad libitum*. Animal experiments were performed as per the regulations specified by the Institute's Animal Ethical Committee (DIPAS/IAEC/2017-14), which were in accordance with the guidelines of the CPCSEA, the Government of India.

2.2 Induction of Immunosuppressed State

The immunosuppressed state was obtained by the administration of hydrocortisone (40 mg/kg body weight, intramuscular) in rats daily for 1 week prior to wound creation and continued till the termination of the experiment to maintain the state of immunosuppression, as explained earlier by our group[5].

2.3 Wound Formation

Animals were anaesthetised by intraperitoneal (i.p.) administration of ketamine-xylazine cocktail (90-10 mg/kg, body weight). The dorsal hair of rats was removed using an electric clipper and a circular (1.5 cm diameter) full-thickness dermal wound was made on the dorsal surface of the rat using the sterile surgical blade and scissor under aseptic conditions. After seven days post-wounding rats were euthanised by administrating overdose of thiopental (90 mg/kg body weight, i.p.) and wound tissues were carefully collected to evaluate the biochemical markers.

2.4 Experimental Design and Laser Treatment

Rats were randomly divided into two groups with 6 animals in each: an immunosuppressed control (ISC) group and a laser irradiated (LI) group. Light irradiation was done using NIR 810 nm Al-Ga-As diode laser (Physiolaser-RJ Laser, Germany) at 40 mW/cm^2 average irradiance; 24 J/cm^2 total fluence; 10 Hz pulse frequency; duty cycle 50 per cent; 10 minutes illumination time once daily for seven days (Table 1). There was a distance of 3.0 cm between the laser probe and the surface of the wound bed to get the optimum irradiance and to cover the wound surface area of rats. ISC group was left untreated to heal naturally.

Table 1. List of NIR 810 nm diode laser parameters for photobiomodulation treatment

Laser Parameters	Value
Wavelength	810 nm
Spot Size	Diameter = 1.5 cm, area = 1.77 cm^2
Average Power	70 mW
Average Irradiance	40 mW/cm^2
Total Energy	42 J
Total Fluence	24 J/cm^2
Mode	Pulse Frequency 10 Hz
Pulse Duration	50 msec
Peak irradiance	80 mW/cm^2
Duty cycle	50%
Illumination Time	10 min, once daily
Treatment Regimen	7 Post-wounding days

2.5 Skin Penetration Ability

The skin penetration profile of 810 nm laser was analysed on rat skin flaps. Laser irradiation of skin flaps was performed with 810 nm laser either at CW or PW (10 Hz)-mode and amount of penetrating light energy was measured and recorded using a 3A-ROHS with Nova-II laser power/ energy meter (Ophir Optronics Solutions Ltd., Israel).

2.6 Wound Area Analysis

The wound area contraction was measured planimetrically on day zero and eighth postwounding and represented in term of square millimeters.

2.7 Evaluation of Oxidative Stress and Antioxidant Markers

The collected wound tissues were processed to evaluate the markers associated with oxidative stress and endogenous antioxidant as per the procedure described earlier[7]. Briefly, tissue homogenate (10 %, w/v) of collected skin sample was prepared in 0.15 M KCL-5 mM EDTA buffer using a tissue

homogeniser (Polytron, PT-3100, Switzerland). Homogenised samples were subjected to sonication (10 pulses of 5 second) using Ultra-Sonicator (VC-505, Sonics Vibra Cell, USA) followed by centrifugation at 9000 x g for 20 min. The collected supernatant was used for the analyses of biochemical markers.

2.7.1 Reactive Oxygen Species Estimation

The estimation of Reactive Oxygen Species (ROS) levels were determined using dichlorofluorescein diacetate (DCFH-DA), as described earlier[9]. Briefly, skin tissue homogenate (150 µl) was allowed to incubate with 100 µM DCFH-DA (10 µl) in dark condition for 30 min. followed by the addition of PBS to make the volume up to 3.0 ml. The fluorescence was measured using a fluorimeter (Perking Elmer, UK) with excitation and emission wavelength at 485 nm and 530 nm, respectively. The results were expressed as fold-change in the generation of ROS.

2.7.2 Nitric Oxide Estimation

The concentration of Nitric Oxide (NO) was measured in skin tissue homogenate using Griess reagent as described earlier[10]. The biological metabolites of NO are nitrite levels. Briefly, 100 µl of the sample was mixed with same amount of Griess reagent and allowed to incubate for 30 min. in dark. The color intensity of chromophore azo-derivative formed upon incubation was measured at 540 nm. Different dilutions of sodium nitrite was taken to prepare the standard curve. The results were represented as µM/ml sodium nitrite.

2.7.3 Superoxide Dismutase Activity Estimation

Estimation of Superoxide Dismutase (SOD) (E.C.1.15.1.1) enzyme activity in the skin tissue homogenate was performed as per the method described earlier[11]. Briefly, 0.1 ml sample was added in 3.0 ml SOD assay buffer (50 mM Tris-cacodylate buffer pH 8.2, 1 mM nitro-blue-tetrazolium salt, 0.01 per cent Triton-X-100) followed by the addition of 0.01 ml substrate (60 mM pyrogallol) and enzyme kinetic activity was monitored (OD per min.) at 540 nm for 3 min. The results of SOD activity have been expressed as U/ mg protein.

2.8 Statistical Analysis

Values are represented as mean ± standard error (SE). The statistical analysis was performed by utilizing GraphPad Prism software (version 6.0). Statistical comparative analysis was performed using the student's *t*-test and $p < 0.05$ was considered significant.

3. RESULTS

Initially, the skin penetration profile of 810 nm laser irradiation was performed to analyze the penetration ability to the depth of sub-dermal region of rat skin flaps either with CW or PW (10 Hz)-mode. The results revealed that skin penetration abilities were significantly ($p < 0.05$) different between the CW and PW (10 Hz)-mode of 810 nm laser. The penetration ability of 810 nm laser at PW (10 Hz) was better and easier than CW-mode and delivered sufficient light energy to influence the tissue repair process. However, both PW and CW delivered stable average power, when there were no obstacles between the laser source and optical power meter (direct, through the air) (Table 2).

Table 2. Skin penetrations profile of 810 nm diode laser for continuous wave (CW) and pulsed (10 Hz) wave (PW)-mode in rat.

Diode Laser	Penetrating Laser Power (mW)	
	Direct (through air)	Through skin flap
810 nm (CW)	70.35 ± 3.88	4.29 ± 0.186
810 nm PW (10 Hz)	69.86 ± 4.21	4.99 ± 0.018*

Data are mean ± SE; N = 6; *$p < 0.05$ compared with CW-mode.

3.1 Effect of PBM with PW 10 Hz 810 nm Laser on Wound Area Contraction

PBM with 810 nm laser with pulsed-mode at 10 Hz frequency showed augmented wound healing as evidenced by the significant ($p < 0.05$) increased wound area contraction in LI group as compared to non-irradiated ISC group (Table 3). Clinical observations did not show any signs of inflammation, pus formation, bleeding, or any incident in wounds.

Table 3. Wound area (mm^2) of non-irradiated immunosuppressed control (ISC) and pulsed laser (810 nm, PW 10 Hz) irradiated (LI) groups after seven days post-wounding in rats.

Group	Average Wound Area (mm^2)	
	0 day	8^{th} day
ISC	180 ± 4.5	135 ± 2.5
LI	182 ± 3.2	85 ± 2.9*

Values are mean ± SE; N = 6; *$p < 0.05$ compared to non-irradiated immunosuppressed control (ISC).

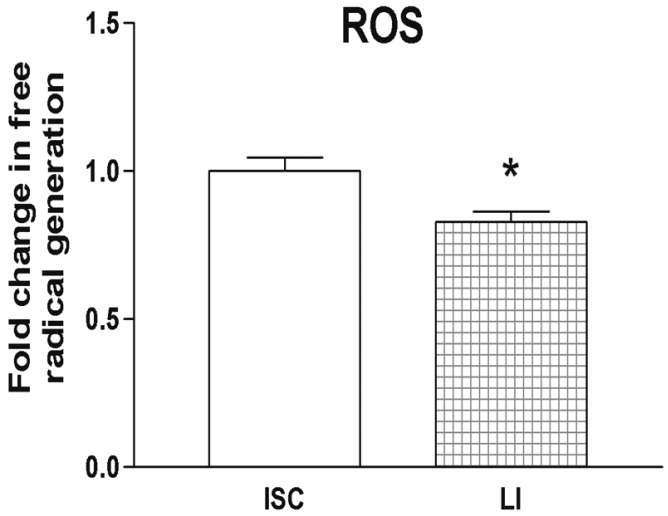

Figure 1. Effect of pulsed (10 Hz) 810 nm laser irradiation (LI) on reactive oxygen species (ROS). Values are mean ± SE. *$p < 0.05$ compared to non-irradiated immunosuppressed control (ISC).

3.2 Effect of PBM with PW 10 Hz 810 nm Laser on ROS Level

Free radicals target the cell membrane and other organelles, which subsequently cause oxidant-induced cellular

damage. The results of this study exhibited that PBM with pulsed 810 nm laser significantly ($p < 0.05$) decreased the ROS levels in LI group as compared to the non-irradiated ISC group (Fig. 1).

3.3 Effect of PBM with PW 10 Hz 810 nm Laser on Nitric Oxide (NO) Level

Nitric Oxide (NO) is very short-lived and their excessive production leads to cellular and molecular alterations. The level of NO was significantly ($p < 0.05$) decreased in LI group when compared to the non-irradiated ISC group (Fig. 2).

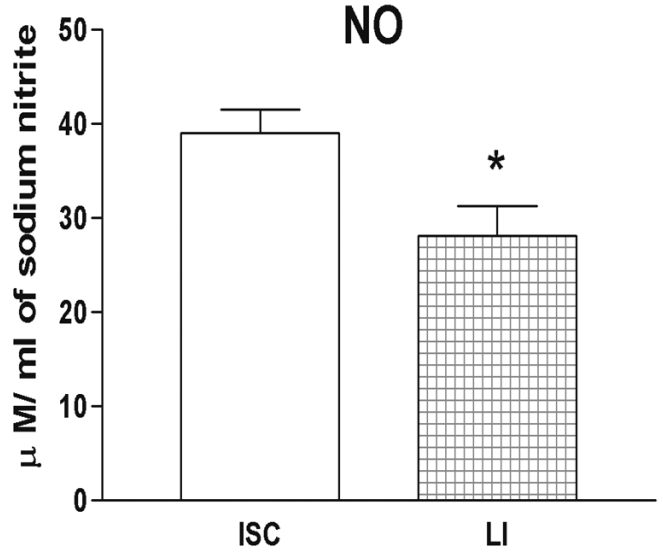

Figure 2. Effect of pulsed (10 Hz) 810 nm laser irradiation (LI) on nitric oxide (NO). Values are mean ± SE. *$p < 0.05$ compared to non-irradiated immunosuppressed control (ISC).

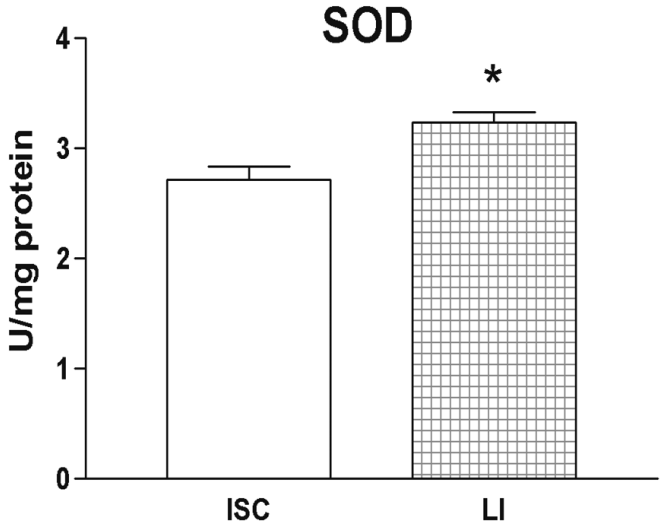

Figure 3. Effect of pulsed (10 Hz) 810 nm laser irradiation (LI) on superoxide dismutase (SOD). Values are mean ± SEM. *$p < 0.05$ compared to non-irradiated immunosuppressed control (ISC).

3.4 Effect of PBM with PW 10 Hz 810 nm Laser on SOD Activity

Cellular oxidative stress created excessive free radicals at the wound site, which can be quenched by endogenous antioxidants; hence inhibit the deleterious effects of oxidant. The present study observed that SOD activities significantly ($p < 0.05$) increased in pulsed 810 nm laser PBM treated group as compared to the non-irradiated control group (Fig. 3).

4. DISCUSSION

Chronic wounds and their treatment lead to physical, mental and social burdens to the affected individuals. Glucocorticoids have been extensively used for immunosuppressed therapy for a prolonged duration, which exerts a deleterious effect on the wound healing process. It impairs the wound healing process by regulating the expression of various cytokines gene at the wound site and thereby prevents the early inflammatory phase, which appears to be essential in wound repair process[6].

PBM has been using for various indications including pain management, inflammation reduction, traumatic brain injury, muscle injury, wound repair and regeneration[12,13]. PBMT is known to be a light-based healing modality, hence its potential benefits substantially depend on various irradiant parameters like wavelength (λ), irradiance (mW/cm^2), fluence (J/cm^2), frequency-mode, treatment regimen and exposure time etc. It has been shown that PBM exhibits biphasic dose-response activity, where low power light-intensity exhibits photobiostimulatory effects, conversely high power light-intensity exhibits inhibitory effects[3]. Red and NIR light has less scattering effect, minimal absorption by non-specific tissue chromophores with deep penetration ability, which makes this spectral region favourable for PBMT for augmented healing processes[3,5]. PBMT typically addresses light-tissue interaction in a wavelength range of red and NIR light (600-1100 nm) using coherent light (laser) and non-coherent (light-emitting diode, LED) sources, which are absorbed by cellular photoreceptive molecules. The photostimulated receptor molecules initiate photochemical and phototransduction process at various biological scales via cellular signaling pathways, increasing DNA, RNA, and protein biosynthesis, which, in turn, constitute the basis of the photo-induced stimulation effect on tissues[13]. PBM ameliorates oxidative stress and regulates redox homeostasis by attenuating the cellular markers pertaining to oxidative stress. Further, it has been shown that PBM triggers tissue repair signaling events inside the cells leading to activation of redox-sensitive enzymes, transcription factors and cell cycle progression[7]. In the present study, we have evaluated the effects of the 810 nm laser with PW 10 Hz on dermal penetration, wound healing and its impact on oxidative stress in hydrocortisone-induced immunosuppressed rats.

The PW-mode of light has been demonstrated to be more effective than CW, in the context of healing of different kinds of injury including deep tissue injuries and stroke management[5]. Our results revealed that the skin penetration profile of CW and PW (10 Hz)-mode of 810 nm laser has a significant difference, where the amount of energy penetrating skin was found to be more in PW-mode than CW. The better penetration ability of PW laser along with quenching period (pulse-on/ off) greatly minimised the thermal effect in the irradiated area as compared to CW. This difference in thermal effect from these two modes of lasers may be due to its variation in skin penetration profile.

The result of present study is convincing with the previous finding, in which 810 nm laser irradiation with PW-mode (10 Hz) was more effective than CW for improving neurological outcomes in traumatic brain injury[14]. Similarly, in another study Ilic et al, reported that CW-mode of light caused a marked neurological deficit on intact rat brain, however, similar power density delivered by PW light produced no neurological or tissue damage[15].

The healing of dermal wounds greatly depends on the rate of wound contraction, which is directly governed by specialised smooth muscle cells, called myofibroblasts[16]. Our current study showed that PBM with PW 810 laser enhanced wound healing by enhancing wound contraction and re-epithelialisation. PBM with NIR 810 nm at PW-10 Hz increased the healing rate as compared to the non-irradiated rats. The finding of the present study is in line with the previous study, where PBM with 810 nm laser irradiation causes faster wound contraction and repair as compared to other wavelengths of the light in the red and NIR region[17]. Dermal wound bed mimics as a hypoxic condition for injured cells and triggers the excessive generation of reactive species. ROS has a role in cellular signaling and regulation of various transcription factors, however, excessive levels of ROS react with different cellular components and exhibited detrimental effects. Similarly, a certain level of NO has a beneficial role in the repair process, such as enhancing angiogenesis and proliferation; however, excessive levels of NO reacts with O2- and produce more oxidant like RNS and create a downstream damaging cascade[18]. In the present study, PBM with PW 810 nm laser markedly decreased both ROS and NO levels in the dermal wound milieu. The earlier findings also corroborate our results and suggest that NIR (904 nm) laser irradiation enhanced the repair of diabetic wound by ameliorating oxidative stress[19]. In another study, 904 nm superpulsed laser PBM significantly decreased the oxidative stress by preventing the excessive generation of free radicals and enhance the activity of endogenous antioxidants to maintain redox homeostasis, which leads to accelerated healing processes in burn wound[7].

The antioxidant defense system plays a vital role in eliminating free radical species (ROS and RNS). The enzymatic antioxidant such as SOD is front line free radicals curbing agent, as it converts superoxide into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). H_2O_2 further breaks into H_2O and O_2 by catalase, which averts its deleterious effect on numerous cellular components[20]. The findings of the present study indicate that PBM with PW 810 nm laser enhanced the SOD activity, which reduced the free-radical induced oxidative damage during dermal wound healing. Our finding made synchronisation with the previous study, which declared increased SOD activity after NIR 904 nm superpulsed laser irradiation on burn wound and led to enhance tissue repair[7].

5. CONCLUSION

Taken together, this study indicates that the penetration ability of 810 nm laser at pulsed-mode 10 Hz was better and easier than continuous-mode and delivered adequate light energy to influence the tissue repair process. Further, the results signify that PBM with 810 nm laser accelerates wound healing by enhancing wound area contraction, reducing nitroxidative stress by decreasing free radical generation, nitric oxide while increasing endogenous antioxidant level. The present findings showed that pulsed (10 Hz) 810 nm PBM led to reduce oxidative stress, enhance antioxidant and maintain redox homeostasis, which accelerates dermal wound healing in immunosuppressed rats.

Conflict of Interest: None

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In the current study, he has conceived the idea, designed the study, analysed and interpreted the results along with preparation of the current manuscript.