Wiley Online Library
Early View publication on www.wileyonlinelibrary.com
(issue and page numbers not yet assigned;
citable using Digital Object Identifier – DOI)

J. Biophotonics 1–13 (2014) / **DOI** 10.1002/jbio.201400058

Journal of BIOPHOTONICS

FULL ARTICLE

Superpulsed (Ga-As, 904 nm) low-level laser therapy (LLLT) attenuates inflammatory response and enhances healing of burn wounds

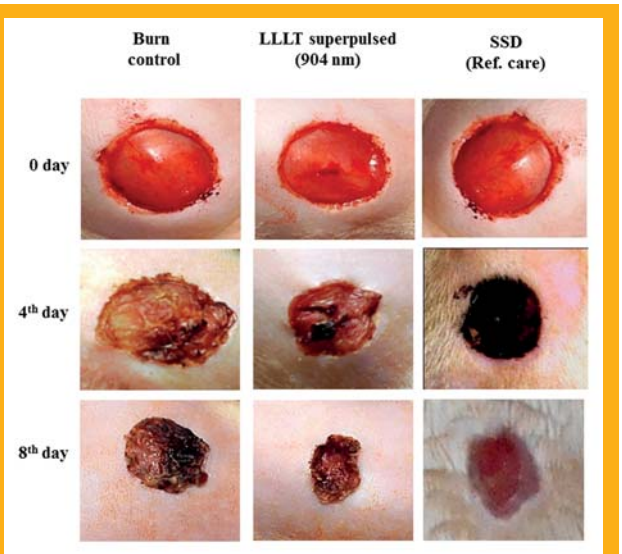
Asheesh Gupta, Gaurav K. Keshri, Anju Yadav, Shefali Gola, Satish Chauhan, Ashok K. Salhan, and Shashi Bala Singh*

Defence Institute of Physiology and Allied Sciences, DRDO, Lucknow Road, Timarpur, Delhi-110 054, India

Received 20 May 2014, revised 11 July 2014, accepted 27 July 2014
Published online 11 September 2014

Key words: Burns, hypoxia inducible factor-1α, nuclear factor-kB, low-level laser (light) therapy (LLLT), photobiomodulation, superpulsed laser (904 nm)

Low-level laser therapy (LLLT) using superpulsed near-infrared light can penetrate deeper in the injured tissue and could allow non-pharmacological treatment for chronic wound healing. This study investigated the effects of superpulsed laser (Ga-As 904 nm, 200 ns pulse width; 100 Hz; 0.7 mW mean output power; 0.4 mW/cm^2 average irradiance; 0.2 J/cm^2 total fluence) on the healing of burn wounds in rats, and further explored the probable associated mechanisms of action. Irradiated group exhibited enhanced DNA, total protein, hydroxyproline and hexosamine contents compared to the control and silver sulfadiazine (reference care) treated groups. LLLT exhibited decreased TNF-α level and NF-kB, and up-regulated protein levels of VEGF, FGFR-1, HSP-60, HSP-90, HIF-1α and matrix metalloproteinases-2 and 9 compared to the controls. In conclusion, LLLT using superpulsed 904 nm laser reduced the inflammatory response and was able to enhance cellular proliferation, collagen deposition and wound contraction in the repair process of burn wounds.



Photomicrographs showing no, absence inflammation and faster wound contraction in LLLT superpulsed (904 nm) laser treated burn wounds as compared to the non-irradiated control and silver sulfadiazine (SSD) ointment (reference care) treated wounds

1. Introduction

Wound repair is a complex, dynamic and multifaceted biological process with the aim of restoration of injured tissue. It proceeds in three overlapping phases viz. inflammation, proliferation and tissue remodeling [1]. Burns are among the most common and devastating forms of trauma, as it causes ruth-

* Corresponding author: e-mail: asheeshgupta2001@gmail.com, Phone: 91-11-23883314, Fax: 91-11-23914790

less pain along with physical impairments and psychological burden to the patients. It may lead to complications such as long-term disability, prolonged hospitalization, loss of body extremities and even death. Currently, research on different pharmacological and non-pharmacological modalities to reduce morbidity and mortality rates following severe burn injury is a developing area in modern biomedical sciences. The therapeutic advantages of low-level laser (light) therapy (LLLT) on tissue repair and regeneration have been the focus of recent studies [2, 3]. LLLT or photobiomodulation refers to the use of photons at a non-thermal irradiance to alter biological activity. LLLT has evolved as a rapidly growing, non-invasive alternative physical approach to many medical conditions, including stimulation of wound healing, prevention of tissue damage, reduction of edema, relief from pain and inflammation [4, 5].

Although the evidences of the beneficial effects of LLLT are increasing, the molecular mechanisms of biomodulation by the irradiation still remain poorly understood. The photobiostimulatory effect of LLLT is thought to be through absorption of red and near-infrared (NIR) light (at wavelengths that can penetrate tissue) by photoacceptors, in particular cytochrome c oxidase (unit IV of the mitochondrial respiratory chain), and perhaps also chromophores in the plasma membrane of cells, thereby increasing mitochondrial respiration, ATP production, additional transport of Ca^{2+} in the cytoplasm and initiating signaling pathways mediated by reactive oxygen species (ROS), nitric oxide, and cyclic AMP, ultimately leading to activation of several transcription factors concerned with cell migration, proliferation, survival, tissue repair, and regeneration [2, 6, 7].

The achievement of clinically desired outcomes with LLLT depends on selection of optimal optical parameters. LLLT uses light typically of narrow spectral width in the red or NIR spectrum (630–1000 nm) and can be in the mode of continuous or pulsed-wave. The photostimulatory effects of LLLT depend upon many parameters, such as the wavelength, pulse frequency, peak power, pulse width/rest period, irradiance, time, energy, energy density etc. [8]. Many studies have reported the positive healing effects of LLLT under continuous-wave (CW) mode [9, 10], however, there is a limited attention for exploring the pulsing effects in LLLT on tissue repair, although some recent studies did find evidence for the enhanced benefits of pulsed laser over CW laser [11, 12]. Superpulsed lasers emit relatively strong but extremely short light pulses sequentially. Along with shorter treatment time, superpulsed laser allows for deeper penetration without the unwelcome effects of CW (thermal damage). One of the exclusive features of superpulsed is that each pulse emits energy for a fixed duration which may be in the order of billionth of a second (10^{-9} s). Hence, the required energy for the purpose of healing can be delivered to the tissue by increasing and decreasing treatment time along with frequency modulation.

The uses of NIR wavelength 904 nm gallium-arsenide (Ga–As) laser have increased recently, owing to greater penetration power in comparison to other types of laser, and therefore used as healing modality for dense connective tissue (musculoskeletal) injuries [13, 14]. In spite of positive effects, the action of superpulsed laser on impaired wound tissues is still not fully understood, and in general it is difficult to establish optimum optical dosimetry parameters to be used in the healing therapies. This study investigated the healing efficacy of LLLT using superpulsed 904 nm laser on experimental full-thickness burn wounds in rats, followed by biophysical, biochemical and molecular analyses and histopathological observations and further explored its possible associated mechanism of action.

2. Materials and methods

2.1 Experimental animals

The animal experiments were performed in accordance with the regulations specified by the Institute's Animal Ethical Committee and conform to the national guidelines on the care and use of laboratory animals, India. Male adult Sprague-Dawley rats (180 ± 20 g; animal colony of DIPAS, Delhi) were used in this study. The animals were maintained under controlled environment at the Institute's animal house at 25 ± 1 °C and 12 h: 12 h light: dark cycle. The animals were housed one per cage with access to food and water *ad libitum.*

2.2 Burn model

The animals were anesthetized by an intraperitoneal (i.p.) injection of a ketamine-xylazine cocktail (90 mg/kg ketamine and 10 mg/kg xylazine) before burn induction and during follow-up treatment. The dorsal surface of the rat was shaved using electric fur clipper, and the underlying skin cleaned with 70% ethanol. To create a full-thickness thermal burn injury, an aluminum metal rod (1.5 cm diameter) was heated to 85 °C and applied to the shaved skin for 20 s as previously described [15]. The temperature of the metal rod was monitored with a fabricated digital computerized multimeter. Hot rod was resting on its own weight of 30 g. No additional pressure was applied on the hand leaded metal rod. Sin-

gle burn wound was created on dorsal part of each rat. After 24 h, dead tissues were excised using sterile surgical blade and scissors. The wound was left uncovered during the whole period of experiment.

2.3 Experimental design

Initially, a study was performed to evaluate the healing efficacy of superpulsed 904 nm laser by selecting a fixed frequency i.e. 100 Hz. and different exposure time i.e. 3, 10 and 15 min. LLLT was applied daily for 7 consecutive post-wounding days. The laser dosimetry study showed that superpulsed 904 nm laser was most effective at a time duration of 10 min only, and other laser parameters were optimized like irradiance 0.4 mW/cm^2 and fluence delivered 0.2 J/cm^2 to illuminate the dorsal surface of the rat with full-thickness burn (spot size, 1.77 cm^2). Further detailed biochemical, cellular and molecular studies were carried out using these optimized superpulsed laser radiant exposure parameters only.

The animals were randomly divided into six experimental groups (six animals in each group) (Table 1). The rats in group 1 and 4 were left untreated and considered as non-irradiated control burn. The rats in group 2 and 5 were treated with a NIR 904 nm superpulsed laser. The rats in group 3 and 6 were treated with topical application of silver sulfadiazine (SSD) cream USP 1.0%, w/w (Ranbaxy laboratories Ltd., India) and considered as reference care. Non-illuminated control and SSD treated rats were kept anesthetized for the same time period as the laser illuminated rats. Half of the animals were sacrificed after 4 days and the other half were sacrificed after 7 days post-wounding.

Table 1 Distribution of rats in experimental group and study period.

Group	Treatment	Study period (days post-wounding)
Group 01	Burn control (non-irradiated)	4
Group 02	LLLT superpulsed 904 nm laser treated	4
Group 03	Silver sulfadiazine ointment (Ref. care)	4
Group 04	Burn control (non-irradiated)	7
Group 05	LLLT superpulsed 904 nm laser treated	7
Group 06	Silver sulfadiazine ointment (Ref. care)	7

Table 2 List of laser parameters.

Laser Parameter	Value
Wavelength	904 nm
Mode	Superpulsed laser
Spot size	Diameter = 1.5 cm, area = 1.77 cm^2
Average irradiance	0.4 mW/cm^2
Total fluence	0.2 J/cm^2
Average power	0.7 mW
Total energy	0.4 J
Illumination time	10 minutes
Pulse frequency	100 Hz
Duty cycle	0.002%
Pulse duration	200 ns
Peak power	35 W
Peak irradiance	19.8 W/cm^2

2.4 Laser irradiation

LLLT was performed using a Ga–As diode superpulsed laser of 904 nm wavelength (NIR) with a frequency of 100 Hz. and 90 W peak power (Physiolaser Olympic Basic, RJ Laser, Germany) to deliver a light spot centered on the dorsal surface of the rat with full-thickness burn. The irradiance was measured using a 3A-ROHS with Nova II laser power/energy meter (Ophir Optronics Solutions Ltd., Israel). The distal tip of the laser probe was set for laser irradiation onto the burn wound at a power density of 0.4 mW/ cm^2. The duration of laser irradiation was 10 min, and the total fluence delivered was 0.2 J/cm^2. The complete laser parameters are given in Table 2. LLLT was applied daily at the same time for 4 or 7 consecutive post-wounding days on the burn wounds, with the first application 1 h after creation of wound.

2.5 Pro-healing parameters

2.5.1 Wound contraction and biochemical markers

The wound size was monitored in digital photographs following photo-recording. The first photo taken on the day of injury (day 0) and subsequent photos were captured on the fourth and eighth day post-wounding. A ruler (in millimeter) was placed next to each wound, and the scale of the ruler image was set before calculating changes in wound areas in each photograph. Fiji (Image J) software (NIH, Bethesda, MD) was used to assess changes in wound area/initial wound area over time. Values were expressed in square millimeters [10]. The granulation tissue excised on eighth day post-wounding was used

to analyze the pro-healing biochemical parameters viz. DNA, total protein, hydroxyproline and hexosamine contents [16–19].

2.5.2 TNF-α level analysis

Wound granulation tissues harvested after 4 and 7 days post-wounding were homogenized in a radioimmune precipitation assay (RIPA) buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 µL/mL protease inhibitor cocktail and 10 mg/mL PMSF (Sigma-Aldrich) in PBS. Wound homogenate was analyzed using ELISA kit per manufacturer's instructions (Invitrogen Corporation, Camarillo, CA) to determine TNF-α protein content. Briefly, 100 µL of properly diluted samples or standard of recombinant rat TNF-α were added to the 96-well plate pre-coated with anti-TNF-α and left for 2 h at room temperature (RT). After washing, 100 µL of biotin conjugated antibody was added and left for 1 h at RT. After washing the plate, 100 µL of streptavidin-peroxidase (HRP) was added and left for 30 min at RT, followed by further washing. The reaction was revealed by addition of 100 µL of stabilized chromogen followed by incubation for 30 min at RT and then reaction stopped by addition of stop solution. Readings were obtained in an ELISA micro plate reader (VERSA Max, Molecular Devices, California, USA) at wavelength of 450 nm. The sample concentrations were calculated from standard curve prepared from recombinant TNF-α. The limit of detection was 11.7–750 pg/mL for TNF-α.

2.5.3 Histopathology

The regenerated skin tissues harvested on fifth and eighth day post-wounding were preserved in 10% buffered formalin. From paraffin-embedded specimens, 4 µm thickness sections were stained with hematoxylin and eosin and observed for the histopathological changes under a light microscope, followed by photomicrography.

2.5.4 Gelatin zymography

Matrix metalloproteinase's (MMPs) expression was studied in the wound granulation tissues by gelatin zymography assay [15]. Briefly, samples were homogenized with Tris-buffer (saline 0.9%, Tris 0.05 M, Triton X-100-0.25%, $CaCl_2$-0.02 M) and centrifuged for 30 min at 4226 × g at 4 °C, and supernatants were collected. Tissue extract (50 µg) was subjected to 10% SDS–PAGE containing 0.1% SDS and 1 g/L gelatin under non-reducing conditions without prior boiling. After electrophoresis, gel was washed in 2.5% Triton X-100 for 30 min to remove SDS and allow protein to renature, and subsequently immersed in activity buffer (50 mM Tris/HCl, pH 8.0, 5 mM $CaCl_2$, 0.2 M NaCl, 0.02% NaN_3) for 21–24 h at 37 °C. The gel was then stained with 2.5% coomassie brilliant blue-R in methanol, acetic acid, and water (4 : 1 : 5) followed by destaining with methanol, acetic acid, and water (4 : 1 : 5). Enzymatic activities were detected as clear bands of gelatin lysis against blue background.

2.5.5 Western immunoblot analysis

Frozen wound tissue was homogenized with a Polytron homogenizer (PT 3100, Kinematica AG, Littau-Lucerne, Switzerland) with 4 strokes of 15 seconds each at interval of 15 sec in ice-cold buffer (A) containing 0.5 M sucrose, 10 mM HEPES, 10 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 10% Nonidet P-40, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, MO). Homogenates were kept on ice for 15 min and then centrifuged for 10 min at 4000 × g at 4 °C. The supernatant with cytosolic fraction was collected, stored (–80 °C), and the pellet was re-suspended in cold buffer (B) (50 mM HEPES, 50 mM KCl, 0.1 mM NaCl, 0.1 mM EDTA, 1.0 mM DTT, 0.1 mM PMSF, 20% glycerol) for the nuclear fraction. It was incubated for 30 min on ice followed by centrifugation for 45 min at 14000 × g at 4 °C. The supernatant containing the nuclear fraction was aliquoted and stored at –80 °C for further analysis. Total protein concentrations in the cytosolic and nuclear extracts were determined according to Lowry et al. [17]; bovine serum albumin served as the standard.

Total protein (50 µg) samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) through 10% gels and then electrophoretically transferred to a nitrocellulose membrane (Protran, Whatman, Germany). The membranes were blocked overnight at 4 °C in tris buffered saline with Tween-20 (TBST) buffer (0.01 M Tris HCl, Ph 7.5, 0.15 M NaCl and 0.05% Tween-20) containing 5% skimmed milk protein. It was followed by primary and secondary antibody incubation at a dilution of 1 : 1000 for 2.5 h and 1 : 10000 for 2 h respectively at RT. The following primary antibodies were used: FGFR-1, fibronectin, HSP-90, HIF-1α, β-actin (#F5421, F3648, H1775, H6536, A1978; Sigma-Aldrich, St. Louis, MO), NF-*k*B (#51-3500, Invitrogen, CA), VEGF, HSP-60 (#SC-152, SC13115; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary anti-mouse/

rabbit-IgG antibodies conjugated to horseradish peroxidase (#A9044, A0545) were obtained from Sigma-Aldrich, St. Louis, MO. The membranes were washed with TBST and immunoreactive bands were developed by enhanced chemiluminescent substrate (Sigma-Aldrich) and exposed to X-ray films (Kodak, Rochester, NY). The band densities were quantified using a scanning densitometric analysis and Fiji (Image J) software program (NIH, Bethesda, MD).

2.6 Statistical analysis

Values are reported as mean ± SE, and statistical significance between experimental and control values was analyzed by one way ANOVA followed by Dunnett's post-hoc test using Graph Pad Prism 2.01 (Graph Pad Software Inc., La Jolla, CA, USA). A *P*-value <0.05 was considered statistically significant. Densitometric data for Western immunoblots are expressed as a percentage of the control mean density after normalization to loading controls.

3. Results

3.1 Visual observations and wound contraction

LLLT with superpulsed 904 nm laser treatment (fluence 0.2 J/cm^2, 100 Hz and fluence rate 0.4 mW/cm^2)

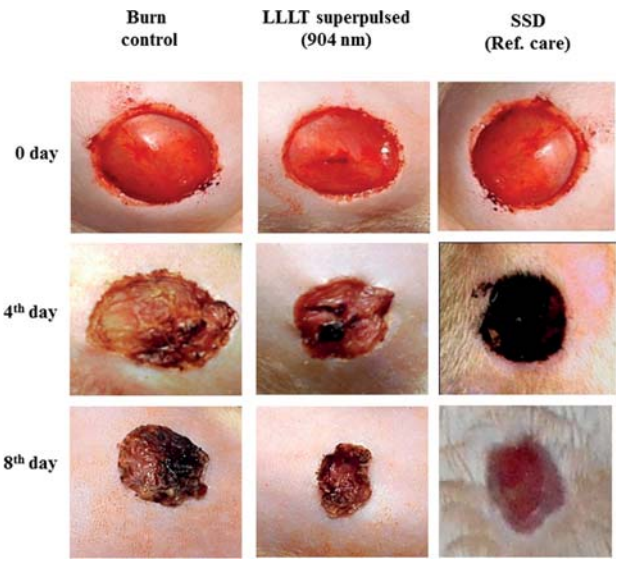


Figure 1 Photomicrographs showing no, absence inflammation and faster wound contraction in LLLT superpulsed (904 nm) laser treated burn wounds as compared to the non-irradiated control and silver sulfadiazine (SSD) ointment (reference care) treated wounds.

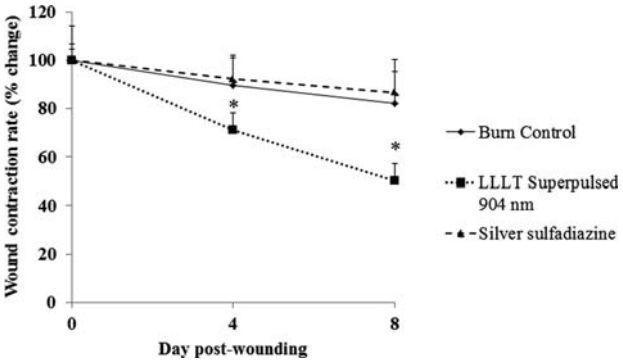


Figure 2 Effect of superpulsed 904 nm laser on wound contraction rate (% change) of burn wound in rats. Wound received no illumination (control), LLLT with superpulsed 904 nm laser (100 Hz, 0.7 mW, 0.4 J) and topical treatment with silver sulfadiazine (SSD) ointment (reference care). Values are mean ± SEM, $n = 6$ animals per group. *$p < 0.05$ compared to the non-irradiated burn controls.

demonstrated augmented healing in burn wounds. It was found that laser treated wounds underwent much faster contraction than the control (non-irradiated) wounds (Figure 1). The irradiated animals showed a significant reduction in wound area (50%, $p < 0.05$) on eighth day post-wounding compared with the non-irradiated control animals (18%, $p < 0.05$) (Figure 2). Reference drug SSD treated wound did not show any significant change in wound contraction. This substantiates the healing efficacy of superpulsed 904 nm LLLT on burn wounds.

3.2 Augmented pro-healing biochemical parameters

Table 3 depicts the effect of superpulsed 904 nm laser treatment for 7 days post-wounding on pro-healing biochemical parameters in burn wound tissue. The laser treatment significantly ($p < 0.05$) increased the cellular proliferation as evidenced by enhanced DNA (28%) and total protein (37%) contents compared with the control animals. Further, laser irradiated wounds also exhibited significant ($p < 0.05$) increased collagen accumulation and stabilization as evidenced by enhanced hydroxyproline (35%) and hexosamine (25%) contents. However, treatment with the SSD did not show any significant changes in pro-healing parameters as compared with the control animals (non-irradiated).

3.3 TNF-α level

ELISA was used to detect the level of pro-inflammatory cytokine TNF-α in response to superpulsed

Table 3 Effect of superpulsed (904 nm) LLLT for 7 days on various biochemical parameters on burn wound healing in rats.

Parameters	Burn control (non-irradiated)	Superpulsed (904 nm) laser treated	SSD (Ref. care)
DNA (mg/g tissue wt.)	3.6 ± 0.2	4.6 ± 0.1*	3.3 ± 0.1
Protein (mg/g tissue wt.)	123.1 ± 11.1	168.9 ± 12.5*	130.9 ± 12.5
Hydroxyproline (mg/g tissue wt.)	42.4 ± 1.8	57.3 ± 2.9*	45.3 ± 2.9
Hexosamine (mg/g tissue wt.)	0.48 ± 0.01	0.60 ± 0.02*	0.51 ± 0.02

Values are mean ± SEM; $n = 6$; *$p < 0.05$ compared with non-irradiated burn control. SSD; silver sulfadiazine.

904 nm laser treated and non-irradiated control burn wounds. A significant reduction in TNF-α level was observed by 19% ($p < 0.05$) after 4 day post-wounding in LLLT treated wounds compared to the non-irradiated control wounds. TNF-α level was further decreased by 40% ($p < 0.05$) after 7 day post-wounding in LLLT treated wounds compared to the control wounds (Figure 3).

3.4 Histopathological examinations

The histopathological examinations showed that tissue regeneration was much faster in superpulsed laser treated wounds than the SSD treated and non-irradiated control groups. The images examined on fifth and eighth days post-wounding demonstrated a denuded epithelium, increased infiltration of inflammatory cells viz. neutrophils, lymphocytes and lesser fibrous tissue at the wound site in the non-irradiated control tissue section. However, wounds treated with superpulsed 904 nm laser were fully covered by epithelial layer, and showed enhanced fibrogenesis, neo-vascularization and high collagen deposition. Meanwhile, the inflammatory infiltrate was less intense in the irradiated wound compared to the SSD treated and control wounds. Furthermore, non-irradiated control and standard care SSD treated wounds showed loosely packed collagen fibers with irregular pattern whereas, collagen fibers were densely packed and parallely arranged in the laser treated wounds (Figure 4A–L).

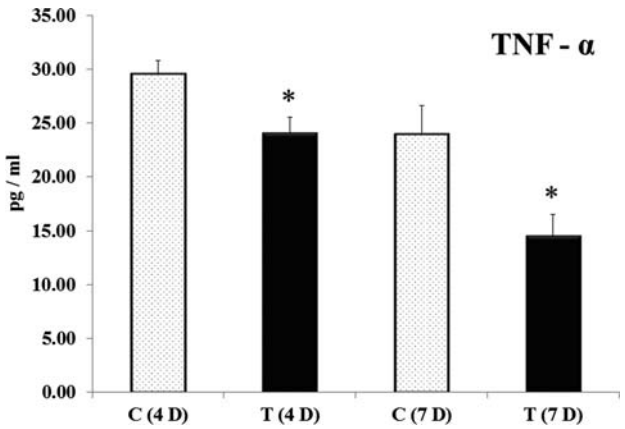


Figure 3 TNF-α levels in LLLT superpulsed 904 laser treated and non-irradiated control burn wounds after four and seven days post-wounding. Values are mean ± SEM, $n = 6$ animals per group. *$p < 0.05$ compared to the non-irradiated burn controls.

3.5 Matrix metalloproteinases (MMPs) expression

The influence of the superpulsed 904 nm laser treatment on the expression of MMPs was studied using gelatin zymography analysis. In the present study, two major pro-MMPs viz. MMP-2 (72 kDa) and MMP-9 (92 kDa) were observed in wound granulation tissues after 4 and 7 days post-wounding. An enhanced expression of MMP-2 and 9 was observed at 4 day post-wounding in superpulsed 904 nm laser treated wounds compared to the non-irradiated control burn wounds (Figure 5). No difference in MMPs expression was observed after 7 days post-wounding.

3.6 Growth factors, cellular and nuclear proteins expression

Western immunoblot analysis of burn wound tissue treated with superpulsed 904 nm laser revealed differential protein expression of growth factors, extracellular matrix (ECM), cellular and nuclear proteins as compared with the non-irradiated control wounds (Figure 6). Analyses of protein content were limited to VEGF, fibronectin, FGFR-1, HSP-60, HSP-90, HIF-1α, NF-κB and β-actin. Figure 6A showed significant ($p < 0.05$) up-regulated protein expression of angiogenesis marker (VEGF) by 75% and 29% in laser irradiated group compared with the control after 4 and 7 days post-wounding, respectively. An up-regulated expression of fibronectin and FGFR-1 was observed in laser treated wounds as compared

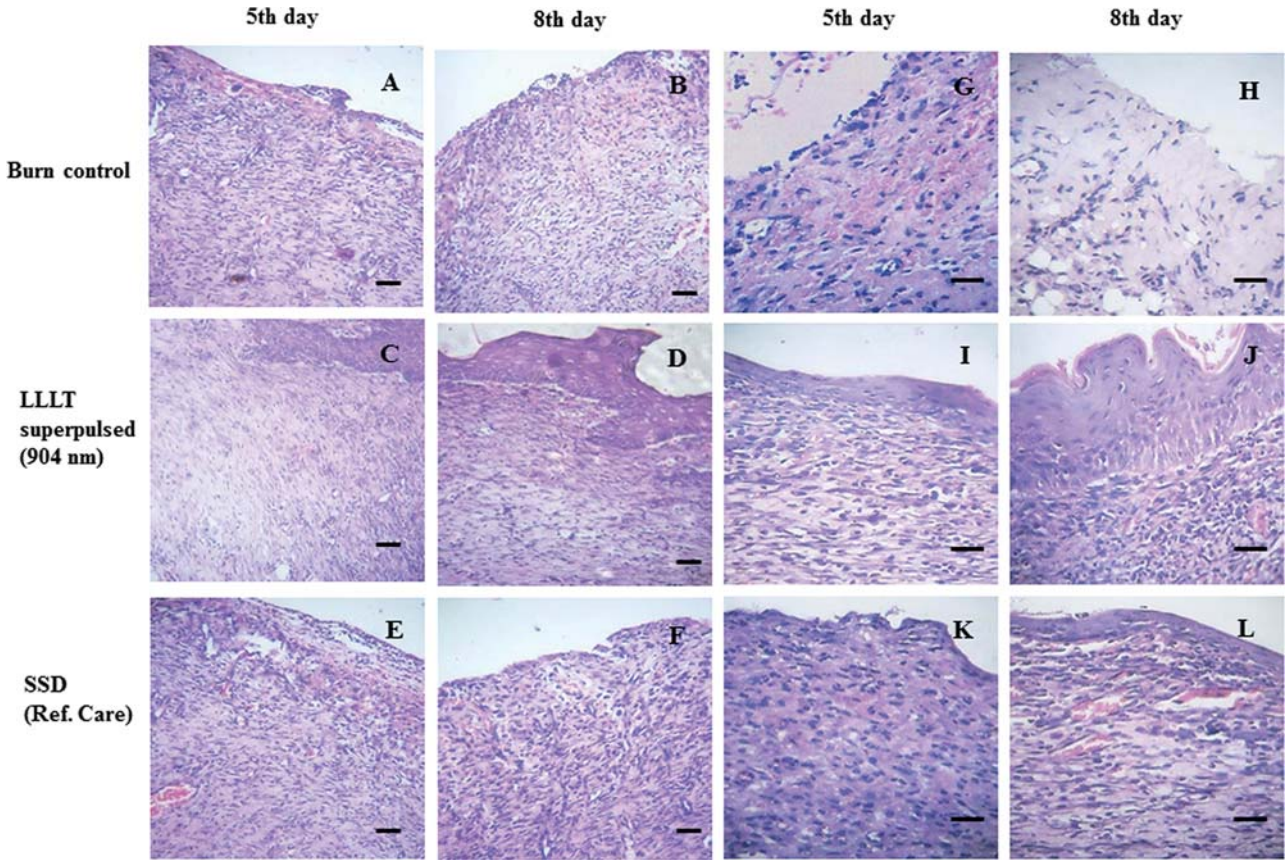


Figure 4 Photomicrographs of histopathological changes on fifth and eighth days post-wounding in non-irradiated control burn wound, LLLT superpulsed 904 nm laser and silver sulfadiazine (SSD) ointment (reference care) treated wound tissues in rats. Lower magnification (**A–F**) of dermal area of control and SSD treatment showed denuded epidermis, infiltration of inflammatory cells, loosely packed collagen and less fibroblasts. LLLT treated granulation wound tissue showing less infiltration of inflammatory cells, new blood vessel formation (angiogenesis) and high fibroblasts with regularly arranged dense collagen deposition and complete re-epithelialization. Scale bar, 100 μm. Higher magnification (**G–L**) of dermal area showing increased fibroblasts, collagen deposition, neo-vascularization and complete re-epithelialization in superpulsed 904 nm laser treated wounds whereas, control and SSD treatment showed non-epithelialized wound surface and infiltration of inflammatory cells. Scale bar, 10 μm.

to the controls (Figure 6B, C). HSP-60 and HSP-90 protein levels were augmented in the laser treated group as compared with the levels observed in the non-irradiated control wounds (Figure 6D, E). Concerning HIF-1α protein expression, as shown in Figure 6F, it was significantly ($p < 0.05$) increased after 4 and 7 post-wounding days compared to the controls. However, LLLT with superpulsed 904 nm laser exhibited significant down-regulated protein expression of NF-*k*B as compared to the controls (Figure 6G).

4. Discussion

The present study investigated the therapeutic effect of superpulsed LLLT (904 nm, 0.7 mW, 100 Hz, 0.4 J) as a non-invasive treatment of full-thickness burn wounds in experimental rats. The red and NIR portion of the optical spectrum open possible therapeutic modulations in living tissues and their effects are dependent on the physiological state of the tissue at the moment of irradiation as well as on the optical parameters of laser. It has been reported that injured or stressed cells appeared to respond better to LLLT than normal cells [20]. The use of the LLLT in burn wounds is based on its anti-inflammatory action with consequent analgesia [21, 22].

Biophysically, the ability of laser to penetrate tissue is dependent on laser's wavelength. Light with a wavelength range of 700–1000 nm is NIR and invisible, and it penetrates tissue better than light in the red wavelengths (600–700 nm). Various studies conducted to determine the healing efficacy of superpulsed 904 nm laser showed that its required effective doses are quite lower than that in its CW mode as well as from 632.8 nm and 780–860 nm lasers [23, 24]. However, a possible satisfactory understanding

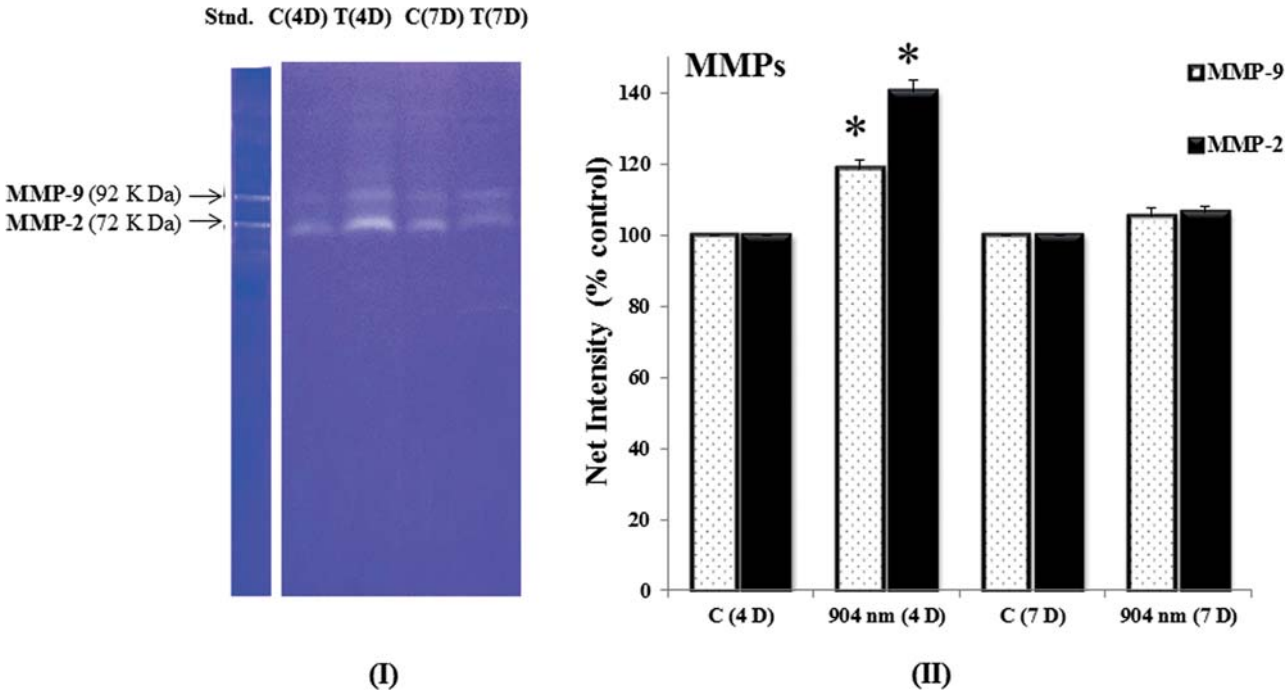


Figure 5 (**I**) Matrix metalloproteinase (MMP-2 and 9) expression by gelatin zymography in LLLT superpulsed 904 nm laser treated and non-irradiated control burn wound tissues after 4 and 7 days post-wounding. (**II**) Densitometric analysis using Fiji software. Change in expression expressed as net intensity (% control). Values are mean ± SEM, $n = 6$ animals per group. *$p < 0.05$ compared with non-irradiated control burn wounds.

of the requirement for differentiated dosing is still lacking. An important aspect other than wavelength dependency is the mode of operating the laser. In a recent systematic review, Hashmi et al. [11] compared continuous with pulsed and superpulsed laser irradiation used for the various therapeutic applications of LLLT. They reported that pulsed lasers generate less heating of superficial tissues because of quench periods (pulse off times) and hence improved the ability of light to penetrate deeper tissues potentially achieving greater treatment effects from direct stimulation of the deeper structures. Their conclusion indicated that overall pulsed-mode may be superior to CW with everything else being equal, owing to increased penetration of photons at the pulsed peak power supported by some of the previous studies [12]. The beneficial pulsing effect could be attributed to increased penetration depth and kinetics of ion channels.

Ga–As diode laser of 904 nm wavelength operates with strong, short pulses (peak power 10–100 W) in a superpulsed mode with nanosecond or picosecond pulse duration. Results from this study provide evidence for the photobiomodulatory effects of 904 nm superpulsed laser treatment on burn wound healing. The study also showed a significant decrease in TNF-α level and increased cellular proliferation and collagen biosynthesis processes after superpulsed 904 nm laser treatment. TNF-α is a pro-inflammatory cytokine derived from monocytes/macrophages and considered to be one of the most vital triggers of the acute inflammatory phase. The present findings suggest that this protocol of laser irradiation is able to down-regulate the inflammatory response and improve the repair process. Similar results previously described corroborate the anti-inflammatory effects and enhanced collagen synthesis mediated by 904 nm as shown in our results [25, 26]. Recently, effects of LLLT with superpulsed 904 nm laser (60 mW mean output power, 700 Hz., 1.67 W/cm^2) have been compared with sodium diclofenac (topical application) and cryotherapy in management of acute skeletal muscle injury. The study demonstrated that LLLT with 1 J dose significantly decreased pro-inflammatory cytokines IL-1β, IL-6 and TNF-α levels and found better effects than diclofenac or cryotherapy in acute inflammatory phase after muscle trauma [27]. Melo et al. [28] studied the effect of 904 nm lasers on sutured wound healing in rats and reported that laser irradiation is able to down-regulate the inflammatory response and favor the acceleration of biological events responsible for the healing process. Lee et al. [29] demonstrated that treatment with NIR 904 nm laser reduces swelling and promotes healing of experimentally infected open dermal wounds in rats. Further, it has been reported that LLLT with 904 nm laser relieves pain and accelerates the reso-

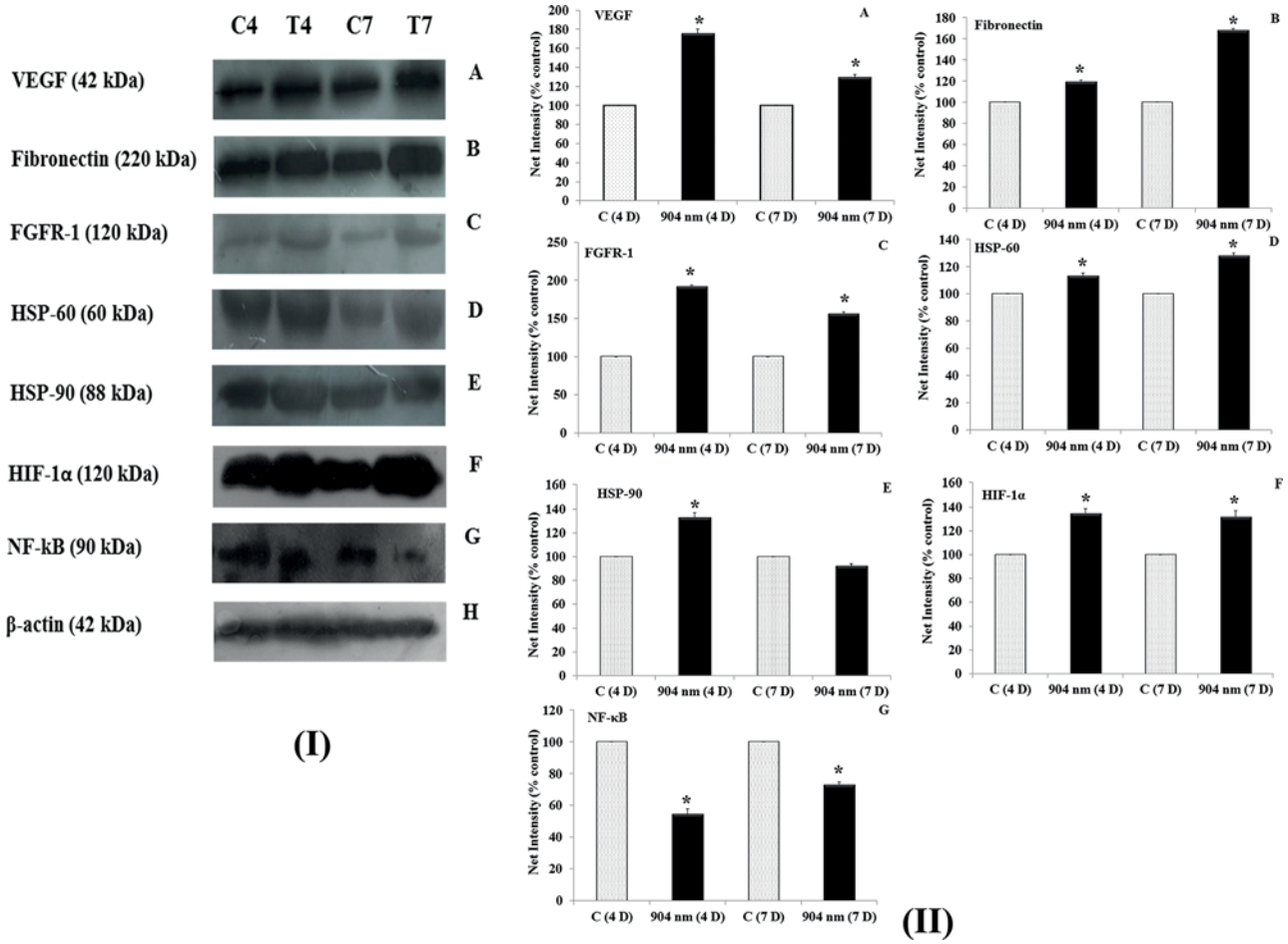


Figure 6 Growth factors, extra cellular matrix (ECM), cellular and nuclear proteins expression levels. (**I**) Immunoblotting for cytosolic proteins viz. vascular endothelial growth factor (VEGF), fibronectin, fibroblast growth factor receptor-1 (FGFR-1), heat shock protein (HSP)-60, HSP-90 and β-actin, and nuclear proteins viz. hypoxia inducible factor (HIF-1α) and nuclear factor (NF)-*k*B in LLLT superpulsed 904 nm laser treated and non-irradiated control burn wound tissues after 4 and 7 days post-wounding. (**II**) Densitometric analysis using Fiji software. Change in expression expressed as net intensity (% control). Values are mean ± SEM, $n = 6$ animals per group. *$p < 0.05$ compared with non-irradiated control burn wounds.

lution of inflammation in management of chronic myofacial pain in the neck under a double-blind and randomized-controlled trial [30]. The work done by Reddy [31] reported that the Ga–As 904 nm laser improves healing of impaired wounds in diabetic rats as measured by increase in wound tensile properties, wound collagen production and collagen maturation.

Consistent with this, in-vivo studies and human trials have shown that LLLT with 904 nm wavelength has modulatory effects on inflammatory markers (TNF-α, IL-1β, PGE2, plasminogen activator), leukocyte activity (macrophages, lymphocytes, neutrophils) and reduced inflammatory process (hemorrhage, edema, necrosis, neutrophil cell influx) [32, 33]. Enhanced activity of mitochondrial respiratory chain (complexes I, II, III, IV and succinate dehydrogenase), ATP synthesis [34] and reduction in the release of ROS and increased antioxidant levels and heat shock proteins have also been found after LLLT with 904 nm laser in traumatized muscle injury [35, 36].

The results of the present investigation demonstrated that LLLT with superpulsed 904 nm can significantly accelerate the wound closure of a full-thickness burn. A significant increase in wound contraction of laser treated burn in comparison with SSD treated and non-irradiated controls showed the effectiveness of superpulsed 904 nm in the healing of burn wounds. Our results are in agreement with the findings of M. Matic et al. [37] that pulsed LLLT significantly enhanced the wound contraction of a surgically induced cutaneous wound. A significant enhanced cellular proliferation and collagen accumulation was evident by increased DNA, total protein, hydroxyproline and hexosamine contents in super-

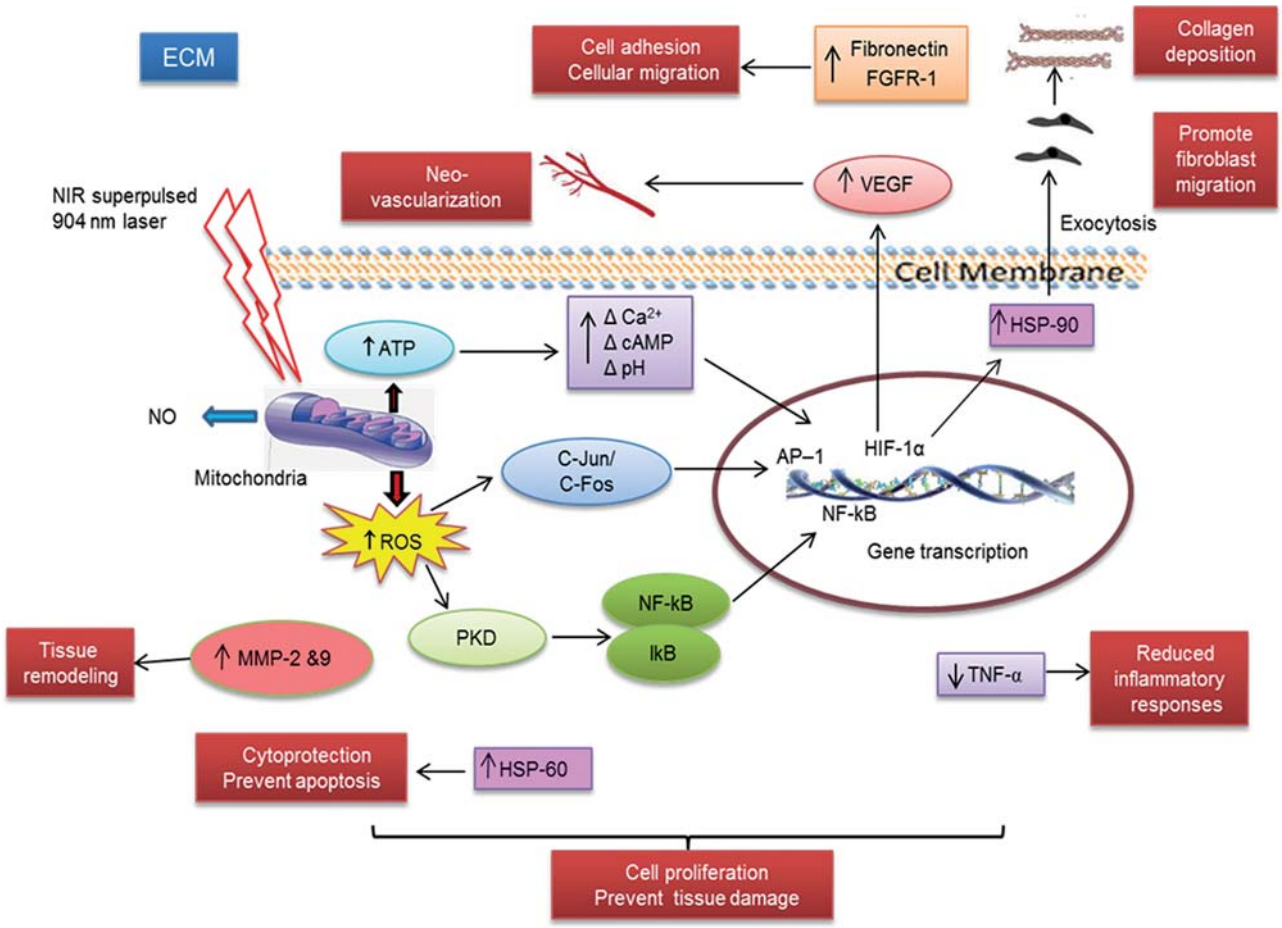


Figure 7 A possible mechanism of action of low-level laser therapy (LLLT) superpulsed (904 nm) laser-induced tissue repair in burn wounds. Light is initially absorbed by mitochondrial chromophore (photoacceptor) and causes increase production of ATP, reactive oxygen species (ROS) and dissociation of nitric oxide (NO), which in turn cause changes in cellular redox potential, Ca^{2+}, K^{+}, cAMP and pH levels and induce several transcription factors (HIF-1α, NF-kB, AP-1). The photosignal transduction and amplification chain induced by photons leads to an increase of cell proliferation, cell motility, production of growth factors and extra cellular matrix (ECM) accumulation. LLLT superpulsed (904 nm) laser with this protocol reduces inflammatory response by decreasing TNF-α levels and NF-kB protein expression, and up-regulates angiogenesis markers (VEGF, HIF-1α), enhances cellular proliferation and cell migration (FGFR-1 and fibronectin), cytoprotection via. molecular chaperons (HSP-60 and 90) and increases collagen accumulation.

pulsed 904 nm treatment. The increased content of hydroxyproline and hexosamine in superpulsed laser treated burn wounds provides the strength to the re-generated wound tissue. However, SSD ointment treated animals did not show any significant changes in pro-healing parameters compared to the non-irradiated control and superpulsed 904 nm laser treated animals. SSD, undoubtedly is a very efficient anti-bacterial agent, however, its effect on wound healing is not very promising. The anti-bacterial mechanism of SSD is thought to act via silver ion-mediated inhibition of DNA replication and modification of cell membrane and cell wall of the pathogens. On the other hand, the accumulated silver may have cytotoxic effects on neutrophils and other immune cells which contribute to local immune dysfunction. Furthermore, the inhibition of cell proliferation by SSD may also impair wound healing by directly inhibiting the proliferation of fibroblasts and keratinocytes [38].

The histological images indicated enhanced granulation tissue, fibrogenesis, neovascularization, complete re-epithelialization and decreased inflammatory infiltrate on the eighth day post-wounding by superpulsed 904 nm LLLT. The results of the biochemical indices were also well-corroborated with the histopathological examinations. These findings were consistent with some previous in-vivo studies suggesting healing efficacy of pulsed LLLT on skin wounds [39–41]. A. Khoshvaghti et al. [22] showed that LLLT with pulsed NIR laser significantly decreased the total numbers of mast cells during the

proliferation and remodeling phases of healing in burn wounds of rats. Servetto et al. [42] demonstrated the effect of LLLT on rats with experimental myopathy induced by injecting adrenaline in the left posterior limb muscle at the same point on 5 consecutive days. They used a He-Ne (632.8 nm) or a pulsed Ga–As (904 nm) laser delivering 9.5 J/cm^2 daily for seven consecutive days with either laser. Both lasers reduced inflammatory biomarkers associated with oxidative stress, fibrinogen, L-citrulline and superoxide dismutase.

MMPs are important members of a proteolytic zinc endopeptidase enzyme family, play an important role in regulating tissue repair processes such as eliminate damaged proteins, destroy the provisional ECM, facilitate cell migration, remodel the granulation tissue and regulate the activity of some cytokines. These enzymes are produced by a variety of cell types including fibroblasts, smooth muscle, endothelial, epithelial and inflammatory cells [43]. In the present study, enhanced expression of MMP-2 and 9 after 4 day post-wounding indicates that LLLT modulates the inflammatory process during the acute phase of burn wound healing. These findings corroborated with previous report that demonstrate the involvement of MMPs in LLLT-induced nerve recovery process [44].

The photobiomodulatory effect of LLLT with superpulsed 904 nm is exhibited by the differential protein expression in regenerated wound tissue of burned rats. LLLT increased angiogenesis as evidenced by histopathology and up-regulated angiogenesis markers (VEGF and HIF-1α expression). VEGF is an important pro-angiogenic cytokine and stimulates multiple components of the angiogenic cascade, however likely also augments collagen accumulation and re-epithelialization [45]. Angiogenesis is a critical component for healing, as it is essential for delivery of oxygen and nutrients to the injured tissue site. Angiogenesis process is initiated by the activation of endothelial cells, followed by ECM degradation, MMPs activation, cellular proliferation, enabling tube sprout formation and growth of the new blood vessel formation. Hypoxia triggers expression of HIF-1α, which is a transcription factor that under hypoxia conditions (like wound microenvironment), accumulates in endothelial cells and can bind to VEGF gene promoter and induce VEGF gene expression and initiation of angiogenesis [46]. Earlier reports have also shown the up-regulated expression of angiogenesis markers (VEGF and HIF-1α) submitted to LLLT with red laser (660 nm) in dermal wound healing [47, 48].

In our study we observed a down-regulated expression of NF-*k*B protein level in burn wound tissue treated with superpulsed 904 nm laser. NF-*k*B is a transcription factor belonging to the Rel family of DNA-binding proteins which is present in the cytoplasm as a dimer in an inactive state, bound with members of the I*k*B (inhibitors of NF-*k*B). Upon stimulation, by compounds such as pro-inflammatory cytokines (TNF-α), ROS, oncogenes or UV irradiation, a kinase signaling cascade results in phosphorylation of I*k*B, signaling ubiquitination-mediated proteasomal degradation, which in turn results into NF-*k*B release, translocation into nucleus and binding to the corresponding DNA sequence of the NF-*k*B target genes [49]. The down-regulated expression of NF-*k*B in LLLT superpulsed laser treated wounds might be attributed to reduced levels of TNF-α which, in turn is able to down-regulate the inflammatory response during acute phase of healing process. Our result support previous finding by Rizzi et al. [36] who found that LLLT with 904 nm laser reduced the inflammatory response induced in traumatized muscle and also able to block the effect of ROS release and activation of NF-*k*B. They further suggested that decreased activation of NF-*k*B could be attributed to reduction of oxidative stress during treatment with LLLT.

The photostimulatory effect of superpulsed LLLT is exhibited by the significant up-regulated protein expression levels of FGFR-1 and fibronectin. FGFR-1 is a member of the tyrosine kinase family of growth factor receptor. Signal transduction by FGFR requires dimerization or oligomerization and autophosphorylation of the receptors through their tyrosine kinase domain and subsequently leads to DNA replication or differentiation. FGFR-1 is highly expressed in developing human tissues including vascular basement membranes, brain and skin [50, 51]. Fibronectin is a multi-functional, ECM glycoprotein and contains several functionally and structurally distinct domains which may bind to cell surface, collagen, fibrinogen or fibrin, glycosaminoglycans, proteoglycans and heparin. Various in-vitro and in-vivo studies have shown that fibronectin enhances cell adhesion and migration and plays an important role in cytoskeletal organization, phagocytosis, hemostasis, embryonic differentiation, and wound healing [52]. The increased cellular proliferation and cell migration in superpulsed 904 nm laser treated wounds might be attributed to the enhanced expression of FGFR-1 and fibronectin.

The cytoprotective role of HSP-60 and HSP-90 has been observed in superpulsed 904 nm laser treated burn wound tissue. Molecular chaperon, HSP is a group of highly conserved proteins having diverse functions including the assembling, refolding of multiple proteins and transport of nascent polypeptide chain across the cell membrane. The role of HSP-90 has been shown in wound healing under the influence of HIF-1α. Increased expression of HIF-1α in turn causes exocytosis of HSP-90. In ECM, HSP-90 promotes the migration of skin fibroblasts that ultimately corresponds to enhanced wound healing pro-

cess. HSP-60 has cytoprotective role as it functions as anti-apoptotic molecule and prevents cell death [35, 53].

Collectively, as depicted in Figure 7, the photobiomodulation of the cytokines, growth factor receptor, cellular and nuclear factors by irradiation with superpulsed 904 nm laser triggers cell networks to accelerate tissue repair. In conclusion, our results demonstrate that superpulsed 904 nm laser therapy is able to promote repair of burn wounds as a result of reduced inflammatory response, and favor the cellular proliferation, collagen accumulation and wound contraction. This study provides insight into the mechanism by which LLLT superpulsed 904 nm laser irradiation accelerates burn wound healing.

Acknowledgements The study was funded by the Defence Research and Development Organization (DRDO), Ministry of Defence, Government of India.

Author biographies Please see Supporting Information online.

References

[1] A. J. Singer and R. A. F. Clark, New Eng. J. Med. **341**, 738–746 (1999).
[2] A. Gupta, P. Avci, M. Sadasivam, R. Chandran, N. Parizotto, D. Vecchio, W. C. de Melo, T. Dai, L. Y. Chiang, and M. R. Hamblin, Biotechnol. Adv. **31**, 607–631 (2013).
[3] H. Abrahamse, Photomed. Laser Surg. **30**, 681–682 (2012).
[4] H. Chung, T. Dai, S. K. Sharma, Y. Y. Huang, J. D. Carroll, and M. R. Hamblin, Ann. Biomed. Eng. **40**, 516–533 (2012).
[5] A. Gupta and M. R. Hamblin, in: M. R. Hamblin and Y. Y. Huang (eds.), Handbook of Photomedicine, (CRC Press, Taylor and Francis, USA, 2013), pp. 43–52.
[6] T. I. Karu and S. F. Kolyakov, Photomed. Laser Surg. **23**, 355–361 (2005).
[7] N. Lane, Nature **443**, 901–903 (2006).
[8] D. Barolet, P. Duplay, H. Jacomy, and M. Auclair, J. Biomed. Opt. **15**, 048005 (2010).
[9] Q. Wu, W. Xuan, T. Ando, T. Xu, L. Huang, Y. Y. Huang, T. Dai, S. Dhital, S. K. Sharma, M. J. Whalen, and M. R. Hamblin, Lasers Surg. Med. **44**, 218–226 (2012).
[10] A. Gupta, T. Dai, and M. R. Hamblin, Lasers Med. Sci. **29**, 257–265 (2014).
[11] J. T. Hashmi, Y. Y. Huang, S. K. Sharma, D. B. Kurup, L. De Taboada, J. D. Carroll, and M. R. Hamblin, Lasers Surg. Med. **42**, 450–466 (2010).
[12] T. Ando, W. Xuan, T. Xu, T. Dai, S. K. Sharma, G. B. Kharkwal, Y. Y. Huang, Q. Wu, M. J. Whalen, S. Sato, M. Obara, and M. R. Hamblin, PLoS One **6**, e26212 (2011)
[13] D. A. Pires-Oliveira, R. F. Oliveira, S. U. Amadei, C. Pacheco-Soares, and R. F. Rocha, Osteoporos. Int. **21**, 2109–2114 (2010).
[14] E. C. Leal-Junior, R. A. Lopes-Martins, P. de Almeida, L. Ramos, V. V. Iversen, and J. M. Bjordal, Eur. J. Appl. Physiol. **108**, 1083–1088 (2010).
[15] N. K. Upadhyay, R. Kumar, S. K. Mandotra, R. Meena, M. S. Siddiqui, R. C. Sawhney, and A. Gupta, Food Chem. Toxicol. **47,** 1146–1153 (2009).
[16] K. A. Burton, Biochem. J. **62**, 315–321 (1956).
[17] O. H. Lowry, N. J. Rosenburgh, A. L. Farr, and B. J. Randell, J Biol Chem. **193**, 265–275 (1951).
[18] J. F. Woessner Jr., Arch. Biochem. Biophys. **93**, 440–447 (1961).
[19] L. A. Elson and W. T. J. Morgan, J. Biochem. **27**, 1824–1828 (1933).
[20] T. I. Karu, Laser Life Sci. **2**, 53–74 (1988).
[21] J. M. de Moraes, D. E. O. de Mendonca, V. B. L. Moura, M. A. P. Oliveira, C. L. Afonso, M. C. Vinaud, M. M. Bachion, and R. S. deLino Jr., Lasers Med. Sci. **28**, 1169–1176 (2013).
[22] A. Khoshvaghti, M. Zibaamnzarmofrad, and M. Bayat, Photomed. Laer Surg. **29**, 597–604 (2011).
[23] J. Joensen, K. Ovsthus, R. K. Reed, S. Hummelsund, V. V. Iversen, R. A. Lopes-Martins, and J. M. Bjordal, Photomed. Laser Surg. **30**, 688–694 (2012).
[24] J. M. Bjordal, C. Couppe, R. T. Chow, J. Tuner, and E. A. Ljunggren, Aust. J. Physiother. **49**, 107–116 (2003).
[25] F. Aimbire, R. Albertini, M. T. Pacheco, H. C. Castro-Faria-Neto, P. S. Leonardo, V. V. Iversen, R. A. Lopes-Martins, and J. M. Bjordal, Photomed. Laser Surg. **24**, 33–37 (2006)
[26] A. B. C. Salate, G. Barbosa, and P. Gaspar, Photomed. Laser Surg. **23**, 470–475 (2005).
[27] P. de Almeida, S. S. Tomazoni, L. Frigo, P. T. C. de Carvalho, A. A. Vanin, L. A. Santos, G. M. Albuquerque-Pontes, T. De Marchi, O. Tairova, R. L. Marcos, R. A. B. Lopes-Martins, and E. C. P. Leal-Junior, Laser Med. Sci. **29**, 653–658 (2013).
[28] V. A. Melo, D. C. Anjos, R. Albuquerque Jr., D. B. Melo, and F. U. Carvalho, Acta Cir. Bras. **26**, 129–134 (2011).
[29] P. Lee, K. Kim, and K. Kim, Laser Ther. **5**, 59–64 (1993).
[30] A. Gur, A. J. Sarac, R. Cevik, O. Altindag, and S. Sarac, Lasers Surg. Med. **35**, 229–235 (2004).
[31] G. K. Reddy, Lasers Surg Med. **33**, 344–351 (2003).
[32] J. M. Bjordal, R. A. Lopes-Martins, and V. V. Iversen, Br. J. Sports Med. **40**, 76–80 (2006)
[33] F. Correa, R. A. Lopes Martins, J. C. Correa, V. V. Iversen, J. Joensen, and J. M. Bjordal, Photomed Laser Surg. **25**, 245–249 (2007).
[34] P. C. Silveira, L. A. Silva, D. B. Fraga, T. P. Freitas, E. L. Streck, and R. Pinho, J. Photochem. Photobiol. B. **95**, 89–92 (2009).
[35] D. Avni, S. Levkovitz, L. Maltz, and U. Oron, Photomed Laser Surg. **23**, 273–277 (2005).
[36] C. F. Rizzi, J. L. Mauriz, D. S. F. Correa, A. J. Moreira, C. G. Zettler, L. I. Filippin, N. P. Marroni, and J. Gonzalez-Gallego, Laser Surg. Med. **38**, 704–713 (2006).

[37] M. Matic, B. Lazetic, M. Poljacki, V. Djuran, A. Matic, and Z. Gojinov, Lasers Med. Sci. **24**, 321–327 (2009).
[38] Z. Aziz, S. F. Abu, N. J. Chong, Burns: J. Int. Soc. Burn Injuries **38**, 307–318 (2012).
[39] F. A. Al-Watban and X. Y. Zhang, J. Clin. Laser Med. Surg. **22**, 15–18 (2004).
[40] H. Demir, H. Balay, and M. Kirnap, J. Rehabil. Res. Dev. **41**, 147–154 (2004).
[41] A. Ezzati, M. Bayat, and A. Khoshvaghti, Photomed. Laer Surg. **28**, 603–611 (2010).
[42] N. Servetto, D. Cremonezzi, J. C. Simes, M. Moya, F. Soriano, J. A. Palma, and V. R. Campana, Lasers Surg. Med. **42**, 577–583 (2010).
[43] A. M. Jessica and G. K. Barbara, Matrix Biol. **25**, 505–514 (2006).
[44] C. C. Alcantara, D. Gigo-Benato, T. F. Salvini, A. L. R. Oliveira, J. J. Anders, and T. L. Russo, Lasers Surg. Med. **45**, 246–252 (2013).
[45] P. Bao, A. Kodra, M. Tomic-Canic, M. S. Golinko, H. P. Ehrlich, and H. Brem, J Surg. Res. **153**, 347–358 (2009).
[46] A. Ahluwalia and A. S. Tarnawski, Cur Medicinal Chem. **19**, 90–97 (2012).
[47] A. C. Renno, A. M. Iwama, P. Shima, K. R. Fernandes, J. G. Carvalho, P. De Oliveira, and D. A. Ribeiro, J Cosmet Laser Ther. **13**, 237–242 (2011).
[48] V. Cury, A. I. S. Moretti, L. Assis, P. Bossini, J. S. Crusca, C. B. Neto, R. Fangel, H. P. Souza, M. R. Hamblin, and N. A. Parizotto, J. Photochem. Photobiol. B: Biol. **125**, 164–170 (2013).
[49] P. V. Uden, N. S. Kenneth, and S. Rocha, Biochem. J. **412**, 477–484 (2008).
[50] R. E. Friesel and T. Maciag, FASEB J. **9**, 919–925 (1995).
[51] M. Meyer, A. K. Muller, J. Yang, D. Moik, G. Ponzio, D. M. Ornitz, R. Grose, and S. Werner, J Cell Sci. **125**, 5690–5701 (2012).
[52] N. S. Greaves, K. J. Ashcroft, M. Baguneid, and A. Bayat, J. Dermatol. Sci. **72**, 206–217 (2013)
[53] W. Li, Y. Li, S. Guan, J. Fan, C. F. Cheng, A. M. Bright, C. Chinn, M. Chen, and D. T. Woodley, EMBO J. **26**, 1221–1233 (2007).