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ORIGINAL ARTICLE

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Monitoring of bactericidal action of laser by in vivo imaging of bioluminescent *E. coli* in a cutaneous wound infection

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Abstract The worldwide rise in antibiotic resistance necessitates the development of novel antimicrobial strategies. This study aimed to evaluate the bactericidal action of an 810-nm diode laser in a cutaneous wound infection. An *Escherichia coli* strain was transformed with a shuttle vector (pRB474) containing firefly luciferase gene from *Photinus pyralis* resulting in a bioluminescent phenotype. Because firefly luciferase is an enzyme and as such is prone to inactivation at elevated temperature, the first phase has consisted in evaluating in vitro the effect of temperature elevation (30, 40, 50, and 60°C for 2 min) on bacteria bioluminescence. The second phase was performed in vivo. Two full-thickness circular, 14-mm diameter wounds (control and laser-irradiated) were induced on rats. Wound infection was carried out using a suspension (50 µl PBS) containing $5x10^7$ cells of bioluminescent *E. coli* (10^9 cells/ml). Thirty minutes later, light irradiation was performed with an 810-nm diode laser (P=10 W, ∅=1.4 cm, fluence: 130, 195, and 260 J/cm^2). Temperature was measured within each wound with a noncontact infrared thermometer. Light emission of the bioluminescent bacteria was monitored in vivo by a bioluminescence imaging system before and at 4, 8, 24, and 48 h after laser irradiation. In vitro, bacteria bioluminescence is not affected when temperature is maintained at 50°C for 2 min. In vivo, bioluminescence imaging showed that at 4 h, the viability of *E. coli* was reduced when compared to the control (CTRL) group (p<0.01). This observation was confirmed at 8 h (p<0.001), at 24 h (p<0.001), and finally at 48 h (p<0.001). Loss of viability of *E. coli* depends on laser fluence. At 48 h, bioluminescent bacteria were not detected (100% loss of viability) in the wound irradiated at 260 J/cm^2. For this fluence, the temperature reached 45°C at the end of the irradiation. This study confirms previous observations on the bactericidal effect of diode lasers. Because a progressive desiccation of the superficial dermis is usually observed when using laser irradiation, the hypothesis that laser irradiation dries out the wound making the wound an inhospitable place for bacteria is much more relevant than a direct effect of infrared light on chromophores inside bacteria. This is confirmed by the fact that in this latter case, one would expect an immediate drop in luminescence followed by an increase as the surviving bacteria started to divide and repopulate the wound. However, the exact mechanism deserves further studies. This study points out the advantage of using bioluminescence imaging to evaluate laser for the treatment of acute infections in vivo, nondestructively, and noninvasively.

Keywords Bioluminescence · *Escherichia coli* · Firefly luciferin · Luciferase · Laser · Wound infection

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Introduction

Laser irradiation, apart from conventional methods, has been shown to have potential to eliminate bacteria. The antimicrobial effect of the Nd:YAG laser has been demonstrated in several in vitro studies. Schultz et al. [1], with a high power (120 W) Nd:YAG laser, reduced the viable counts of bacteria in aqueous suspension in microtiter plates. Using the same wavelength, Grönqvist et al. [2] have shown *Staphylococcus epidermidis* growth inhibition on agar plates. Ward et al. [3] observed a bactericidal action of high power Nd:YAG laser light on transparent and turbid *Escherichia coli* bacterial suspensions. Similarly, Lee and Pinheiro have used a CO_2 laser to treat bacteria-infected cutaneous wounds [4].

Optical techniques have recently been proposed for rapid monitoring of the effectiveness of therapeutic strategies used in wound infection. The method of optically monitoring bacterial numbers and viability, in real time, in living animals by use of genetically engineered bacteria

that emit luminescence, together with ultrasensitive photon-counting cameras, has been demonstrated in several models [5–9]. Quantification of the luminescence images can determine, in real time, the extent of infection in living animals and thereby can provide both temporal and spatial information about the labeled bacteria and their metabolic activities. Using this technique, we have recently demonstrated that antibiotic effects can be detected directly, nondestructively, and noninvasively in vivo in a cutaneous wound model [10].

This study aimed to evaluate the bactericidal action of an 810-nm diode laser in a cutaneous wound infection using a bioluminescence imaging technique. Because firefly luciferase is an enzyme and as such is prone to inactivation at elevated temperature, in vitro studies were performed first to evaluate the potential role of heat on bacteria bioluminescence.

Materials and methods

Bacterial strain

A relatively nonpathogenic strain of *E. coli*, which lacks virulence factors necessary to cause invasive infection, was used [11]. *E. coli* TOP10F′, a facultative anaerobe (ref C615-00) in the normal intestinal flora of humans and animals [12], was obtained from Invitrogen (Invitrogen SARL, Cergy Pontoise, France) and routinely grown at 37°C. Furthermore, TOP10F′ is a recombination negative strain designed for stable replication of high copy number plasmids [13]. Bioluminescent *E. coli* was generated by transforming the strain TOP10F′ with a firefly luciferase gene (Promega (E1781) using the shuttle vector pRB474 [14]. pRB474 was introduced into the cells by electroporation as previously described [10]. Transformants were plated onto agar plates containing 50 μg /ml of ampicillin and grown for 1 day at 35°C. Bioluminescent colonies were selected using a luminometer (Lumat LB 9501 Berthold, Berthold France SA, Thoiry, France).

In vitro studies

One hundred microliters of fresh Luria Broth containing the bioluminescent bacteria in growth phase (OD_{600}: 0.6) was placed in a cuvette and was incubated for 2 min at 30°C (n=4), 40°C (n=4), 50°C (n=4), and 60°C (n=4) using a thermostated circulating bath (Ministat, Huber, Rimsting, Germany). The temperature was controlled inside the cuvette using a 0.2-mm thermocouple probe (Mini-Hypodermic Thermocouple Probe Model HYP-0, Omega, Guyancourt, France). The aliquots were transferred to a 96-well black sides plate for bioluminescence imaging up to 100 h postincubation. The temperature of the bioluminescence imaging chamber was maintained at 30°C. No heated aliquots (n=4 maintained at 20°C) were used as control. Before each measurement, 10 μl of substrate solution (1 mM of D-luciferin to 0.1 M phosphate–citrate buffer) was added to the well. Serial dilution and plating were used to determine the number of live bioluminescent bacteria present in the sample after heating at different temperatures at 4 and 8 h. Counts produced after plating of serially diluted product were reported as "colony-forming units" (cfu) as described by Jett [15].

Animals

All animal experiments conformed to the Ministère de l'Agriculture et de la Forêt Resolution on the use of animals in research and were approved by the Subcommittee on Research Animal Care of the Lille Medical University (protocol 2003-35).

Male Sprague–Dawley rats (Charles River France, Les Oncins, France), weighing between 200 and 300 g, were used for this study. The back of the rat was shaved, and a depilatory cream was applied to remove any remaining hair. Two full-thickness circular, 14-mm diameter wounds were created using surgical scissors on the back of the rats while they were under general anesthesia (140 mg of ketamine per kilogram of body weight and 3 mg of chlorpromazine per kilogram). The positions of these wounds were 4 and 9 cm caudal, respectively, to the ears and placed on the midline. A Teflon chamber similar to the chamber developed by Balazs was applied around each wound and was glued into the edge of the skin with Histoacryl (B. Braun Surgical GmbH, Melsungen, Germany) and sutured (Ethicon 4-0 sutures) [16]. A sterile glass window (GF-C Whatman) was placed inside the chamber to protect the wound against other infections.

Bioluminescence imaging system

Light emission of the bioluminescent bacteria was detected in vivo by a bioluminescence imaging system (IVIS 50, Xenogen, Alameda, USA). The animal was placed inside a light tight chamber on a heated moveable platform. The field of view was adjusted to measure simultaneously the control and the laser-irradiated wounds.

Two images were taken on each animal. The first image was a 0.2-s exposure of the animal illuminated by lights located in the top of the imaging chamber. This image was referred to as a "photographic image" and was displayed as a grayscale image. The second image was a 15-s exposure of the animal taken in darkness to record low-level luminescent emission. This image was referred to as a "luminescent image" and was displayed as a pseudocolor image overlaid on the photographic image. This procedure and the acquisition parameters were kept constant for all measurements. A color bar shows the relationship between the pseudocolors of the luminescent image and the numerical values of the image data. The numerical value is proportional to the number of photons detected in each pixel. It is referred to as relative luminescence units (RLU).

Regions of interest (ROIs) were used to quantify the amount of light emission detected within each wound. The

ROIs encompassed the entire surface area of each wound. In each image, two equivalent ROIs (1.5 cm^2) were created to quantify the amount of light emission. Each wound was imaged at 0, 4, 8, 24, and 48 h postinfection using the bioluminescent imaging system. Before each measurement, 50 µl of substrate solution (1 mM of D-luciferin to 0.1 M phosphate–citrate buffer) was added exogenously to the wound. The difference between CTRL wounds and laser-irradiated wounds was evaluated using the Student's *t* test.

Laser

The laser irradiation was performed with an 810-nm diode laser (OPC-B015-FCTS, Opto Power, Tucson, AZ, USA) coupled into a 600-µm fiber to produce a 14-mm ($1/e^2$) circular spot on tissue. The diameter and homogeneity of the spot was controlled using thermal paper (Linargraph direct print, Kodak, Rochester, USA). The laser power was measured with a power meter (Ophir Optronics, Jerusalem, Israel).

Before the present study, several radiant powers (W/cm^2) and fluences (J/cm^2) were evaluated on the wound without bacterial infection since it had been demonstrated that the application of the 810-nm diode laser may cause damage to collateral tissues when radiant power is too high [17]. Only radiant powers below 8 W/cm^2 and fluences below 400 J/cm^2 did not induce cell damage in a cutaneous wound (data not shown). For this reason, the maximum dose used in this study was restricted to 260 J/cm^2. Irradiation was performed using a power of 10 W (radiant power: 6.5 W/cm^2) and irradiation times of 20, 30, and 40 s, which produced fluences of 130, 195, and 260 J/cm^2, respectively.

Infection model

Three groups of three rats were used (18 wounds). A suspension (50 µl PBS) containing $5x10^7$ cells of mid-log phase bioluminescent *E. coli* (10^9 cells/ml) was inoculated into each wound. Thirty minutes postinoculation (duration required by the bacteria to attach to the tissue) and immediately before imaging, 50 µl of substrate solution (1 mM of D-luciferin to 0.1 M phosphate–citrate buffer) was added to the wound.

Bacterial loading was controlled using the imaging system to confirm that it was equivalent in each wound. On each rat, one wound was not irradiated (CTRL) (n=9). After 30 min, nine wounds (one of each rat) were irradiated: 20 s (n=3), 30 s (n=3), and 40 s (n=3).

Wound temperature changes after exposure to light

Because heat is thought to be a key factor in bacterial killing, temperature at the surface of the wound was measured with a noncontact infrared thermometer (KT17, Heimann GmbH, Wiesbaden, Germany) before, during, and after laser irradiation.

Results

The bioluminescence signal of *E. coli* measured by the luminometer was linearly proportional to bacterial cfu (as determined by serial dilution and plating) from 10^3 to 10^7 organisms (data not shown).

In vitro studies

Figure 1 shows the bacteria viability measured by the bioluminescence imaging system (from 0 to 100 h). Figure 2 shows the bacteria viability measured by cfu 4 and 8 h after heating with a thermostated circulating bath for 2 min at 30°C (n=4), 40°C (n=4), 50°C (n=4), and 60°C (n=4).

When compared to control, bacteria bioluminescence is not affected after heating at 30, 40, and 50°C for 2 min. Because the bacteria are in growth phase, they are able to multiply in culture medium for 25 h where a maximum bioluminescence emission is reached. Then, a continuous decline is observed due to the consumption of the culture medium.

An important reduction of bioluminescence emission from bacteria is noted in the samples heated at 60°C. However, this temperature is not sufficient to destroy all bacteria in the culture medium. This observation is confirmed by standard plate count method (Fig. 2). Figure 3 displays the bioluminescence image of the 96-well black sides plate recorded 20 h after heating.

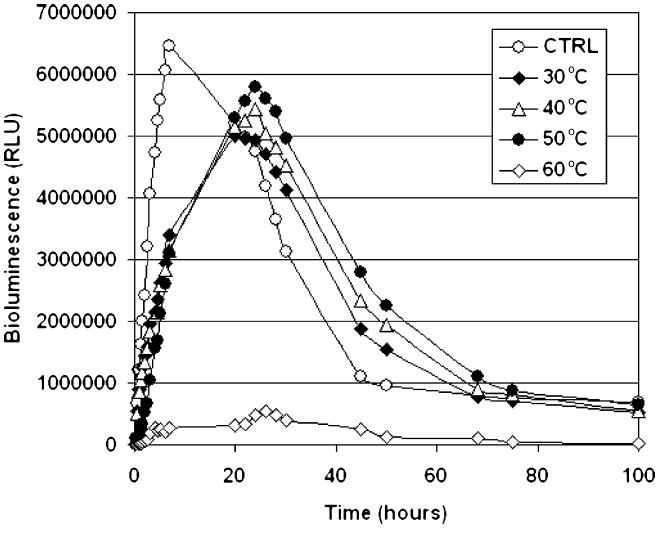

Fig. 1 Effect of the heating by thermostated bath on the viability of *E. coli* suspensions containing approximately 10^5 cfu/ml in Luria Broth. Bacterial samples were heated at different temperatures (30, 40, 50, and 60°C) during 2 min, and the loss of viability was determined by bioluminescence imaging system. Bioluminescence was recorded for 100 h

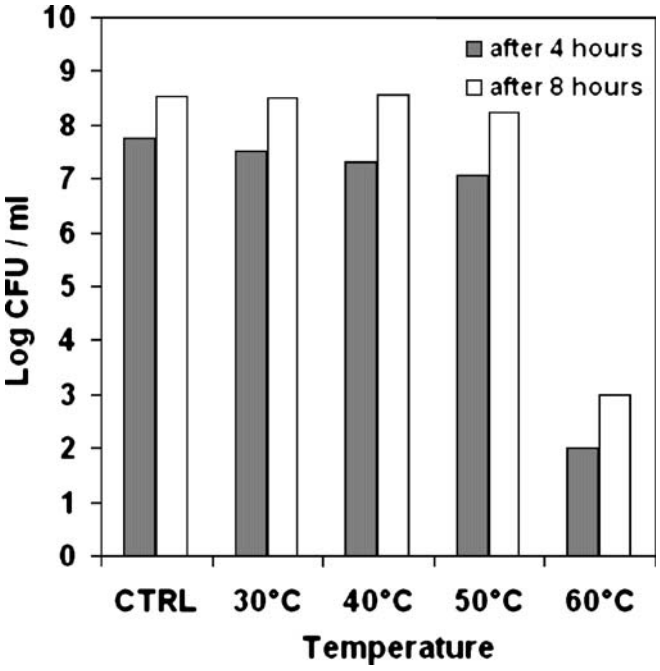

Fig. 2 Effect of the heating by thermostated bath on the viability of *E. coli* suspensions containing approximately 10^5 cfu/ml in Luria Broth. Bacterial samples were heated at different temperatures (30, 40, 50, and 60°C) during 2 min, and the loss of viability was by colony-forming unit assay at 4 and 8 h

Animal studies

Figure 4 displays the bioluminescence signal as a function of fluences and delay after irradiation. CTRL group shows a slight decrease of the bioluminescence signal as a function of time. For the three laser groups, bioluminescence imaging showed that at 4 h, the viability of bioluminescent

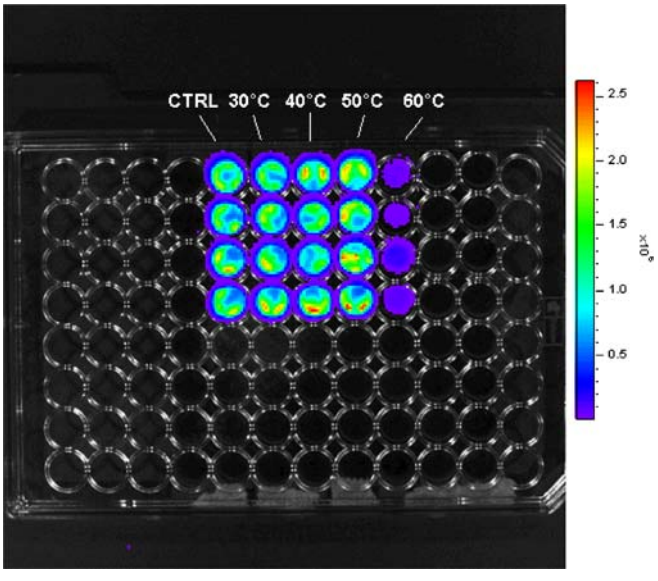

Fig. 3 One hundred microliters aliquots of bioluminescent bacterial suspensions recorded 20 h after heating at 30°C (n=4), 40°C (n=4), 50°C (n=4), and 60°C (n=4). Four aliquots were not heated. *Control* CTRL

E. coli was reduced when compared to the CTRL group (p<0.01). This observation was confirmed at 8 h (p<0.001), at 24 h (p<0.001), and finally at 48 h (p<0.001). Loss of *E. coli* viability depended on laser fluence. At 48 h, bioluminescent bacteria were not detected (100% loss of viability) in the wound irradiated at 260 J/cm^2.

Figure 5 shows that temperature elevation was linearly proportional to the fluence used. After irradiation, surface temperatures reached 38±2°C for 130 J/cm^2, 40±2.5°C for 195 J/cm^2, and 45±2°C for 200 J/cm^2. The regression curve has the following equation:

$$T(^\circ C) = 0.0585 \times F + 29.75, r^2 = 0.95.$$

Figure 6a,b shows two images recorded, respectively, before laser irradiation and 48 h later (CTRL and laser: 260/cm^2). At 48 h, the laser-irradiated wound did not show any viable bacteria.

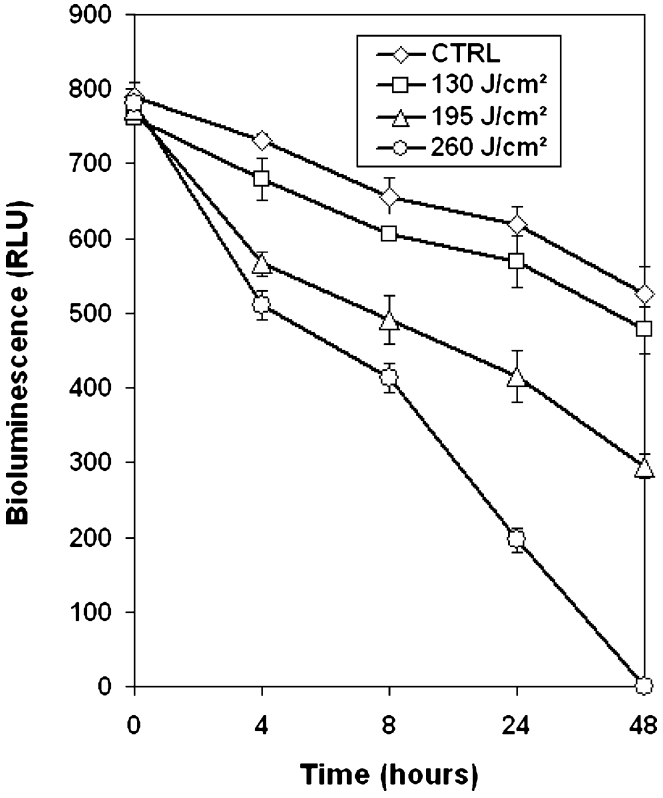

Fig. 4 In vivo monitoring of *E. coli* bioluminescence in the cutaneous wound. Each wound was inoculated with a suspension (50 µl PBS) containing 5x10^7 cells of mid-log phase bioluminescent *E. coli* (10^9 cells/ml). Before each measurement, 50 µl of substrate solution (1 mM of D-luciferin to 0.1 M phosphate–citrate buffer) was added exogenously to the wound. Each set of data represents the mean (±SD) number of relative light units (RLU) (for each laser group: n=3, for CTRL group: n=9)

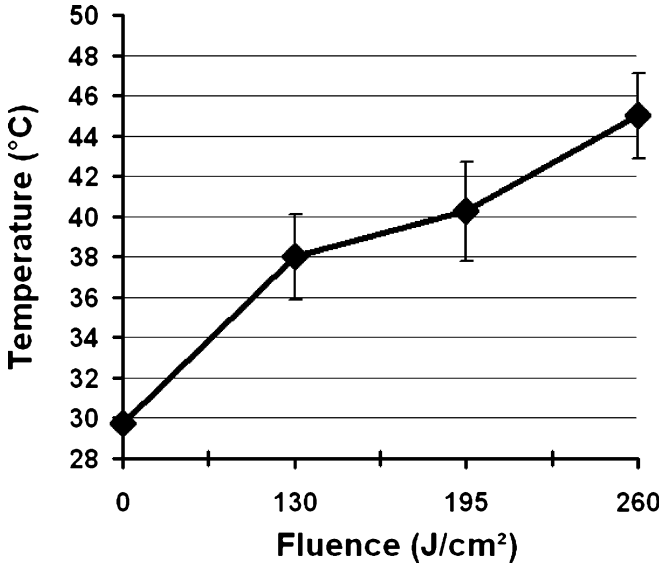

Fig. 5 Wound temperature as a function of light exposure (J/cm^2). The wounds infected with bioluminescent *E. coli* were illuminated with three different fluences: 130, 195, and 260 J/cm^2. (λ=810 nm, *P*=10 W, ∅=14 mm)

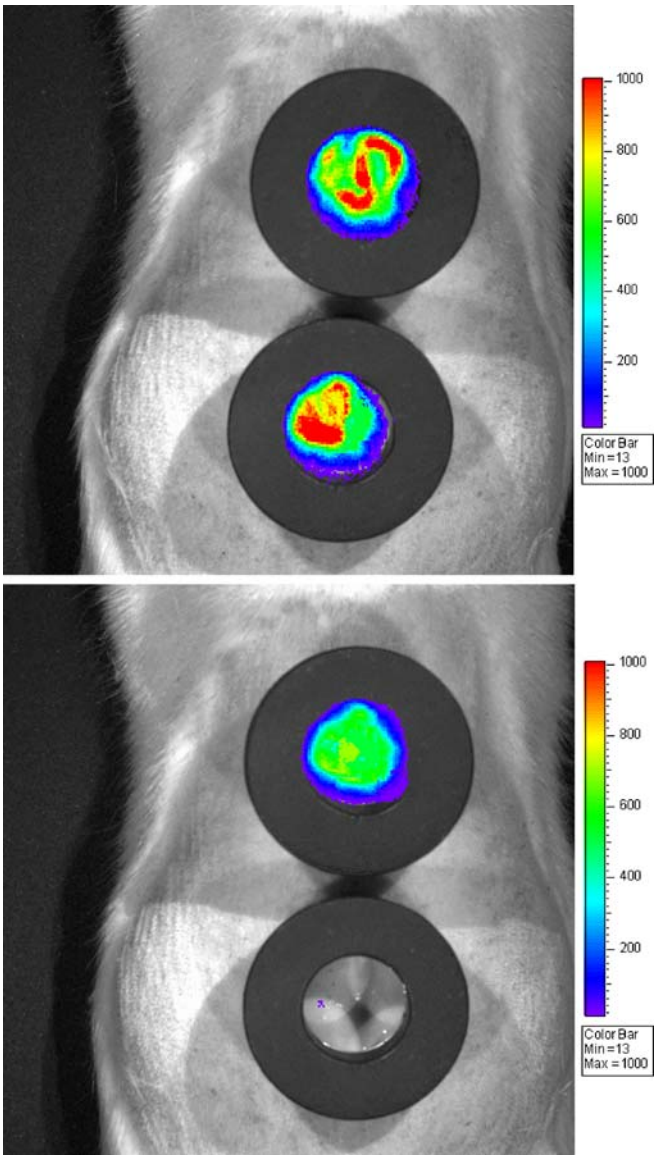

Fig. 6 Overlay images recorded **a** before laser irradiation (*above*) and **b** 48 h later (*below*). Each wound was inoculated at *T*=0 with a suspension (50 µl PBS) containing 5x10^7 cells of mid-log phase bioluminescent *E. coli* (10^9 cells/ml). Before each measurement, 50 µl of substrate solution (1 mM of D-luciferin to 0.1 M phosphate–citrate buffer) was added exogenously to the wound

Discussions

In the present work, we have examined the effects of an 810-nm diode laser irradiation on a cutaneous wound infected with bioluminescent *E. coli* (pRB474). The firefly luciferase gene from *Photinus pyralis* used in this study is a single polypeptide. The light production is started by the addition of the substrate, D-luciferin. Therefore, the metabolic stress caused by light production (and ATP consumption) takes place only at the luminescence measurement stage. Recently, other bioluminescent reporter systems such as bacterial luciferase operon luxCDABE have also been used to provide a means of detecting bacterial viability [5–7, 18]. The bacteria containing operon luxCDABE produce light continuously and are under constant metabolic stress caused by light emission and/or production of five polypeptides. These reasons may explain the difference in the light production power of the firefly luciferase and the bacterial luciferase operon [19].

Using the same animal model, we have previously demonstrated that antimicrobial activity of sulfamethoxazole–trimethoprim was associated with similar changes in bioluminescence. Similarly, the slight decrease of the bioluminescence signal as a function of time observed in the CTRL group was in accordance with this previous study [10].

The in vitro study shows that the light emission of the bioluminescent bacteria was not affected by temperature increase up to 50°C maintained for 2 min. This observation was confirmed by standard plate count method. Because the bacteria were in growth phase, they were able to multiply in culture medium up to 25 h. Then, a continuous decline was observed due to the consumption of the culture medium. In addition, the bioluminescent bacteria maintained at 30°C entered a stationary growth phase more quickly than control bacteria incubated at 20°C [20, 21] A factor influencing the effectiveness of a heat treatment is the composition of the environment surrounding the bacteria. Dry and/or acidic environments increase the rate of killing at a given temperature due to the damaging effects acid has on the cell. Conversely, proteins in a solution such as medium culture have a protective effect. The *D*-value, which denotes the decimal reduction time, and which is considered to be the time required at a specific temperature and under specified conditions to reduce a microbial population by one decimal, has been quoted as 10 min at 54°C for *E. coli* [3, 22]. Consequently, this in vitro study is in agreement with previous observations. These observations confirm that temperature lower than

50°C and lasting for 2 min or less does not inactivate firefly luciferase, and thus reduction of bacteria bioluminescence must be due to bacteria inactivation or to an inappropriate medium affecting the bacterial ability to reproduce.

The in vivo study shows that bacterial killing depends on laser fluence. At 260 J/cm^2, the bioluminescent bacteria were not detected in the wound after 48 h postirradiation.

It is difficult to compare the results of our study with previous laser studies because of differences in the laboratory settings and irradiation constants. Because wavelength, spot diameter, exposure time, and operating mode (CW or pulsed) are different, it is impossible to compare the lethal effect of the laser only considering the total delivered energy.

Kreisler et al. [17] have examined the cellular effects of the diode laser with a wavelength of 810 nm on human periodontal tissues. They observed that the power range (0.5–2.5 W) was not decisive for survival of cells, but rather the time of exposure. Schoop et al. [23] have evaluated various laser systems, namely, the Nd:YAG, the diode, the Er:YAG, and the Er:YSGG laser. They have demonstrated that for all the wavelengths investigated, using radiant powers greater than 1.5 W was capable of significant reductions of *E. coli*.

It is usually considered that the bactericidal action of the laser is due to thermal heating. Grönqvist [2], when using a Nd:YAG laser at 1,000 J/cm^2, has obtained agar temperature of approximately 70°C. Because it is known that temperatures above 60°C can cause thermal damage and killing of bacteria, killing at an exposure of 1,000 J/cm^2 is likely explained by photothermal mechanisms.

The *D*-value of *E. coli* at 54°C has been quoted as 10 min [22]. However, in our experiments, the maximum surface temperature reached after laser irradiation was only 45°C and was maintained for less than 1 min. Therefore, the specific question to be answered here was whether the microbial killing was simply due to heating to a lethal temperature or whether other mechanisms might contribute. Two hypotheses can be formulated:

(1) Laser irradiation could induce local temperature increase inside the bacteria higher than the temperature increase measured at the surface of the wound. It has been demonstrated that chromophores inside bacteria are sensitive to infrared light. Consequently, local heating inside bacteria or light-induced modulation of enzymatic activity could be responsible for bacterial killing [24, 25]. Ward et al. also observed extensive killing with the Nd:YAG laser [3]. Temperature recording has shown that the maximum temperature was 50°C after a 23-s exposure time. Scanning electron microscopy was performed and showed that the *E. coli* cell surface had been injured by the laser exposure at 50°C, whereas conventional heating did not have the same effect. However, if this hypothesis were valid, one would expect an immediate drop in luminescence followed by an increase as the surviving bacteria started to divide and repopulate the wound.

(2) A progressive desiccation of the superficial dermis is usually observed when using laser irradiation [26]. A dry wound surface would make the wound an inhospitable place for bacteria [27] and might explain the bacterial killing. This hypothesis seems to be more relevant because the decrease in bioluminescence is similar to the decrease observed in vitro after 25 h.

However, this later mechanism deserves further investigation. An assessment of laser-induced tissue damage should be completed with an investigation of the actual effect on the bacterial structure.

Conclusion

This study demonstrates that bioluminescent *E. coli* could serve as a biosensor of antibacterial activity for in vivo studies. The use of bioluminescent imaging strategies to reveal the real-time effects of potential therapeutic agents or devices on bacterial infections in cutaneous wound animal models would greatly accelerate the analyses of compounds under development. The use of diode laser irradiation on a cutaneous wound infected with bioluminescent *E. coli* in living animal could be proposed as an alternative antimicrobial strategy. This technique could circumvent the problems associated with the use of antibiotics such as development of resistance in target organism, including the emergence of bacterial strains that are resistant to all available antibacterial agents or permitting the colonization of opportunistic pathogens.

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