Effects of low intensity laser irradiation during healing of infected skin wounds in the rat

Ethne L. Nussbaum*,

Departments of Physical Therapy, Mount Sinai Hospital, University of Toronto, 500 University Ave, Toronto, ON M5G 1X5, Canada

Facundo Las Heras,

Department of Laboratory Medicine and Pathobiology, Mount Sinai Hospital, University of Toronto, 500 University Ave, Toronto, ON M5G 1X5, Canada

Kenneth P.H. Pritzker,

Department of Laboratory Medicine and Pathobiology, Mount Sinai Hospital, University of Toronto, 500 University Ave, Toronto, ON M5G 1X5, Canada

Tony Mazzulli, and

Department of Microbiology, Mount Sinai Hospital, University of Toronto, 500 University Ave, Toronto, ON M5G 1X5, Canada

Lothar Lilge

Department of Medical Biophysics, University of Toronto, 610 University Avenue, Rm. 7–418, Toronto ON M5G 2M9, Canada

Abstract

Background and objective—Low intensity laser irradiation remains a controversial treatment for non-healing wounds. This study examines the effect of low intensity light on healing of infected skin wounds in the rat.

Materials and methods—Wounds on the rat dorsum were inoculated with *Pseudomonas aeruginosa*. Wounds were irradiated or sham-irradiated three times weekly from day 1 to 19 using 635-nm or 808-nm diode lasers delivering continuous wave (CW) or intensity modulated (3800 Hz) laser radiation, all at radiant exposures of 1 and 20 J/cm². Wound area and bacterial growth on the wound surface were evaluated three times a week. Histological and immunohistochemical analyses were performed at day 8 and 19.

Results—Wounds that were irradiated using a wavelength of 635 nm (1 and 20 J/cm²) or intensity modulated 808-nm laser light at 20 J/cm² were smaller in area at day 19 than the sham-irradiated controls (achieved significance level=0.0105–0.0208) and were similar to controls in respect of bacterial growth. The remaining light protocols had no effect on wound area at day 19 although they increased *Staphylococcus aureus* growth across the time line compared with controls ($p<0.0001$ to $p<0.004$). CW 808-nm light at 20 J/cm² significantly delayed half-heal time. Histological and immunohistochemical analyses supported wound closure findings: improved

*Corresponding author: Ethne L. Nussbaum, e.nussbaum@utoronto.ca.

healing was associated with faster resolution of inflammation during the acute phase and increased signs of late repair at day 19. Significant inflammation was seen at day 19 in all irradiated groups regardless of radiant exposure, except when using 635 nm at 1 J/cm².

Conclusions—Red light improved healing of wounds. Only one 808-nm light protocol enhanced healing; lack of benefit using the remaining 808-nm light protocols may have been due to stimulatory effects of the light on *S. aureus* growth.

Keywords

bacteria; histology; immunohistochemistry; phototherapy; wound healing

1 Introduction

Low intensity laser light remains a controversial treatment for non-healing human wounds. Although there is considerable anecdotal support for light treatment of wounds, amongst the several controlled trials only a few small studies have demonstrated benefit [1–11]. There are many factors involved in delayed wound healing: infection is a key element [12]. Patients with infected wounds are at risk of developing bacteremia and osteomyelitis which can have catastrophic consequences; at the least, hospital length of stay and healthcare costs are greatly increased [13].

Open wounds are readily colonized by the normal flora that reside on periwound skin, including such organisms as coagulase-negative staphylococci, streptococci, *Bacillus* species and *Corynebacterium* species. Wound colonization at low concentration (<10⁵ organisms per gram of tissue) will usually not impede wound healing as long as host resistance and bacterial activity remain in balance. In fact, bacterial presence at low concentration may stimulate granulation tissue formation thereby improving healing [14, 15]. Further, by binding to available receptor sites on the wound surface, normal flora protect wounds from colonization by environmental organisms that are potentially more dangerous, for example, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Cutaneous wounds normally heal in an orderly process comprising three overlapping phases: inflammation, proliferation and remodeling.

- Phase 1 (0–7 days) is defined by early edema followed by the infiltration of inflammatory cells (neutrophils followed by macrophages and lymphocytes) which debride the wound of damaged cells and foreign material and, in turn, release growth factors and biochemical compounds that activate tissue regeneration.
- Neovascularization and provisional matrix formation (3–14 days) define phase 2 with new blood vessels growing in primarily from tissues subjacent to the wound. Provisional matrix is characterized first by proteoglycan secreted by connective tissue cells, followed by collagen synthesis by activated fibroblasts. The early collagen fibers are relatively thinner and aligned parallel to blood vessels (perpendicular to the wound surface). During this time, particularly in

rodents, there is connective tissue contraction and epidermal growth over the wound surface.

- Phase 3 is a period of wound remodeling (10 days–6 months) which is characterized by obliteration of new blood vessels, reduction of proteoglycan matrix, and collagen remodeling characterized by resorption of early collagen and formation of new collagen fibers oriented along stress lines [16, 17].

There is clear evidence in preclinical literature that low intensity light (LIL) affects bacterial growth [18–21]. Dependent on radiant exposure, growth may be increased or decreased; clearing wounds of bacteria is possible using some combinations of wavelength and radiant exposure. In our laboratory, wounds in the rat were cleared of bacteria using red light at radiant exposure of 20 J/cm²; importantly, these sterile wounds healed more slowly than control wounds [22]. We found that 635-nm light at radiant exposure of 1 J/cm² produced the most beneficial effects on wound healing, interestingly, without disturbing the normal wound bioburden [22]. The major difference between the prior study and the work reported here is that previously the wounds were exposed to opportunistic environmental organisms whereas in this study wounds were infected using a standardized bacterial inoculum.

There is scarce literature on the effects of LIL on healing of wounds that are colonized by pathogenic bacteria, such as *S. aureus* and *P. aeruginosa*. Kim's group [23] irradiated *S. aureus*-inoculated wounds in the rat using 904-nm light at low radiant exposure (0.0076 J/cm²) and observed improved healing on the seventh day. The authors did not attempt to quantify bacterial growth in the wounds or examine light effects on the underlying tissue. As a result, they were unable to suggest whether laser stimulation of the host tissue predominated over stimulation of bacterial growth in the wounds or the laser restrained the spread of infection in the wounds allowing healing to occur. Hamblin et al. [24] examined the effects of photodynamic therapy (665-nm light; 240 J/cm²) on *P. aeruginosa*-infected wounds in mice. They observed a high bacterial kill rate but no effects on healing rate.

A common clinical approach using LIL is to irradiate wounds three times weekly usually without the use of photosensitizing drugs. There may be a potential advantage of using light over antibiotic therapy for infected wounds: because laser effects are mediated via excited states of a receptor molecule it is considered unlikely that the organism *per se* could develop resistance due to prolonged usage [25]. However, further investigation is indicated because particular frequent or intense light might be toxic to tissues and could inhibit cell processes essential for wound healing.

Accordingly, the goals of the present study were: a) to determine whether healing in laser-irradiated infected wounds in the rat was associated with modification of the bacterial load and whether the effects were dependent on wavelength and radiant exposure; and b) to identify light-mediated differences in host tissue histology during early and late wound healing to develop hypotheses towards the underlying mechanisms.

2 Materials and methods

2.1 Subjects

One hundred and eight, male, Sprague-Dawley rats (Charles River Inc., St. Constant, Quebec, Canada) weighing 250–300 g were obtained for this study. Animals were housed separately without bedding, and were maintained on a 12-h light/dark cycle with food and water ad libitum. All procedures and assessments were performed under isoflurane inhalation (2% in air). The study was approved by the Mount Sinai Hospital Research Institute Animal Care Committee.

2.2 Study design

Prior to surgery on day 1, animals were assigned to one of six possible irradiation groups (14 animals per group) or to a sham irradiation group (24 animals in total) (Figure 1). Eight sham-irradiated animals were included in each cycle of experiments, four being assigned to each radiant exposure. Animals were euthanized immediately after excision of the wound tissue at day 8 or day 19. Day 8 was selected because the early inflammatory processes should have resolved by this time. Day 19 was selected in order to conclude the study at a standardized time for all animals and when above 70% wound closure could be expected; this was based on pilot work and a previous study [22].

2.3 Wound inducement

On day 1, dorsal hair over the lower back was removed and the skin was washed with 70% alcohol. Using a 1×1 cm template for consistency, full-thickness skin was removed down to, but not through, the panniculus carnosus.

Prior to each laser treatment, excluding day 1, wound surfaces were gently rinsed using 20 ml of sterile saline. Wound dressings were not applied.

2.4 Wound inoculation

All wounds were inoculated using a calibrated pipette with 200 µl of a suspension of *P. aeruginosa* (American Type Culture Collection #27853, Rockville, MD, USA) in sterile saline, equivalent to 10⁶ colony forming units (cfu)/ml. The isolate does not produce toxins and it is not a particularly resistant strain to the broad-spectrum antibiotics that would normally be used to treat it clinically. We selected *P. aeruginosa* because it is frequently associated with deteriorating wounds and it has intrinsic resistance to some widely used antibiotics [26]. In addition, it produces exotoxins that act against commensal bacteria [27]. The conditions for bacterial inoculation were chosen to balance the ability to establish an infection with the risk of inducing invasive disease to the point where the animals died due to overwhelming infection.

2.5 Irradiation procedures

Wounds were irradiated or sham-irradiated three times weekly beginning on day 1 immediately post-inoculation. This regimen was chosen because the common clinical approach to laser therapy for wounds is three exposures a week at 48-h intervals. Wounds were not irradiated prior to euthanasia on day 8 or day 19.

Irradiation was performed using fiber-coupled solid-state diode lasers operating at 808 nm (B&W TEK, Inc., Newark, DE, USA) and 635 nm (B&W TEK, Inc.). Red light was selected because in previous work we found that red light at low radiant exposure improved healing during the inflammatory phase of wound repair and that at higher radiant-exposure red light had a bactericidal effect [22]. Selection of 808-nm light was also based on previous work demonstrating that 808-nm light produced undesirable proliferation of *S. aureus* [22]. We wanted here to determine whether similar effects occurred when these protocols were applied in experimentally inoculated wounds. To further understand the effects of 808-nm light on infected wounds we tested 808-nm light in both continuous and intensity modulated modes of irradiation because in previous *in vitro* studies we had found that 810-nm light-induced effects on bacterial growth were different in continuous compared with intensity modulated mode [18].

Power output of the lasers was confirmed prior to, midway through and after the study using a thermopile power meter (TPM-300-CE Power Meter, PS-310; GENTEC Inc. Ste-Foy, Quebec, Canada).

The optical fiber was fitted with a beam expander at the distal end to irradiate a circular area of diameter 3.0 cm (area, 7.1 cm²; irradiance, 0.028 W/cm²) which incorporated the wound and some surrounding intact skin. Irradiance was based on previous *in vitro* work in our lab which showed that *P. aeruginosa* growth was significantly more inhibited using an irradiance of 0.03 W/cm² compared with 0.015 W/cm². The distal end of the fiber was mounted in a metal holder which positioned the fiber perpendicular to and at a standardized distance from the wound surfaces. Each wavelength was studied at radiant exposures of 1 J/cm² and 20 J/cm² (36- and 710-s exposure, respectively). Sham irradiation was applied for 36 s. Each wavelength was studied in continuous wave (CW); in addition 808-nm light was studied at intensity modulation frequency of 3800 Hz with 50% duty cycle at 1 and 20 J/cm².

Sham and laser-irradiated animals were anesthetised and handled identically to ensure that any observed effects on wound closure or morphology could not be due to procedural differences. The study design is illustrated in Figure 1.

2.6 Wound area measurement

Wound status was photodocumented three times a week, beginning immediately post wounding on day 1. The digital camera (Canon Powershot G5, Melville, NY, USA) was mounted on a stand which positioned the lens perpendicular to the tissue surface, and a metric reference for image calibration was positioned immediately adjacent to the wound edge. Images were filed using code numbers to conceal treatment groups. Wound area was measured directly from the number-coded images using computerized technology (Image-J Software for Windows, developed by the National Institutes of Health, USA and downloadable at <http://rsb.info.nih.gov/ij/download.html>) and without reference to any previous images or measurements. Wound area was expressed as a percentage of the wound area measured on day 1.

2.7 Bacterial growth assessment

Our culture method was designed to follow standard laboratory practice for processing wound swabs. The presence of bacteria was determined using a semi-quantitative swab technique, beginning on day 3 and continuing three times a week until the end of the study (day 8 or day 19). Swabs were stored in Amies transport medium with charcoal and were delivered to the lab coded by number to ensure that the microbiology assessor was blinded to treatment groups. Swabs were streaked onto blood and MacConkey agar culture plates using routine methods and the plates were incubated for 20 h at 35°C in 5% CO₂. Plates were then examined and the results were reported as no growth or by listing the specific types of bacteria that grew (opportunistic bacterial growth such as normal skin flora, *S. aureus*, and *Escherichia coli* in addition to *P. aeruginosa*). Bacteria were identified using the VITEK II system (bioMérieux Canada, Inc., St-Laurent, Quebec).

2.8 Histologic assessment

Wound tissue was collected from eight experimental animals in each irradiation group, four on day 8 and four on day 19, and four sham-irradiated animals, one on day 8 and three on day 19. Animals were assigned randomly to this procedure using a table of random numbers prior to wound inducement on day 1. Wounds were excised with a volume equal to the original wound size plus a surrounding circumference of 2 mm. Specimens were numbered to ensure blinding of the assessor. Specimens were bisected and fixed in 10% neutral buffered formalin and subsequently dehydrated in alcohol, cleared in xylene and embedded in paraffin. Five µm-sections were cut and stained, using hematoxylin and eosin (H&E), Gram stains for microorganisms and Picro Sirius red after papain digestion for presence of collagen fibers. Sections were graded for wound healing according to seven parameters related to acute inflammatory response and repair: edema, polymorphonuclear leucocytes, macrophages, granulation tissue, fibroblasts, collagen deposition, and evidence of epithelialization. Each feature was semi quantitatively evaluated (from 0=absent, to 3=prominent) based on well defined and reproducible histological parameter classification reported by us previously [16, 22].

In an attempt to more closely determine the cell behavior induced by light, immunohistochemical analysis was performed on a representational number of day-8 and day-19 specimens to determine proliferation index using antiMib-1 antibodies (LabVision, Fremont, CA, USA; dilution 1/200) and to determine apoptosis index using Caspase 3 (BioVision, Mountain View, CA, USA; dilution 1/25). Rat spleen and thymus were used as positive-control tissue for optimization of Mib-1 and Caspase-3, respectively. Internal positive control for Mib-1 was observed in the basal layer of the epidermis adjacent to the wound area.

2.9 Statistical analysis

2.9.1 Outliers—Boxplot tests were used to detect any possible outliers within each day's observations of wound area percentage. An animal was considered an outlier of the group if it had more than four outlier readings along the observation period. A convenient definition of an outlier is a point which falls more than 1.5 times the inter quartile range, above the third quartile or below the first quartile.

2.9.2 Wound area percentage at day 19—Because a *t*-test might not give very accurate results with the relatively small number of subjects remaining in each group at day 19 (*n*=10) (see Figure 1), the bootstrap method (with 10,000 bootstrap replications) was implemented. Bootstrap is a computer-based method for assigning measures of accuracy to statistical estimates. The null hypothesis was that the mean area (%) of irradiated wounds was equal to the mean area (%) of sham-irradiated wounds (controls). Achieved significance level (ASL) was considered significant at 5% (ASL=0.05).

2.9.3 Wound healing over time—A non-linear growth curve in the form of log-logistic function was derived to describe the healing curve of each group over the 19-day observation period using SAS NLIN procedure (SAS version 8.3; SAS Institute Inc., Cary, NC, USA). Comparison of predicted and measured data and the value of coefficient of determination confirmed that the model fitted the data well. Representative variables of the curves were estimated and each treatment group was compared with control for maximum wound area (mean wound area was >100% after day 1), rate of healing, and time when half-heal was reached (with respect to maximum wound area). For each variable, the significance of differences and 95% confidence intervals (CI) were calculated ($\alpha=0.05$).

2.9.4 Bacterial growth—In each group, the counts of sterile wounds and of each bacterial species were expressed as percentages of the total number of samples examined during the observation period (eight swabs from animals euthanized on day 19 and three swabs from animals euthanized on day 8). Fischer's exact test was performed for the null hypothesis that the percentages of wounds with no growth, normal skin flora, *P. aeruginosa*, *S. aureus*, and *E. coli* in irradiated groups were equal to that of the control (sham) group.

2.9.5 Histological assessment—Principal component analysis (PCA) was used as a variable reduction procedure in order to summarize the original data with fewer and new scores. In the original data seven variables were assessed with a 4-level scale, with higher levels corresponding to stronger presence.

The SAS procedure PROC FACTOR was used to perform the analysis. Two new variables were defined that together accounted for 80% of the variances in the original dataset. The new scores for each animal were then used to calculate average scores by group and day (day 8 and 19) for subsequent analysis.

The bootstrap method (with 10,000 bootstrap replications) was implemented to test the null hypothesis that scores at day 8 and day 19 in each irradiated group were equal to that of control. ASL was considered significant using the criteria of 0.05.

3 Results

3.1 Outliers

Results showed that one treatment group (635 nm, 1 J/cm²) contained an outlier based on the finding of one mild and four extreme outlier readings in percentage wound closure along the time line. All data for this animal were removed prior to further analysis.

3.2 Wound area percentage at day 19

Wound healing by group is shown in Figure 2. Table 1 shows the results of analysis. The control group wounds were on average 21.7% of their initial size. The wounds irradiated using wavelengths of 635 nm (1 and 20 J/cm²) and intensity modulated 808 nm at 20 J/cm² were significantly smaller than controls (ASL=0.0105–0.0208).

Wounds irradiated using CW 808-nm light (1 and 20 J/cm²) and intensity modulated 808-nm light at 1 J/cm² were similar to controls.

3.3 Wound healing over time

Table 2 shows results of model fitting and estimates with 95% CI for three parameters of healing. The last column of the table gives the value of coefficient of determination (R²), a value that measures how well the model explains the raw data. The fitted log-logistic curve explains 80–93% of the variance in the raw data in this study, indicating that the model fits the data well.

On average wound areas were 20–37% larger on day 3 than at baseline. Compared with control wounds there was no significant effect of any light protocol on preventing the initial wound from worsening.

Figure 2 shows that during the early period all wounds irradiated using 808-nm light healed on average more slowly than control wounds. Wounds irradiated using CW 808-nm light at 20 J/cm² reached half-heal point 1.6 days later than controls which was significantly different ($p<0.05$; 95% CI 0.5, 2.6); however, differences in time to reach half-heal point between the remaining 808-nm light treatments and controls were not significant ($p>0.05$). During the late period, from about day 8 onwards, all 808-nm irradiated wounds showed accelerated healing giving these wounds significantly deeper healing trajectories than control wounds.

3.4 Bacterial growth

In total, 92 samples were collected in each active treatment group and 172 samples in the control group. Bacterial data in percentages and results of analysis are shown in Tables 3 and 4, respectively. For purpose of clarity, the non-significant results for *E. coli* have been omitted from Table 4.

Overall, *P. aeruginosa* was detected in a low percentage of wounds being present more commonly on days 3 and 5 (13% and 16% of control wounds, respectively), and seldom from day 10 onwards. For clarity the bacterial profiles across the time line are not shown. When using CW 808-nm light at 20 J/cm² no wounds were positive for *P. aeruginosa* ($p<0.06$).

Wounds irradiated using 635-nm light (1 J/cm² and 20 J/cm²) and modulated 808-nm light (3800 Hz, 20 J/cm²) were similar when compared with controls in respect of each bacterial species as well as the percentage of wounds that were sterile. As noted above, wounds in these three groups were significantly smaller than control wounds at day 19.

Bacterial growth was significantly different in wounds irradiated using CW 808-nm light (1 and 20 J/cm²): an increased percentage of wounds were colonized by skin flora ($p=0.005$ and $p<0.02$) and by *S. aureus* ($p=0.003$ and $p<0.0001$) whereas the percentages of sterile wounds decreased ($p<0.0001$ and $p<0.0001$) compared with controls. As noted above, wound healing up until half-heal time was slow for these two groups, significantly so for the group irradiated at 20 J/cm².

Wounds irradiated using modulated 808-nm light (3800 Hz) at 1 J/cm² also showed significantly increased *S. aureus* growth compared with controls ($p<0.004$).

3.5 Histological assessment

PCA resulted in two new components containing weighted values of the original seven factors of the histological assessment. Component I was considered to represent mostly late repair because it had highly negative weightings for edema and inflammation and high positive weightings for fibroblasts, collagen and epithelialization. Component II was considered to represent mostly acute repair because granulation tissue had a high positive weighting. It should be noted that the two components are uncorrelated and hence are measuring different aspects of the original data. Results for the comparisons of scores between each treatment group and control are shown in Table 5.

Tissue specimens were photographed at two different magnifications in order to show the relationship of the wound to the surrounding normal tissue (low magnification) and the predominant cell component of the repair process (high magnification) at day 8 (Figure 3) and day 19 (Figure 4).

3.5.1 Day 8—Wounds irradiated using 635-nm light at 1 J/cm² were similar to control wounds for markers of inflammation and signs of late repair. Wounds irradiated using 635-nm light at 20 J/cm² showed marginal evidence of a reduced inflammatory process compared with controls (ASL = 0.055). CW 808-nm light (1 J/cm²) increased both the inflammatory response (ASL = 0.028) and the development of late repair (ASL = 0.033) compared with controls. Increased development of late repair was also marginally significant using modulated 808-nm light (3800 Hz) at 20 J/cm² (ASL = 0.048).

3.5.2 Day 19—Wounds irradiated using 635-nm light (1 J/cm²) were similar to control wounds and showed that the earlier inflammation had subsided and instead there were strong signs of late repair: as noted above, the irradiated wounds were on average smaller in area than controls at day 19. Wounds irradiated using 635-nm light at 20 J/cm² showed an increased inflammatory response (ASL = 0.037); notwithstanding, these wounds were also significantly smaller than controls at day 19 (see above).

All 808-nm irradiated wounds showed a greater inflammatory response than controls (ASL = 0.006–0.039); however, only in wounds irradiated using CW 808-nm light at 20 J/cm² was this associated with a significant decrease in the presence of late repair elements (ASL = 0.004).

3.5.3 Immunohistochemical analysis—At day 8 the proliferative index by MIB-1, i.e. the percentage of cells that was positive for proliferation, was high for controls, 635-nm (20 J/cm²) and CW 808-nm (20 J/cm²) irradiated wounds (30%, 40% and 60%, respectively). The high proliferative index was associated with increased apoptosis, likely reflecting the accelerated turnover of cells (1–3%, 1–2% and 4% for controls, 635-nm and CW 808-nm irradiated wounds, respectively).

At day 19 there was a mild to moderate increase in proliferating cells labeled by MIB-1 (3–20%, 20% and 10–15% for control, 635-nm and CW 808-nm irradiated wounds, respectively) representing a reduction for each group when compared with day 8. For control and 635-nm irradiated wounds, this was associated with a very low rate of apoptosis (0% and 0–1%, respectively). Interestingly, apoptosis index remained high at 4% for CW 808-nm irradiated wounds, the group that performed worst according to histological evaluation at day 19.

4 Discussion

The use of LIL for wound healing remains controversial likely due to the negative results of some controlled human studies [4–11]. Although infection is a common factor in delayed wound healing, to date the clinical trials on low intensity laser effects have neglected bacterial growth as a possible confounding factor.

This study examined the healing of *P. aeruginosa* inoculated wounds in the rat. Overall a low percentage of wounds was positive for *P. aeruginosa* and it was present more commonly on days 3, 5 and 8 rather than during late healing. It is not clear why *P. aeruginosa* was not found more frequently in our wound model. The explanation might lie in the fact that the wound surfaces were dry and *P. aeruginosa* attachment favors a moist environment [28, 29]. Zhao et al. [30] inoculated wounds with *P. aeruginosa* and subsequently found, as we did, a combination of *P. aeruginosa* and *S. aureus* in the wounds; however, more than 99% of the bacteria were localized in the scabs rather than growing on the wound surface. Malic et al. [31] noted the ability of bacteria to form biofilms in both acute and chronic wounds. They examined multi-species biofilms *in vitro* and found that *P. aeruginosa* was detected throughout the biofilm in depth, while *S. aureus* was generally concentrated towards the surface of the biofilm. Thus, it is possible that *P. aeruginosa* was in fact present in our wound model but was not detected because we only measured using surface swab methods.

Gram-negative bacteria, including *P. aeruginosa*, cause damage, even when in a stationary phase, by secreting cytotoxic metabolites that destroy host cells and by releasing toxic lipopolysaccharides during cell death [29]. Thus inoculation of the wounds probably explains why control wounds in the present study were on average larger on day 3 and less healed at day 19 than control wounds in our prior study which did not include inoculation. Commensal bacteria are also sensitive to *P. aeruginosa* released toxins which likely explains why the percentage of wounds positive for normal skin flora was lower here than in our prior study.

In the current study effects on healing were examined using red and infrared light, the latter in continuous as well as intensity modulated mode, and using two levels of radiant exposure. Wound closure improved significantly using red light (1 and 20 J/cm²) and intensity modulated infrared light at 20 J/cm². Analysis of bacterial growth showed that the light protocols that accelerated wound closure had no effect on wound bioburden when compared with the sham-irradiated controls. This suggests that the healing advantage of these protocols was as a result of direct effects on the host tissue rather than through a killing or inhibitory effect on bacteria. Similarly in our prior work (non-inoculated model) low dose red light (1 J/cm²) improved wound healing according to wound closure along the time line and according to histological end points, and had no effect on wound bioburden [22].

In contrast, certain 808-nm light protocols used in this study (CW 1 and 20 J/cm², intensity modulated 1 J/cm²) delayed wound healing in the acute phase and significantly altered bacterial profile. It is not clear whether the delayed early healing was a result of increased *S. aureus* growth alone or whether the strong inflammatory response associated with these protocols played a role. The immune system is normally activated in response to signals from pathogens such as *P. aeruginosa*. However, additional light-induced inflammatory effects were demonstrated in this study for all irradiated groups except when using red light at 1 J/cm². Pro-inflammatory effects of light have been reported previously and it is generally thought that an enhanced inflammatory response during acute wound healing is beneficial [32, 33]. However, the inflammation produced here was intense and also unregulated in the sense that it persisted into the late healing phase. Notwithstanding that irradiated wounds were on average similar or smaller in surface area than controls at day 19, the wounds irradiated using CW 808-nm light (1 and 20 J/cm²), and to a lesser extent intensity modulated 808-nm light at 1 J/cm², comprised histologically immature repair tissue compared with controls suggesting some adverse effects had been produced.

Increased *S. aureus* growth and decreased presence of normal flora in association with delayed wound healing using CW 808-nm light was demonstrated in our prior work (non-inoculated model) [22]. It was unclear in that study whether CW 808-nm light killed normal skin flora thereby providing an opportunity for *S. aureus* attachment to wound surfaces or whether the 808-nm light stimulated *S. aureus* growth thereby crowding out the normal skin flora. In the present study CW 808-nm light significantly increased the growth of both *S. aureus* and normal flora when compared with control wounds; this suggests there were direct effects of CW 808-nm light on *S. aureus* growth. Interestingly, although intensity modulated 808-nm light at 1 J/cm² also increased presence of *S. aureus* this particular light protocol had no effect on normal skin flora again pointing to direct effects of the light on bacterial growth.

Results of this study suggest that red and infrared light activated cellular processes differently. At day 8 the percentage of MIB-1 positive proliferating cells using CW 808-nm light (20 J/cm²) was 50% higher than when using 635-nm (20 J/cm²) light; the apoptotic index was also higher corresponding to the increased cell turnover. At day 19, although the percentage of MIB-1 proliferating cells had decreased using 808-nm (20 J/cm²) light (similar to or lower than for controls or red light) the percentage of apoptotic cells remained comparatively high; this raises the possibility that the poor result using 808-nm (20 J/cm²)

light might be related to an increase pool of cells being precipitated into an apoptotic pathway. However, further research is needed to confirm this.

5 Conclusion

We have demonstrated that infrared light stimulates growth of *S. aureus*, an organism that is commonly associated with delayed wound healing. Thus our study might help explain why infrared light has failed to produce a healing advantage in some human wounds.

In conclusion, we have shown that irradiating infected wounds in the rat three times weekly using red light (1 and 20 J/cm²) or intensity modulated infrared light (20 J/cm²) improves wound healing and does not produce unwanted stimulation of bacterial growth. In contrast, CW 808-nm light (1 and 20 J/cm²) or intensity modulated 808-nm light at low radiant exposure (1 J/cm²) may delay wound healing as a result of light-induced effects on *S. aureus* growth and possible negative effects on host tissue, suggesting that these particular infrared protocols should not be considered for treating *S. aureus* infected wounds.

Acknowledgments

This work was made possible by a grant from the Canadian Institutes of Health Research IMH-67522.

References

1. Kaviani A, Djavid GE, Ataie-Fashtami L, Fateh M, Ghodsi M, Salami M, Zand N, Kashef N, Larijani B. A randomized clinical trial on the effect of low-level laser therapy on chronic diabetic foot wound healing: a preliminary report. *Photomed Laser Surg.* 2011; 29(2):109–14. [PubMed: 21214368]
2. Caetano KS, Frade MA, Minatel DG, Santana LA, Enwemeka CS. Phototherapy improves healing of chronic venous ulcers. *Photomed Laser Surg.* 2009; 27(1):111–8. [PubMed: 19196110]
3. Minatel DG, Frade MA, França SC, Enwemeka CS. Phototherapy promotes healing of chronic diabetic leg ulcers that failed to respond to other therapies. *Lasers Surg Med.* 2009; 41(6):433–41. [PubMed: 19588536]
4. Kopera D, Kokol R, Berger C, Haas J. Low level laser: does it influence wound healing in venous leg ulcers? A randomized, placebo-controlled, double-blind study. *Br J Dermatol.* 2005; 152(6): 1368–70. [PubMed: 15949018]
5. Taly AB, Sivaraman Nair KP, Murali T, John A. Efficacy of multiwavelength light therapy in the treatment of pressure ulcers in subjects with disorders of the spinal cord: a randomized double-blind controlled trial. *Arch Phys Med Rehabil.* 2004; 85(10):1657–61. [PubMed: 15468027]
6. Lucas C, van Gemert MJ, de Haan RJ. Efficacy of low-level laser therapy in the management of stage III decubitus ulcers: a prospective, observer-blinded multicentre randomised clinical trial. *Lasers Med Sci.* 2003; 18(2):72–7. [PubMed: 12928815]
7. Franek A, Król P, Kucharzewski M. Does low output laser stimulation enhance the healing of crural ulceration? Some critical remarks. *Med Eng Phys.* 2002; 24(9):607–15. [PubMed: 12376047]
8. Gupta AK, Filonenko N, Salansky N, Sauder DN. The use of low energy photon therapy (LEPT) in venous leg ulcers: a double-blind, placebo-controlled study. *Dermatol Surg.* 1998; 24(12):1383–6. [PubMed: 9865208]
9. Nussbaum EL, Biemann I, Mustard B. Comparison of ultrasound/ultraviolet-C and laser for treatment of pressure ulcers in patients with spinal cord injury. *Phys Ther.* 1994; 74(9):812–23. discussion 824–5. [PubMed: 8066108]
10. Lundeberg T, Malm M. Low-power HeNe laser treatment of venous leg ulcers. *Ann Plast Surg.* 1991; 27(6):537–9. [PubMed: 1793238]

11. Malm M, Lundeberg T. Effect of low power gallium arsenide laser on healing of venous ulcers. *Scand J Plast Reconstr Surg Hand Surg*. 1991; 25(3):249–51. [PubMed: 1780722]
12. Sarvis CM. Infected wounds: tipping the balance with STONES. *Nursing*. 2007; 37(7):62.
13. Cannon BC, Cannon JP. Management of pressure ulcers. *Am J Health Syst Pharm*. 2004; 61(18): 1895–905. quiz 1906–7. [PubMed: 15487879]
14. Robson MC. Wound infection. A failure of wound healing caused by an imbalance of bacteria. *Surg Clin North Am*. 1997; 77(3):637–50. [PubMed: 9194884]
15. Laato M, Lehtonen OP, Niinikoski J. Granulation tissue formation in experimental wounds inoculated with *Staphylococcus aureus*. *Acta Chir Scand*. 1985; 151(4):313–8. [PubMed: 2412377]
16. McMinn, R. Volume tissue repair. New York and London: Academic Press; 1969. Skin and subcutaneous tissues.
17. Clark, RAF. Wound repair: overview and general considerations. In: Clark, RAF., editor. The molecular and cellular biology of wound repair. 2. New York: Springer; 1996. p. 3-50.
18. Nussbaum EL, Lilge L, Mazzulli T. Effects of 810 nm laser irradiation on in vitro growth of bacteria: comparison of continuous wave and frequency modulated light. *Lasers Surg Med*. 2002; 31(5):343–51. [PubMed: 12430152]
19. Nussbaum EL, Lilge L, Mazzulli T. Effects of 630-, 660-, 810-, and 905-nm laser irradiation delivering radiant exposure of 1–50 J/cm² on three species of bacteria in vitro. *J Clin Laser Med Surg*. 2002; 20(6):325–33. [PubMed: 12513919]
20. Bayat M, Vasheghani MM, Razavi N, Taheri S, Rakhshan M. Effect of low-level laser therapy on the healing of second-degree burns in rats: a histological and microbiological study. *J Photochem Photobiol B*. 2005; 78(2):171–7. [PubMed: 15664505]
21. Kim K-S, Lee P-Y, Lee J-H, Kim Y-K. Effects of low level laser irradiation (LLLI) with 904 nm pulsed diode laser on *Staphylococcus aureus*. *Laser Therapy*. 1997; 9(3):121–9.
22. Nussbaum EL, Mazzulli T, Pritzker KP, Las Heras F, Jing F, Lilge L. Effects of low intensity laser irradiation during healing of skin lesions in the rat. *Lasers Surg Med*. 2009; 41(5):372–81. [PubMed: 19533760]
23. Lee P, Kim K, Kim K. Effects of low incident energy levels of infrared laser irradiation on healing of infected open skin wounds in rats. *Laser Therapy*. 1993; 5(2):59–64.
24. Hamblin MR, Zahra T, Contag CH, McManus AT, Hasan T. Optical monitoring and treatment of potentially lethal wound infections in vivo. *J Infect Dis*. 2003; 187(11):1717–25. [PubMed: 12751029]
25. Wilson M, Yianni C. Killing of methicillin-resistant *Staphylococcus aureus* by low-power laser light. *J Med Microbiol*. 1995; 42(1):62–6. [PubMed: 7739027]
26. Valencia IC, Kirsner RS, Kerdel FA. Microbiologic evaluation of skin wounds: alarming trend toward antibiotic resistance in an inpatient dermatology service during a 10-year period. *J Am Acad Dermatol*. 2004; 50(6):845–9. [PubMed: 15153882]
27. Biswas L, Biswas R, Schlag M, Bertram R, Götz F. Small-colony variant selection as a survival strategy for *Staphylococcus aureus* in the presence of *Pseudomonas aeruginosa*. *Appl Environ Microbiol*. 2009; 75(21):6910–2. [PubMed: 19717621]
28. Mitik-Dineva N, Wang J, Truong VK, Stoddart P, Malherbe F, Crawford RJ, Ivanova EP. *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* attachment patterns on glass surfaces with nanoscale roughness. *Curr Microbiol*. 2009; 58(3):268–73. [PubMed: 19020934]
29. Abraham M, Venter P, Lues JF, Ivanov I, de Smidt O. Influence of selected antimicrobials on the viability, endotoxicity and lipopolysaccharide composition of *Pseudomonas aeruginosa* in vitro. *Int J Antimicrob Agents*. 2009; 34(5):419–23. [PubMed: 19656661]
30. Zhao G, Hochwalt PC, Usui ML, Underwood RA, Singh PK, James GA, Stewart PS, Fleckman P, Olerud JE. Delayed wound healing in diabetic (db/db) mice with *Pseudomonas aeruginosa* biofilm challenge: a model for the study of chronic wounds. *Wound Repair Regen*. 2010; 18(5):467–77. [PubMed: 20731798]

31. Malic S, Hill KE, Hayes A, Percival SL, Thomas DW, Williams DW. Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid fluorescent in situ hybridization (PNA FISH). *Microbiology*. 2009; 155(Pt 8):2603–11. [PubMed: 19477903]
32. Young S, Bolton P, Dyson M, Harvey W, Diamantopoulos C. Macrophage responsiveness to light therapy. *Lasers Surg Med*. 1989; 9(5):497–505. [PubMed: 2811573]
33. Byrnes KR, Barna L, Chenault VM, Waynant RW, Ilev IK, Longo L, Miracco C, Johnson B, Anders JJ. Photobiomodulation improves cutaneous wound healing in an animal model of type II diabetes. *Photomed Laser Surg*. 2004; 22(4):281–90. [PubMed: 15345169]

Figure 1.

Diagram showing subject flow through the study. Wounds were irradiated or sham-irradiated three times weekly from day 1 to day of euthanization. Semi-quantitative bacterial analysis and photographic wound area measurements were performed three times weekly. On day 8 or 19 animals were euthanized and wound tissues were excised and processed for later blinded analysis.

Figure 2.

Wound area percentages by group from day of wounding (100%) to end of study at day 19.

Data points are means and standard errors of the mean. *ASL<0.05.

Figure 3.

Wound histology at day 8. (A) Sham-irradiated control wound showing surface necrotic tissue (dashed arrow) with early repair tissue composed of acute inflammatory cells and granulation tissue (arrow) (H&E, 4×). (B) The same wound as in (A), showing tissue edema and focal fibrin deposition (dashed arrow), together with polymorphonuclear cells and early granulation tissue (arrow), composed of activated fibroblasts, and few capillaries (H&E, 10×). (C) Irradiated wound (635 nm laser, 20 J/cm²) showing surface necrotic tissue and early initial granulation tissue (arrow) similar to the control wound in (A) (H&E, 4×). (D) The same wound as in (C), showing presence of granulation tissue composed of activated fibroblasts (dashed arrow) adjacent to the scab. Focal collagen deposition by the repair cells is noted (arrow) (H&E, 10×). (E) Irradiated wound (808 nm laser, 20 J/cm²) showing surface necrotic tissue composed of necrotic debris, fibrin and large presence of polymorphonuclear cells (arrow) (H&E, 4×). There is scarce evidence of areas of granulation tissue. (F) The same wound as in (E), showing tissue debris, fibrin and acute inflammation. In the centre of the field a cluster of spherical basophilic microorganisms (arrow) is evident, corresponding to bacterial colonies (H&E, 10×). An enlarged view of the nest of colonies is shown in the insert (H&E, 20×).

Figure 4.

Wound histology at day 19. (A) Sham-irradiated control wound showing complete re-epithelialization (arrow) and mature repair tissue composed of fibroblasts and collagen deposition (H&E, 4×). (B) The same wound as in (A), showing elongated properly aligned fibroblasts (thick arrows) and a large amount of collagen matrix (dashed arrow). A few capillaries and a few chronic inflammatory cells (lymphocytes; thin arrows) are still present (H&E, 10×). (C) Irradiated wound (635 nm, 1 J/cm²) showing complete re-epithelialization (arrow) and mature repair tissue similar to the control wound in (A) (H&E, 4×). (D) The same wound as in (C). Similar to control wound in (B), the dominant cells are elongated fibroblasts together with a large amount of properly aligned collagen matrix (dashed arrow) that exceeds that seen in the control (B). Very few capillaries or lymphocytes (arrows) are present showing evidence of a more mature repair than in (B) (H&E, 10×). (E) Irradiated wound (635 nm, 20 J/cm²) showing re-epithelialization. The superficial dermis reveals repair tissue composed of fibroblasts and early collagen deposition. However, as a difference to (C), several capillaries and groups of inflammatory cells are easily observed (arrows) (H&E, 4×). (F) The same wound as in (E) showing at the lowest part of the field mature repair tissue with fibroblasts that are focally cohesive and arranged in parallel, associated with large amount of collagen (dashed arrow). However, in the upper part of the specimen a more immature repair tissue composed of dis-cohesive fibroblasts and focal stromal edema is observed (thick arrow). Groups of chronic inflammatory cells are also identified (thin arrow) (H&E, 10×). (G) Irradiated wound (808 nm laser, 20 J/cm²) showing incomplete

wound closure with surface necrotic tissue (thick arrow) and underlying granulation tissue with a pronounced inflammatory cell infiltrate and many capillaries (thin arrow) compared with (A) and (C). Stromal edema is also noted (dashed arrow) (H&E, 4×). **(H)** The same wound as in (G) showing immature activated fibroblasts associated with little collagen compared with (B) and (D). However, large numbers of capillaries (thick arrow) and lymphocytes (thin arrow) are identified, and stromal edema (dashed arrow) is intermingled with the granulation tissue. These changes are associated with early repair.

Table 1

Wound area percentage at day 19.

Laser group	Average wound area (%)	Diff. (%)	ASL
Control	21.73	–	–
635 nm, 1 J/cm ²	4.72	–17.01	0.0123 *
635 nm, 20 J/cm ²	7.10	–14.64	0.0208 *
808 nm, 1 J/cm ²	12.48	–9.26	0.0973
808 nm, 20 J/cm ²	18.22	–3.51	0.3163
808 nm, 1 J/cm ² , 3800 Hz (intensity modulated)	10.79	–10.95	0.0682
808 nm, 20 J/cm ² , 3800 Hz (intensity modulated)	4.60	–17.13	0.0105 *

Diff., difference between treatment group and control.

* Significantly different when compared with control (ASL = 0.05)

Table 2

Model fitting by group showing variable estimates, approximate 95% confidence intervals (CI), significant differences and the values of R^2 .

Laser group	Maximum area		Healing rate			Half-heal time		R^2 (%)
	Estimate (%)	95% CI	Estimate β	95% CI	Diff. (95% CI)	Estimate (days)	95% CI	
Control	129	(120, 138)	2.90	(2.36, 3.44)	–	10.4	(9.6, 11.3)	–
635 nm, 1 J/cm ²	137	(125, 148)	3.57	(2.73, 4.41)	NS	8.3	(7.6, 9.0)	NS
635 nm, 20 J/cm ²	136	(125, 146)	3.90	(2.96, 4.85)	1.0 (–0.1, 2.1)**	10.2	(9.6, 10.8)	–1.1 (–2.2, 0.03)**
808 nm, 1 J/cm ²	125	(120, 131)	5.19	(4.36, 6.01)	2.3 (1.1, 3.5)*	11.3	(10.9, 11.7)	0.9 (–0.1, 1.8)**
808 nm, 20 J/cm ²	133	(127, 139)	4.58	(3.77, 5.39)	1.7 (0.6, 2.7)*	12.0	(11.4, 12.6)	1.6 (0.5, 2.6)*
808 nm, 1 J/cm ² , 3800 Hz (intensity modulated)	126	(119, 133)	4.83	(3.85, 5.80)	1.9 (0.7, 3.1)*	10.9	(10.4, 11.5)	NS
808 nm, 20 J/cm ² , 3800 Hz (intensity modulated)	120	(112, 127)	4.93	(3.79, 6.07)	2.0 (0.7, 3.3)*	10.2	(9.6, 10.8)	NS

Diff., difference between treatment group and control; R^2 , Coefficient of determination; NS, Not significant

β Healing rate estimate is derived from the log-log plot and hence it is unit less.

* Significantly different when compared with control ($p < 0.05$).

** Not significant but marginally different: with a larger sample the result might be different.

Table 3

Wound culture results expressed as a percentage of the total number of wounds examined in each group.

Laser group	No growth (%)	Skin flora (%)	Bacterial growth (%)		
			<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
Control	56	24	21.5	5.2	2.3
635 nm, 1 J/cm ²	43	30	26.2	4.8	6.0
635 nm, 20 J/cm ²	60	25	20.6	2.2	0.0
808 nm, 1 J/cm ²	20	45	43.0	4.0	1.4
808 nm, 20 J/cm ²	15	41	53.0	0.0	0.0
808 nm, 1 J/cm ² , 3800 Hz (intensity modulated)	42	17	37.5	6.8	3.4
808 nm, 20 J/cm ² , 3800 Hz (intensity modulated)	65	22.5	12.5	3.8	2.5

Table 4

Bacterial growth analysis showing the *p*-value from Fisher's exact test and the estimated odds ratio (OR) with 95% confidence intervals (CI) for each treatment group compared with control.

Laser group	No growth			Skin flora			<i>S. aureus</i>			<i>P. aeruginosa</i>		
	<i>p</i> -Value	OR	(95% CI)	<i>p</i> -Value	OR	(95% CI)	<i>p</i> -Value	OR	(95% CI)	<i>p</i> -Value	OR	(95% CI)
Control	—	—	—	—	—	—	—	—	—	—	—	—
635 nm, 1 J/cm ²	0.87	1.08	(0.54, 2.15)	1	0.95	(0.28, 3.17)	1	1	(0.52, 1.94)	0.50	0	(0, 5.82)
635 nm, 20 J/cm ²	0.77	1.14	(0.61, 2.14)	1	1.06	(0.51, 2.20)	1	0.94	(0.43, 2.02)	0.44	0.39	(0.04, 2.45)
808 nm, 1 J/cm ²	<0.0001*	0.19	(0.09, 0.38)	0.005*	2.54	(1.30, 5.07)	<0.003*	2.75	(1.39, 5.59)	1	0.79	(0.15, 3.82)
808 nm, 20 J/cm ²	<0.0001*	0.14	(0.06, 0.29)	<0.02*	2.23	(1.14, 4.45)	<0.0001*	4.07	(2.06, 8.26)	<0.06**	0	(0, 1.07)
808 nm, 1 J/cm ² , 3800 Hz (intensity modulated)	<0.08	0.57	(0.30, 1.06)	0.36	0.67	(0.30, 1.46)	0.03*	2.1	(1.05, 4.29)	1	1.21	(0.30, 5.22)
808 nm, 20 J/cm ² , 3800 Hz (intensity modulated)	0.29	1.44	(0.76, 2.73)	1	0.94	(0.45, 1.97)	0.17	0.54	(0.22, 1.26)	0.72	0.59	(0.22, 1.26)

E. coli results are not shown.

* Significantly different when compared with control.

** Not significant but marginally different; with a larger sample the result might be different.

Table 5

Histological principal component scores averaged by group and day.

Laser group	Day 8						Day 19					
	Average comp. II	Diff.	ASL	Average comp. I	Diff.	ASL	Average comp. II	Diff.	ASL	Average comp. I	Diff.	ASL
Control	0.207	–	–	–0.845	–	–	–0.656	–	–	0.988	–	–
635 nm, 1 J/cm ²	0.018	–0.189	0.32	–0.785	0.060	0.40	–0.921	–0.265	0.30	0.830	–0.158	0.32
635 nm, 20 J/cm ²	–0.839	–1.046	<0.06	–0.821	0.024	0.47	0.507	1.164	<0.04*	0.995	0.007	0.49
808 nm, 1 J/cm ²	1.096	0.889	0.03*	–0.446	0.399	<0.03*	0.455	1.111	0.01*	0.890	–0.099	0.22
808 nm, 20 J/cm ²	0.781	0.574	0.11	–0.606	0.239	0.13	0.975	1.631	0.006*	0.048	–0.940	0.004*
808 nm, 1 J/cm ² , 3800 Hz (intensity modulated)	0.252	0.044	0.48	–0.340	0.505	0.12	0.297	0.953	0.02*	0.852	–0.137	0.18
808 nm, 20 J/cm ² , 3800 Hz (intensity modulated)	0.309	0.101	0.41	–0.458	0.387	<0.05*	0.416	1.072	<0.04*	1.153	0.165	0.23

Average Comp., average component; Diff., difference between treatment group and control.

* Significant difference between the treatment group and control.