



Effect of Low-Level Laser Therapy on Bacterial Counts of Contaminated Traumatic Wounds in Dogs

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Abstract

Introduction: The clinical effect of low-level laser therapy (LLLT) on canine wounds is still under debate. The aim of this pilot study was to evaluate the potential influence of LLLT on the bacterial loads of wounds, using two different energy densities or doses of laser light as an adjuvant therapy for traumatic contaminated wound management.

Methods: A prospective, randomized, blinded, placebo-controlled pilot clinical trial was used to evaluate the effect of two different doses of LLLT as an adjuvant treatment of contaminated traumatic wounds on the bacterial load and wound scoring in dogs. Fourteen dogs with traumatic bites or laceration wounds were randomly assigned to one of the three groups. Animals in groups A and B received a dose of LLLT of 6 and 2 J/cm² respectively. Four wavelengths were used simultaneously: 660 nm, 800 nm, 905 nm, and 970 nm. Animals in group C received placebo LLLT. Bacterial burden and clinical wound scores were evaluated.

Results: A statistically significant reduction in the average count of colony forming units was observed in group B (2 J/cm²) when compared to placebo group C. Group B also showed improved wound scores. No clinically adverse effects were observed in the patients treated with LLLT.

Conclusion: LLLT, with the parameters used in this pilot trial, decreased bacterial loads of contaminated wounds in dogs and improved wound scores, especially when using a dose of 2 J/cm². This is the first time the effect of LLLT on bacterial load has been investigated in a clinical setting using traumatic wounds in canine patients.

Keywords: Laser therapy; Wounds; Bacterial count/bacterial load.

Introduction

Low-level laser therapy (LLLT) is a form of photobiomodulation (PBM) that results in a biological effect from a light source. It is a non-invasive physical modality, both versatile and safe, and is becoming a popular and valuable tool for clinicians.^{1,2} Nevertheless, questions concerning some of the biological effects such as its clinical impact on wounds, especially contaminated and infected wounds, remain unanswered.

The mechanism of action of LLLT is based on chromophores, i.e., molecules, such as water, hemoglobin, or cytochrome-C oxidase, which are stimulated when they absorb the energy emitted by the laser in the form of photons.³⁻⁵ When this happens, local blood flow, oxygenation, and metabolism are enhanced, thus increasing the efficiency of the mitochondrial respiratory chain, ATP production, and the oxidative burst of

neutrophils and macrophages.⁶ Intracellular signaling and secondary reactions eventually translate as DNA and RNA synthesis, cell multiplication, release of growth factors and neurotransmitters, and increased collagen production.⁷ This can be summarized as an improvement of tissue metabolism and functionality.⁸

The most documented effects of LLLT are the ones on wound healing, inflammation, and pain. To date, most research has been performed on experimental animals, but a growing body of evidence includes clinical studies in humans and a few studies in domestic animal species.⁹⁻¹²

Bacterial proliferation in wounds can negatively affect healing and has a significant impact on treatment costs.^{13,14} This, together with growing concerns about antibiotic resistance, explains why there is an urgent and increasing need for alternative treatments. Authors who have focused their research on the effect of LLLT on

bacteria have performed either in vitro experiments^{15,16} or in vivo studies using experimental animals.¹⁷⁻¹⁹ Results have varied depending on the methodology, especially the irradiation and treatment parameters such as dose (J/cm²), wavelength (nm), and power density (W/cm²).²⁰ To the authors' knowledge, no clinical trial using laser therapy for canine patients has been published that concerns acute traumatic, contaminated/infected wounds in particular. The aim of this pilot study was to evaluate the potential influence of LLLT on the bacterial loads of wounds, using two different doses or energy densities of laser light as an adjuvant therapy for contaminated wound management.

Materials and Methods

Study Design

A prospective, randomized, blinded, and placebo-controlled pilot clinical trial was performed in three parallel groups. This study received the approval of the Ethics and Animal Experimentation Committee of the two universities, located in Madrid, Spain. Patient owners were given information about the protocol to be followed and they signed an informed consent form to include their dogs in the study.

Simple randomization was used to allocate each patient to one of the groups (A, B or C) using the statistical software package Stata 13.0 (Stata Corp., College Station, TX, USA). Patient owners, the colony forming units (CFUs) evaluator, and the data analyst were blinded to the treatment group. The only person who knew which group patients belonged to was the clinician who applied the laser treatment.

Sample Population

Patients were included in the study after meeting inclusion criteria. Dogs presenting with traumatic wounds with signs of contamination or infection and no previous treatment were considered. According to the Center for Disease Control and Prevention, contaminated wounds include open, fresh, accidental wounds as well as incisions with acute non-purulent inflammation²¹; patients with clinical signs of infection or some degree of devitalized tissue were also included. The initial inclusion of the patients was reassessed 24 hours later, after laboratory confirmation of wound contamination.

Laser Treatment

The patients received their assigned treatment on days 0 and 1, according to the experimental group they had been allocated to. Two samples were obtained from each patient for their microbiological analysis: the first sample was taken on day 0, before the first laser treatment, and the second sample on day 2 (Table 1).

Groups A and B received an energy dose of 6 J/cm² and 2 J/cm² respectively. Group C received the placebo laser

treatment (Table 2). The therapeutic laser was a class IV type, a K-laser[®] Cube 4 (Eltech K-Laser, Treviso, Italy), which was tested for optical output before and after the end of the trial. This device allowed us to use different frequencies, as well as continuous wave (CW), over the course of the treatment. In particular, an initial CW phase was followed by three pulsed light phases at 500 Hz, 5000 Hz and 20 000 Hz. The device delivered 25% of the energy using CW, and the remainder was equally divided between the pulsed phases. In the pulsed mode, the device had a 50% duty cycle, which meant that while the average power was kept at 2 W, the peak power was 4 W. Treatments were performed in non-contact mode, and the diameter of the laser beam over the patient was adjusted to 10 cm². Therefore, the average power density was 0.2 W/cm². Four wavelengths were used simultaneously: 660 nm, 800 nm, 905 nm, and 970 nm. Treatment time was adapted to the size of the treatment area and to the applied dose. The device also had a placebo mode that produced a 4 mW red light and a beeping sound similar to the treatment mode. This placebo mode was used for the control group.

The treatment area for each wound included the wound itself plus a margin of 2 to 4 cm of healthy tissue. The area to be treated and sampled was delimited using a series of pre-cut sterile silicone templates (Figures 1 and 2) of 25, 50, and 100 cm². This allowed us to calculate the bacterial load for each patient in a uniform way.

Standard Management

Once irradiated, wounds were lavaged with Lactated Ringer's solution, using 50 cc for each cm² of the wound, with an average pressure of 300 mm Hg. Pressure control was achieved with a pressure infuser bag. Both the laser treatment and wound management were repeated on day 1 of the experiment.

For the more severe or complicated wounds, the

Table 1. Study Design: Timeline of Interventions

↓	→→→→→→→→		
↓	Day 0	Day 1	Day 2
↓	Microbiology sample named "before treatment"(BT)		Microbiology sample named "after treatment" (AT)
↓	LLLT	LLLT	LLLT
↓	Wound management	Wound management	Wound management
↓	Antibiotic		

Table 2. Treatment Parameters for Each Group

Group	Dose	Wavelength	Frequency	Power
A	6 J/cm ²	660 nm	CW	2 W Pa 4 W Pp
		800 nm	50 Hz	
		905 nm	5000 Hz	
B	2 J/cm ²	970 nm	20000 Hz	
C	Placebo: 5 mW, 660 nm			

CW, continuous wave; Pa, average power; Pp, peak power.

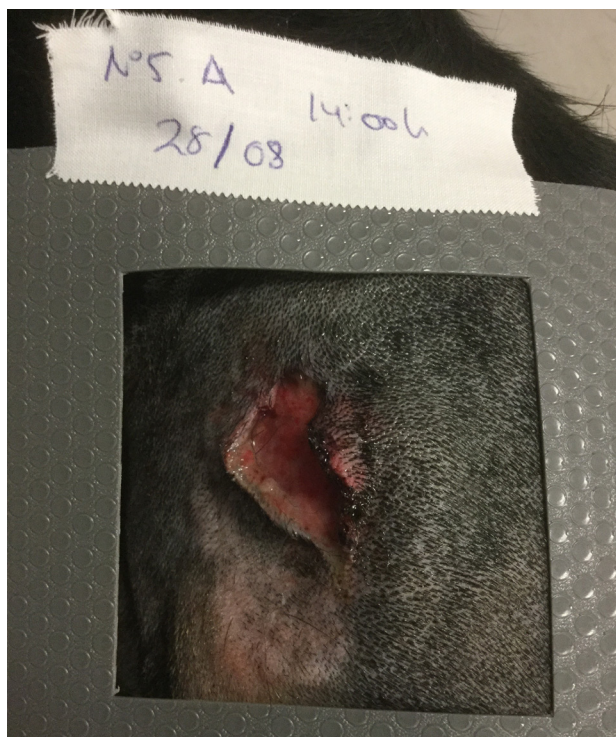


Figure 1. A sample of the pre-cut silicone templates of different sizes used in the study.

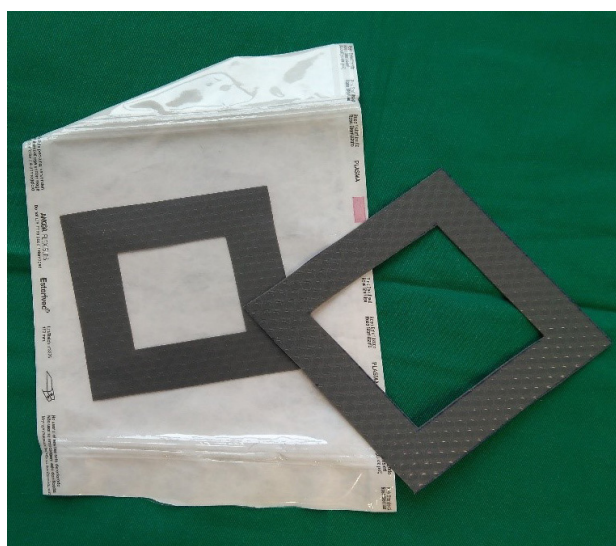


Figure 2. Silicone template over a traumatic wound, limiting the area to be treated and subsequently sampled for bacteriology.

patients were sedated on day 0 for treatment and sampling whenever it was clinically indicated.

All patients received standardized medical treatment after laser therapy. This included a subcutaneous injection of a third-generation cephalosporin dose (cefovecin, 8 mg/kg of body weight), so the only treatment difference among groups was the LLLT dose. Each dog wore an E-collar for at least the first 72 hours to prevent wound licking.

The patients were rechecked on a daily basis for the first

72 hours, and subsequent visits were planned according to each patient's needs until wound healing was complete. All data concerning clinical progression were recorded.

Microbiology

The samples were collected from the wounds using a sterile cotton swab, previously immersed in 1 cc of phosphate buffered saline. Once in the laboratory, and starting with 0.1 cc of the original sample, three progressive dilutions were performed, each ten times more diluted than the previous dilution. Then, 0.1 cc of each of the four dilutions was cultured using both Columbia blood agar and McConkey agar culture plates, which were incubated in aerobic conditions for 24 hours at 37°C and read after incubation. Plates containing between 30 and 300 CFUs were selected as reading plates. The bacterial load (CFUs/cm²) in each wound of each patient was calculated from these reading plates.

Each morphologically distinct strain was isolated and stored at -40°C. The identification of bacterial species was performed at the Centre for Veterinary Sanitary Surveillance of the Complutense University of Madrid (VISAVET), using mass spectrophotometry, with Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF).

Data Collection

The Neperian logarithm of CFUs (LNCFUs) after treatment (AT) in the Columbia blood agar and McConkey agar culture plates was selected as the outcome variable. Independent variables were classified into clinical variables (breed, age, reproductive status, and body weight), wound-specific variables (number of wounds, origin, and area), treatment-specific variables (treatment area, experimental group, additional treatments, and time between first and second sampling) and microbiological variables (LNCFUs before treatment).

Wound Scoring

The pictures of the wounds were taken on day 0, day 1 and day 2 before laser therapy. Wound scoring was performed in a blinded way by two evaluators with more than 15 years of experience in soft tissue surgery. The scoring system used was a modification from the scale proposed by Falanga et al.²² In this way, scoring could range from 2 to 12 since at least a positive culture was necessary on day 0 for the patient to be included in the study (Table 3).

Statistical Analysis

Continuous variables are presented as means and standard deviations, or medians and interquartile ranges. Categorical variables are expressed as frequencies and percentages.

To analyze the influence of different variables in the LNCFUs AT, a multivariate linear regression model was

Table 3. Wound scoring scale

Clinical Parameter	0	1	2
Exudate amount	Severe or purulent type	Moderate	None/mild
Edema or swelling	Severe	Moderate	None/mild
Peri-wound dermatitis	Severe	Moderate	None or mild/minimal
Hematoma	Severe	Moderate	None/minimal
Necrotic or potentially non-viable tissue	>25% of wound surface	0-25%	None
Bacterial culture	Positive		Negative

created. Variables with $P < 0.100$ were considered clinically relevant and included in the multivariate linear regression analysis. The final model was built with a stepwise forward selection and backward elimination technique. The significance levels for forward selection were $P < 0.050$ and for backward elimination were $P < 0.100$.

The results are shown as the ratio of estimated means for each variable. In the quantitative variables, this ratio expresses the relative increase of the mean per each one-unit increase in the independent variable. In the qualitative variables (groups), the ratio expresses the average increase of patients treated in group A or B, while using group C as the reference group. The ratio is obtained by exponentiation to base e of the coefficient of the linear regression model (β).

All tests were two-sided, and differences were considered statistically significant at $P < 0.025$. Bonferroni adjustments were used to correct for multiple comparisons. Statistical analyses were performed using Stata software version 13.0 (Stata Corp) and the Statistical Package for the Social Sciences (SPSS 15.0, IBM, NY, USA).

For wound scoring, inter-rater agreement was evaluated using Cronbach's alpha coefficient. Total scoring for each wound each day was the average of both evaluators' scoring. Total scores for days 0 and 2 were compared using a t-test for independent measures for groups A, B and C.

Results

Fourteen canine patients initially met the inclusion criteria. None of these animals had to be excluded since bacterial contamination was confirmed in all cases. Four patients were randomly assigned to group A, while groups B and C had five patients each. Group A included two Jack Russell Terriers (a male and a female, both intact), a Burgos Pointer (spayed female) and a Bodeguero Andaluz (intact male). The median age in group A was 5 years (1.2–7.5 years) and the median weight was 9.5 kg (10–15 kg). Group B included a Greyhound (spayed female), a Jagdterrier (spayed female) and three mixed-breed dogs (two spayed females and one intact male) with a median

age of 8 years (4–9 years) and a median weight of 15 kg (8–23 kg). Group C included two Greyhounds (both spayed females), a German Shepherd (neutered male), an Ibizan Hound (neutered male) and a mixed-breed spayed female with a median age of 7 years (5–10 years) and a median weight of 23.6 kg (20–32 kg). Three patients from group A had bite wounds. The fourth patient presented with a traumatic cut. Groups B and C included three patients each with bite wounds and two with traumatic cuts. Each patient had between one and four wounds (average 1.5 wounds), but only one wound from each patient was included in the study. In group A, laser treatment and sampling were done over 25 cm² for three patients and over 100 cm² for the fourth dog. In group B, three patients were treated and sampled from 25 cm², and two patients from 50 cm². Group C included three patients who were treated and sampled from 25 cm² and two patients from 100 cm².

Sedation was performed in ten patients on day 0 according to their needs for appropriate wound management. None of the patients had to be sedated again or undergo further surgical management for the treated wound, and no dog needed additional antibiotic therapy during the days of the trial.

Results from bacterial identification are shown in Table 4. Among the 77 isolated bacterial strains, 8 could not be identified due to lack of survival related to problems during storing, defrosting and /or sample processing.

Statistical analysis did not reveal any influence of the clinical variables or wound-specific variables over the number of CFUs AT in Columbia blood agar. Conversely, we observed a relative decrease of 79% (mean ratio 0.21) and 93% (mean ratio 0.07) was observed in the average growth of LNCFUs in Columbia blood agar from day 0 to day 2 for groups A and B respectively. The analysis was adjusted by the time between samples and by the number of LNCFUs in Columbia blood agar. This change was statistically significant in group B ($P < 0.050$). The mean of LNCFUs after treatment increased ($P = 0.018$) 1.47 times the mean of the initial number of LNCFUs in Columbia blood agar, adjusted by the time between the samples and the group. The mean of LNCFUs after treatment increased ($P < 0.001$) 3.76 times per additional hour between the samples, adjusted by the LNCFUs before treatment and the group. This linear regression model is shown in Table 5. No statistically significant results were retrieved from the McConkey agar plates (Table 6).

No adverse effects were observed in the patients treated with LLLT. Cronbach's alpha coefficient between the two evaluators was 0.793. There were no differences in wound scores on day 0 between the groups. All patients improved their wound score from day 0 to day 2. In group A (6 J/cm²), the difference between means from day 0 to day 2 was 1.63 (95% CI: 0.06–3.31; $P = 0.056$). In group B (2 J/cm²), the difference between means from day 0 to day 2

Table 4. Microbiology Results: Bacterial Identification

No. bacteria	Patient	Culture	Bacteria	Gram
1	1	BT	Staphylococcus pseudintermedius	+
2	1	BT	Aerococcus viridans	+
3	1	BT	Neisseria zoodegmatis	-
4	1	AT	Bacillus licheniformis	+
5	1	AT	Escherichia coli	-
6	1	AT	Escherichia coli	-
7	1	AT	Staphylococcus pseudintermedius	+
8	1	AT	Staphylococcus pseudintermedius	+
9	2	BT	Aerococcus viridans	+
10	2	BT	Neisseria zoodegmatis	-
11	2	BT	Neisseria weaveri	-
12	2	AT	Staphylococcus pseudintermedius	+
13	3	BT	Rothia nasimurium	+
14	3	BT	Neisseria zoodegmatis	-
15	3	BT	Actinobacillus pleuropneumoniae	-
16	3	AT	Staphylococcus spp.	+
17	3	AT	--	--
18	3	AT	Staphylococcus spp.	+
19	4	BT	Streptococcus sp.	+
20	4	BT	Staphylococcus aureus	+
21	4	AT	Streptococcus sp.	+
22	4	AT	Staphylococcus aureus	+
23	4	AT	Staphylococcus hominis	+
24	5	BT	Micrococcus spp.	+
25	5	BT	Streptococcus sp.	+
26	5	BT	Rothia nasimurium	+
27	5	AT	--	--
28	5	AT	--	--
29	5	AT	Acinetobacter baumannii	+
30	5	AT	Kocuria rhizophila	+
31	5	AT	Micrococcus luteus	+
32	6	BT	Staphylococcus aureus	+
33	6	BT	Staphylococcus pseudintermedius	+
34	6	AT	Staphylococcus pseudintermedius	+
35	6	AT	Staphylococcus spp.	+
36	7	BT	Pasteurella multocida	-
37	7	BT	Actinobacillus pleuropneumoniae	-
38	7	BT	Pasteurella canis	-
39	7	AT	Neisseria flavescens	-
40	7	AT	--	--
41	8	BT	Neisseria canis	-

Table 4. Continued.

No. bacteria	Patient	Culture	Bacteria	Gram
42	8	BT	Neisseria flavescens	-
43	8	AT	Staphylococcus warneri	+
44	8	AT	Staphylococcus pseudintermedius	+
45	9	BT	Staphylococcus epidermidis	+
46	9	BT	Micrococcus spp.	+
47	9	BT	Acinetobacter spp.	+
48	9	BT	Neisseria zoodegmatis	-
49	9	AT	Enterococcus faecium	+
50	10	BT	Neisseria weaveri	-
51	10	BT	Bergeyella zoohelcum	-
52	10	BT	Streptococcus hyointestinalis	+
53	10	BT	--	--
54	11	BT	Acinetobacter spp.	+
55	11	BT	Staphylococcus spp.	+
56	11	BT	Neisseria zoodegmatis	-
57	11	AT	Bacillus sp.	+
58	11	AT	Staphylococcus delphini	+
59	11	AT	Staphylococcus epidermidis	+
60	11	AT	Streptococcus salivarius	+
61	12	BT	Staphylococcus hominis	+
62	12	BT	Corynebacterium afermentans	+
63	12	BT	Mannheimia haemolytica	-
64	12	BT	Corynebacterium tuberculostearicum	+
65	12	AT	Staphylococcus hominis	+
66	12	AT	Arthrobacter sp.	+
67	12	AT	--	--
68	13	BT	--	--
69	13	BT	Acinetobacter spp.	+
70	13	BT	--	--
71	13	AT	Staphylococcus spp	+
72	14	BT	Bergeyella zoohelcum	-
73	14	BT	Acinetobacter spp	+
74	14	BT	Neisseria zoodegmatis	-
75	14	AT	Staphylococcus spp	+
76	14	AT	Acinetobacter spp	+
77	14	AT	Acinetobacter spp.	+

BT, before treatment. AT, after treatment.

was 2.9 (95% CI: 1.56-4.2; $P=0.002$). In group C (placebo), the difference between means from day 0 to day 2 was 2.00 (CI 95%, 1.39-2.61; $P<0.001$). When the results on day 2 were compared for different groups, the difference between means between groups B and C was 1.30 (95% CI: 0.67-1.93, $P=0.001$); the difference between means between groups A and C was 1.00 (95% CI: 1.29-1.33, $P=0.853$), and the difference between means between

Table 5. Linear Regression Model of LNCFUs AT in Columbia Blood Agar Plates in Relation to LNCFUs BT, Time Between Samples, and Treatment Groups

Columbia Blood Agar (LNCFUs AT)		Coefficient (β)	Mean Ratio	95% CI Lower	95% CI Upper	P Value
Columbia Blood Agar (LNCFUs BT)		0.38	1.47	0.07	0.70	0.018*
Time between samples		1.33	3.76	0.80	1.85	<0.001*
Laser	Group C	1				
	Group A	-1.55	0.21	-3.91	0.82	0.210
	Group B	-2.73	0.07	-4.36	-1.09	0.001*

LNCFUs AT, Neperian logarithm of colony-forming units after treatment; LNCFUs BT, Neperian logarithm of colony-forming units before treatment.

*Statistically significant

Table 6. Linear Regression Model of LNCFUs AT in McConkey Agar Plates in Relation to LNCFUs BT, Time Between Samples, and Treatment Groups

McConkey Agar (LNCFUs AT)		Coefficient (β)	Mean Ratio	95% CI Lower	95% CI Upper	P Value
McConkey Agar (LNCFUs BT)		-0.003	1.00	-0.051	0.045	0.886
Time between samples		-0.003	1.00	-0.025	0.019	0.780
Laser	Group C	1				
	Group A	-0.003	1.00	-0.092	0.086	0.939
	Group B	-0.014	0.99	-0.102	0.074	0.731

LNCFUs AT, Neperian logarithm of colony-forming units after treatment; LNCFUs BT, Neperian logarithm of colony-forming units before treatment.

groups A and B was 1.20 (95% CI: 2.44-0.04, $P=0.056$).

Discussion

In the canine species, the use of PBM has been described in individual clinical cases²³ and in studies with clean surgical wounds,^{12,24-26} but this is the first pilot clinical trial in canine patients with traumatic wounds. To the best of the authors' knowledge, it is also the first one with canine patients, in which PBM with infrared light is used as an adjuvant therapy to fight tissue infection, an effect that has been described in experimental studies in rats²⁷⁻³¹ and rabbits.³² More recently, a different type of PBM, using visible fluorescent light and a transducer gel was used in the treatment of canine pyoderma.³³ In human patients, some studies report the use of LLLT to treat infected wounds in skin and mucosas.³⁴ Investigating new tools to fight infection is a priority nowadays since antibiotic resistance has become a global public health threat with a significant impact on mortality, morbidity and the financial costs of medical care.^{13,14}

The main result from our pilot clinical trial is that LLLT, or PBM, decreased the average number of bacteria in the post-treatment blood agar plates when compared to the placebo group. Although there is a difference between the bacterial load and tissue infection, a higher bacterial load, especially from some pathogenic bacteria, can contribute to the development of tissue infection and interferes with wound healing.^{35,36} The bacterial load was used as the main diagnostic criteria to objectively diagnose infection, although these criteria were later modified.³⁵ The high positivity of bacteriology and the presence of polymicrobial flora were in accordance with what has been described in the literature, especially concerning bite wounds.^{36,37}

The effect of PBM on infected tissue can be explained

by the effect on bacteria themselves, i.e., their survival and multiplication, by the effect in the host's immune response, or by a combination of both. Previous studies have contributed to answers to the question of what type of effect the treatment has on bacteria using both in vitro and in vivo research studies, but the lack of standardized parameters over a variety of microorganisms has produced uneven conclusions.

Some in vitro studies show an antibacterial effect,^{15,18,38} and other studies describe no effect or even an increase in bacterial multiplication.³⁹⁻⁴¹ One of the direct antibacterial mechanisms that has been proposed is based on the action of the light on the pathogen's endogenous porphyrins.^{6,42} These porphyrins are thought to be stimulated by laser light, setting off a release of free radicals, which then damages cytoplasmic protein membranes and DNA. This bactericidal or bacteriostatic effect occurs when cellular damage overcomes repair mechanisms and microbial metabolism is inhibited. Some authors describe differences in efficacy when various pathogens are irradiated.^{15,43,44} Gram-negative bacteria seem to be more vulnerable. This could be due to differences in the peptidoglycan wall. This wall is thicker in gram-positive bacteria and therefore could account for more limited photon penetration.^{15,41} In the present study, however, McConkey agar plates, which favor Gram-negative bacteria, did not show a decreased bacteria load in response to LLLT, while Columbia blood agar plates did.

Our results are in accordance with those of other authors that have described an inhibition of bacterial growth on rat models of wounds,⁴⁵ burns^{19,27} and osteomyelitis.²⁸ In addition to the potential direct effect on bacterial survival,^{39,41,43} several phenomena in the host's response are modulated by laser therapy and may contribute to the indirect antimicrobial effect, such as the increase in local

blood flow and oxygenation.⁴⁶⁻⁵¹ This facilitates the arrival of white blood cells and an improvement in the patients' immune responses.^{52,53}

The difference between groups in the present study was the randomly assigned laser treatment, but otherwise patient management was similar. Wound lavage and management were standardized for all patients as suggested by Balsa and Culp.⁵⁴ Cefovecin was selected as the antibiotic treatment because of its broad spectrum and its long-lasting and constant therapeutic levels in tissues after a single dose.⁵⁵ This eliminated potential fluctuations in antibiotic concentration below the minimum inhibitory concentration values for the pathogens, as well as lack of treatment compliance by patient caretakers.⁵⁶ The heterogeneity of laser technical and treatment parameters used in LLLT studies is a major challenge when comparing research results. This pilot study was performed in such a way that, dose (0, 2 or 6 J/cm²) was the only variable between the experimental groups. These doses (2-6 J/cm²) are similar to what is done in clinical practice with acute wounds⁵⁷ and within the range of other studies for wound management.^{24,26,58}

In this pilot clinical trial, the results favored the use of a lower dose (2 J/cm²). This is in accordance with the recommended dose for acute wounds in clinical practice (1-4 J/cm²), although both our 2 J/cm² and our 6 J/cm² doses produced a decrease in CFU counts. An inhibition of *Staphylococcus aureus* growth with a dose of 5 J/cm² was previously reported by Gomes et al³⁰ and Santos et al²⁹ and also with lower dosages (1.2 and 2.4 J/cm²) on second-degree skin burns in rats according to Bayat et al.²⁷ An inhibition of bacterial counts after LLLT was also described in an osteomyelitis model in rats by Kaya et al,²⁸ who used higher dosages in their trial (7.64-22.9 J/cm² vs. 2-6 J/cm²) although with lower power density (0.127 W/cm² vs. 0.2 W/cm²).³⁸ On the contrary, Araujo et al did not observe any antibacterial effect on a model of septic arthritis in rats, but it should be noted that although a similar dose was used (2 J/cm²), the intra-articular penetration of the 660-nm wavelength that was used was very limited.⁵⁹ Vasheghani et al did not observe an antimicrobial effect on third-degree burns in rats either, but in their case, a much lower dose (0.396 J/cm²) was used.⁶⁰ The use of doses above 10 J/cm² for wounds remains controversial. Some authors report a lack of stimulatory effect when higher doses are used, or even an inhibitory effect for in vitro cell metabolism,^{61,62} but others have reported positive effects in vivo with 30 J/cm².⁶³ In clinical practice, doses in the 10-30 J/cm² range are sometimes used, especially in chronic wounds.

The selected power was within the usual range for acute wound treatment with class IV lasers.^{12,57} Power density was kept to an average of 0.2 W/cm² and this is within the 0.1-0.5 W/cm² of the recommended range set for wounds by the World Association for Laser Therapy. This

value was also congruent with that used by De Sousa et al in their 2016 study.³⁸ While a certain amount of power density is needed to achieve a biological effect of PBM, an excessive power density can cause discomfort and tissue damage through unwanted photothermal effects. While the power of a laser can be focused on a power density that can kill bacteria due to a thermal effect, the aim of LLLT, as a form of PBM, is to modulate the metabolism of the tissue to enhance the process of healing. This has to be achieved at physiologic temperatures, which are safe and comfortable for the patient. Wavelength also has a direct influence on microbial chromophores and has been proposed as a potential mechanism for the antibacterial effect of light. Although blue light (380-475 nm) lacks some of the biological effects of red and infrared light on inflammation and wound healing, its antimicrobial properties have often been reported.^{38,64,65} These antimicrobial effects are still under discussion in the case of the red and infrared light used in PBM. We decided to combine all four wavelengths available in this device (660, 800, 905, and 970 nm) to reproduce what is usually done in practice when such combinations are available and to maximise the spectrum of cellular targets, similar to that done by Renwick et al.²

Our device allowed a pulsing range from 1 to 20000 Hz, and we used a combination of CW, low, medium and high frequencies. The use of CW has been most commonly reported in the literature,^{28,41,66} but other authors have also used pulsed light up to 20000 Hz.^{39,67} The significance of pulsing is still controversial, although it does seem to be of clinical benefit.⁶⁸

Our results with wound scores seemed to be consistent with a progression in time and with the microbiological changes between the groups. Improved wound healing was also described in other clinical studies. Wardlaw et al. reported improved wound healing in canine patients treated with PBM after hemilaminectomy surgery¹² using 8 J/cm² and a combination of 850 nm and 670 nm. Perego et al,²⁵ using 808 nm, described visible clinical improvement of post-operative healing of elective ovariectomy surgical wounds; although the global difference was not statistically significant, there was a statistically significant reduction in the exudate in the areas that had been treated. On the other hand, two other studies using dogs subjected to experimental surgical wounds found no difference in wound healing. Their parameters were very different though; while Kurach et al²⁴ used a class II laser at a wavelength of 635 nm, Gammel et al²⁶ used a class IV laser set at 2-3 W, a 980-nm wavelength and a dose of 5 J/cm².

The present trial included a limited number of dogs with a variety of wound types, and this could limit and challenge the interpretation of our results. Further, the different etiopathogeneses of the investigated wounds could have influenced a different clinical progression, even under the same therapeutic protocol. Breed, sex,

reproductive status, or body weight did not seem to influence the results, and the difference in bacterial growth achieved statistical significance.

Although the results of the clinical progression scores of the wounds were consistent with progression in time and with the reduction of the bacterial load, these results are limited by the low number of cases, the short time lapse during which wounds were scored and by the scoring method itself.

In the future, the inclusion of one or more groups in which antibiotic treatment would not be provided, anaerobic cultures and a longer period of scoring and microbiological sampling could retrieve more results and help to interpret our findings.

In summary, LLLT as an adjunctive treatment was able to decrease the bacterial burden in the wounds in both treatment groups; the effect was more significant with a dose of 2 J/cm². The results from this pilot clinical trial are specific to the described treatment parameters and methodology, but they reinforce the growing scientific background supporting the clinical use of LLLT. Furthermore, they constitute a promising step forward in finding new strategies to treat infected wounds.

Ethical Considerations

This study received the approval of the Ethics and Animal Experimentation Committee of both Complutense University and Alfonso X el Sabio University, Madrid, Spain.

Conflict of Interests

MSR has received honoraria for K-Laser and AVANVET-sponsored speaking engagements. None of these circumstances has had an inappropriate influence on the content of this paper. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of this paper. The therapeutic laser used in this study was loaned to us by AVANVET, the Spanish distributor of K-Laser, who did not play any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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