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Effects of low intensity laser in *in vitro* bacterial culture and *in vivo* infected wounds

Efeitos do laser de baixa intensidade em cultura bacteriana in vitro *e ferida infectada* in vivo

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A B S T R A C T

Objective: to compare the effects of low intensity laser therapy on in vitro bacterial growth and in vivo in infected wounds, and to analyze the effectiveness of the AsGa Laser technology in in vivo wound infections. **Methods**: in vitro: *Staphylococcus aureus* were incubated on blood agar plates, half of them being irradiated with 904 nm wavelength laser and dose of 3J/cm^2 daily for seven days. In vivo: 32 male Wistar rats were divided into control group (uninfected) and Experimental Group (Infected). Half of the animals had their wounds irradiated. **Results**: in vitro: there was no statistically significant variation between the experimental groups as for the source plates and the derived ones (p>0.05). In vivo: there was a significant increase in the deposition of type I and III collagen in the wounds of the infected and irradiated animals when assessed on the fourth day of the experiment (p=0.034). **Conclusion**: low-intensity Laser Therapy applied with a wavelength of 904nm and dose 3J/cm^2 did not alter the in vitro growth of S. aureus in experimental groups; in vivo, however, it showed significant increase in the deposition of type I and III collagen in the wound of infected and irradiated animals on the fourth day of the experiment.

Key words: Infection. Laser therapy. Laser therapy, low-level. Wound healing. In vitro.

INTRODUCTION

Laser therapy is a noninvasive treatment with several effects and applications in clinical practice, including on each of the stages of tissue healing[1-7].

The application of low-level laser in wound healing sounds interesting and promising; however, its use in the management of extensive soft tissue injuries with bacterial contamination was not yet the subject of proper investigation.

The influence of low-level laser on the phenotypic modulation of different bacterial populations has been little discussed in the literature. Since infection is a common cause of delayed wound healing, it is important to understand the effect of low-level laser therapy in bacterial growth[8].

The literature is controversial with regard to the effects of Low- Level Laser Therapy (LLLT) on bacterial growth. In some countries this therapy is still viewed with some skepticism due to the publication of controversial articles, showing both beneficial and no effects[9-12].

It is also very common that research fails to demonstrate a particular effect because of the existence of poorly controlled trials, mostly *in vitro*, with incorrect parameters choice and relatively small sample[13-17].

Given this scenario and the lack of studies analyzing the effectiveness of low-level laser therapy in infected wounds, the continued research in this area is justified.

The objectives of this study were to compare the effects of low-level laser therapy on *in vitro* bacterial growth and on *in vivo* infected wounds, and to analyze the effectiveness of GaAs (gallium arsenide) Laser technology on healing and infection in infected wounds *in vivo*.

METHODS

The study was approved by the Ethics Committee of Animal Use (CEUA) of the Pontifical Catholic University of Paraná under registration number 509.

The use of animals followed the code of ethics of the Council for International Organizations of Medical Sciences for animal experimentation and the principles of the Brazilian College of Animal Experimentation (COBEA) and national legislation on procedures for use of experimental animals (Federal Law 11,794, of October 9, 2008).

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Bacterium

We chose the bacterium *Staphylococcus aureus* (S. aureus), as it is commonly associated with wound infections and it is the most common gram positive bacterium in wound infections, besides being the most virulent species of its genus.

The preparation for the *in vitro* and *in vivo* experiments was initiated with the activation of the bacteria using a standard strain of S. aureus derived from the ATCC 25923. The bacteria grown in BHI (Brain Heart Infusion) was kept alive through a serial dilution of the bacteria and was used to make a suspension equivalent to 10^{-2} colony forming units (cfu)/ml.

Laser Parameters

Irradiation was carried out using a gallium arsenide laser with a wavelength of 904 nm. The equipment is in infrared electromagnetic spectrum and is set on pulsed emission of 0.0120 W average power, 200 ns pulse duration, maximum peak power of 30 W, beam diameter 4 mm, emission frequency of 2000 Hz. The equipment was checked and calibrated before starting the experimental procedure.

In vitro Experimental procedure

The *in vitro* sample consisted of 45culture plates. A sterile swab was used to inoculate the bacterium S. aureus in Müller-Hinton agar plate using a method of striae to allow the counting of bacterial colonies. Antibiogram was performed for the bacterium S. aureus with the BHI mean adjusted using the turbidity of the bacterial suspension to 0.5 equivalent of Mac Farland scale to assess sensitivity and resistance of the bacterial strain against the antibiotic.

Three disks of antibiotics specific for S. aureus (gentamicin, norfloxacin and amoxicillin) were placed on the study positive control plate. The reading of an antibiogram was made by the measurement of the diameter of the inhibition zone of each disk using a millimeter ruler. All antibiotics showed inhibitory action against the bacterial strain tested. This means that the strain had inhibition zones above the recommended values for the seven antibiotics, and the use of these antibiotics would be effective to treat it.

Three source plates were inoculated with the chosen dilution and were identified and divided into three groups: LASER 1, CONTROL 1 and ANTIBIOTIC. All plates were incubated for 48 hours at 35°C. After incubation, the three most isolated colonies from the LASER 1 plate were irradiated. Immediately after irradiation, three colonies were removed from the plate and diluted in three separate test tubes containing sterile saline and then replicated, maintaining the same method of seeding by quantification for the three new blood agar plates. In the CONTROL 1 (negative control) plate no treatment was employed. The three most isolated colonies were diluted and replicated using the same method used in LASER 1 plates. All plates were incubated for 24 hours at 35°C.

After the incubation period, all plates had only one more isolated colony (Figure 1) chosen to be irradiated and replicated in the LASER group and only replicated in the CONTROL group. The routine of irradiations and replications was repeated as described above, every 24 hours and at the same time of day, until seven days of the experiment, to assess a possible cumulative effect of the low-level laser therapy. All plates were photographed in the periods of 24 (sources) and 48 hours (derivative) after replication.

The employed technique was punctual application on each of the three selected colonies. The probe remained perpendicular to the plate at a distance of 1cm from it. The probe was fixed with an adjustable bracket with rod and claws for test tubes, and the dose was $3J/cm^2$.

In vivo Experimental Procedure

The *in vivo* sample comprised 32 male, adult Wistar rats, weighing between 251- 358g. All were kept in individual cages with controlled room temperature (21°C), light / dark cycles of 12h, commercial solid chow and water *ad libitum*. The animals were randomly divided into two groups of 16 animals. The first eight animals from each subgroup were identified by number and belonged to NICG (Non-Infected Control Group) and the other eight belonged to the IG group (Group Infected) group. Each group had four animals treated with LLLT and the other half received no treatment.

All animals were subjected to the following surgical procedures: anesthesia with ketamine 10% (60

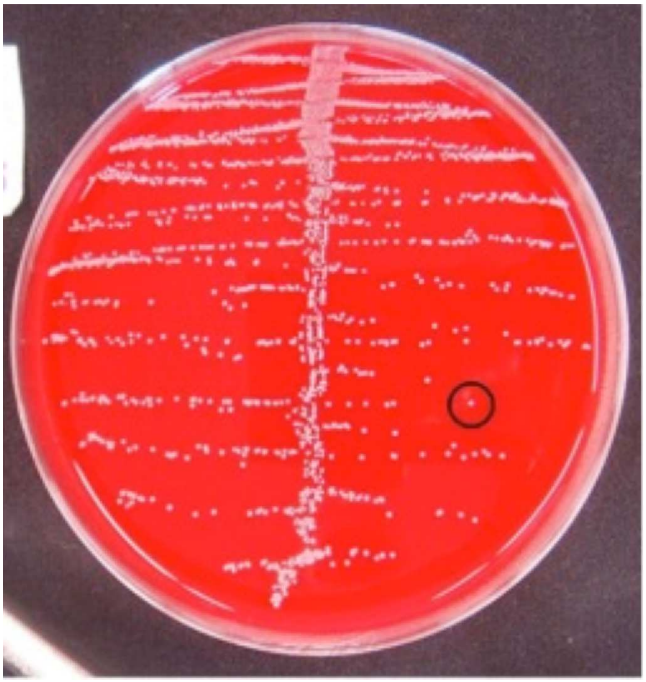

Figure 1 - *Colonized plate; isolated colony chosen for irradiation is circled.*

to 80mg/kg) and xylazine 2% (8 to 15mg/kg), shaving in the dorsal region and a linear, cranial-caudal incision made in the middle third of the dorsal region (an inch below the line between the clavicles), with 3cm of length and depth to the visualization of muscle fascia, using surgical scalpel. The control group had the edges of the wound sutured with two simple stitches of 3 0 mononylon suture and the infected group had the wounds inoculated with a suspension containing the activated S. aureus using a sterile swab; all wounds were then also approximated and sutured.

The laser technique used was the punctual application method, on three points of the wound, keeping 1cm distance between them. The probe remained situated perpendicular to the wound of the animal, keeping a distance of 1cm between the laser beam output and the wound. In the untreated animals we performed a simulation of laser irradiation, with the apparatus turned off. The animals were irradiated every 24 hours on the same schedule until completing seven days of treatment, to show the possible cumulative effect of LLLT. On the fourth day of the experiment, half of the treated animals (two in each group) were sacrificed. The other two were sacrificed on the eighth day of the experiment, using CO_2 gas chamber to rodents.

All skin samples were collected and immersed in vials containing 10% formalin. The determination of the bacterial growth was made using wound secretions collected from two different infected animals which were on the eighth day of the experiment. All tests (inoculation on MacConkey agar, blood agar and mannitol salt agar, the catalase test and coagulase) confirmed the presence of *Staphylococcus aureus*.

In vitro: all photographed plates were examined and the reading procedure abided by the following standardization: determine the total field of the plate; pictures of the source and derived plates individually submitted to manual calibration using a color selection, where the system has recognized the area of blood agar by the green color and the rest of the contents of the plate, composed of the bacterium colonies, was set to be recognized by the color yellow.

In vivo: all skin samples were dehydrated in alcohol, cleared in xylene and placed in paraffin. Then they were cut into 4 ìm fragments thick and stained with picrosirius (PS). The reading of PS slides was performed by obtaining two photomicrographs per slide in an optical microscope with capture cameras, using polarized lens.

The images were captured by a digital camera and subsequently frozen and digitized by a digitizer board, whose images were sent to a computer. For each histological section, we carried out the reading of two random fields at 400x magnification to evaluate the percentage of type I and III collagen. The percentage of area occupied by red (collagen type I) and green (type III collagen) fibers was calculated.

Statistical Analysis

To compare the groups with respect to quantitative variables, we used the non-parametric Mann-Whitney. In comparing the results in consecutive days of assessment within each group, we used the non-parametric Friedman test; p values <0.05 were considered statistically significant.

RESULTS

In vitro: the results of measurements obtained in relation to the area/background (A/B) comparing the laser and control groups during each day of the experiment, considering the source and derived plates, showed no significant variation between the means of the A/B between the experimental groups (p>0.05). In other words, groups had similar growth characteristics, with low mean dispersion over the entire experimental period. The source plates reached even higher values than the derived ones, reaching a value of "1" on the second and third days of experiment. Throughout the implementation of the experimental protocol, in each group (intragroup) we tested the hypothesis that there was a cumulative effect of low-level laser therapy, considering the source and derived plates. The test affirmed the equality of results over the days of the experiment.

There was no statistically significant difference between groups in relation to the area under the curve (AUC) when assessing all the reviews on consecutive days on the source plates and derived ones. In absolute values, the groups had the area under the curve (AUC) with normal distribution (Figures 2 and 3).

Percentage of variation of the variable A/B for each two consecutive days of evaluation throughout the experiment were calculated in the two experimental groups, including the source and derived plates. The percentages showed no statistically significant difference in the variation (p>0.05). In the source plates the largest percentage change for the groups occurred between the second and third day (34.07% Laser and 20.76% Control) and between the sixth and seventh day (31.73% Laser and 26, 76% control). In the derived plates the highest percentage occurred between the second and third day (45.98% Laser and 85.67% Control).

In vivo: two rats from the infected group (treated with laser irradiation) died after surgery. No other complications occurred during the experiment. To compare the groups as for the area of collagen, we tested the null hypothesis that the results are the same in both groups compared against the alternative hypothesis of different results. The result of the collagen comparison between the CONTROL and INFECTED groups for each valuation date indicated a significant difference only between the control group and the one infected and treated with low-level laser therapy when assessed on the fourth day of experiment

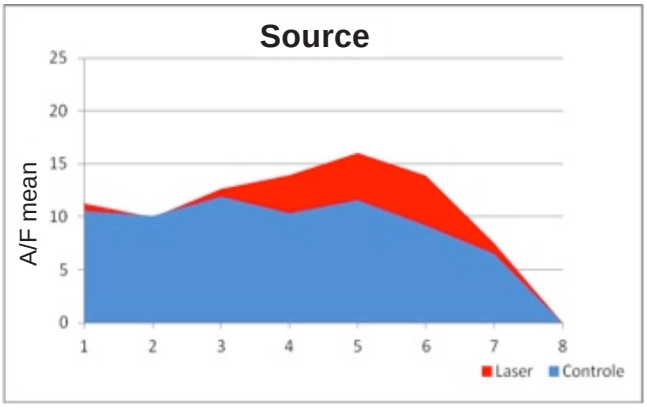

Figure 2 - *Area under the curve on the source plates.*

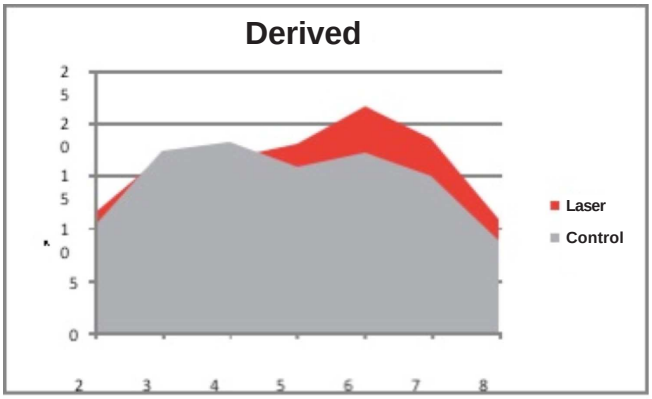

Figure 3 - *Area under the curve on the derived plates.*

(p=0.034). The other groups showed no significant variation in any of the two variables (collagen types I and III) in any of the time intervals (Table 1).

As for collagen in each group (intragroup), regarding the time of assessment (4th and 8th day of the experiment), we tested the null hypothesis that the means of deposition of type I and III collagen are equal for the two moments assessed, versus the alternative hypothesis that at least one time has an average different from the others. At no time there were significant differences, indicating that the values are similar at all time points in the same group.

In the evaluation at the fourth and eighth day for type I and III collagens in the irradiated control group, the value of p reached 0.867; in the non-irradiated control group, p returned 0.491; in the infected group, p was 0.104; in and the uninfected group, p reached 0.094.

The results of the comparison of collagen in relation to low-level laser therapy within the control group at each evaluation point showed no statistically significant difference. The control group treated with LLLT showed no greater or lesser effectiveness on the deposition of type I and III collagens in relation to the non-irradiated, yielding a p=0.867 when assessed on the fourth day and p = 0.590 when evaluated on the eighth day. When comparing the effectiveness within the group of infected animals in each evaluation point, there was no statistically significant difference, though with a slight increase of collagen deposition that can be observed in a larger value of the mean. Infected animals treated with LLLT showed no greater or lesser effectiveness deposition of collagen types I and III in relation to the non-irradiated group, obtaining a p=0.101 when assessed on the fourth day and p = 0.224 when evaluated on the eighth day.

Figures 4 and 5 demonstrate the variation of the quantified averages in percentage of variables collagen types I and III in their different groups (control vs. infected), subgroups (irradiated vs. non-irradiated) and evaluation times (fourth and eighth day of the experiment).

DISCUSSION

Infection results in destruction of the affected tissue and also in delaying wound healing and scar formation. By the findings from previous studies, the effectiveness of LLLT on tissue repair was observed in various protocols[4-9,18]. So far, nonetheless, no work has validated the use of laser therapy when there is the presence of bacterial infection in the wound.

In the references surveyed on the use of LLLT in the healing process of infected wounds, is noteworthy the variability in the methodology used, as well as the results found.

The standardization of equipment and choice of the laser parameters employed, such as wavelength, period, frequency, intensity for tissue repair with concomitant bacterial infection, show great variation in the literature[10,12,15,19-24].

The gallium arsenide laser was chosen to be one of most used lasers in physical therapy practice, capable of

Tabela 1 - Controle x Infectados irradiados com terapia a laser avaliados no quarto dia de experimento.

Variável	Grupo	n	Media	Mediana	Mínimo	Maximo	Desvio padrão	Valor de p
COL I	GC I 4D	16	82,02	88,18	60,15	97,97	12,42	
	GI I 4D	14	91,27	91,57	83,28	98,102	39,77	0,034
COL III	GC I 4D	16	17,97	11,81	20,24	39,84	12,42	
	GI I 4D	14	87,26	84,26	18,93	16,71	39,77	0,034

producing significant changes in skin ulcers and surgical wounds of humans. It has been used with different doses. Best results were observed with relatively low doses. Doses of 3 or 4 J/cm^2 produce better results than 5J/cm^2 when cell growth and collagen synthesis in cultured fibroblasts are assessed, and doses above 4J per point can inhibit the activity of fibroblasts[25]. These studies demonstrate that very high doses of energy do not seem to provide the best effects on tissue repair.

Laser therapy was applied daily for seven days in the *in vitro* and *in vivo* studies. The literature suggests that the stimulation of bacterial growth is significantly enhanced by repeated irradiation when compared with single exposure.

Laser treatment is usually applied to wounds three to five times a week. Thus, on infected wounds one must consider the cumulative effects of treatment[26].

The choice of *in vitro* research method was based on previous studies[13,19,24] that performed several experiments evaluating the effects of laser on different types of bacteria, using variations in wavelength, dose and frequency[13,19,21,22,24]. Most studies practiced irradiation of the plates in a single session (common methodological practice in this type of study)[21,24] and bacterial colonies counts were made up to 24 hours afterwards[19,23,24].

For this experiment, we developed a research design aimed to reproduce, *in vitro*, conditions common in the current practice of physical therapy. In the pre-existing research, authors almost unanimously suggest, for further investigations, the need of a larger number of exposures of the plates to the laser light, in order to capture a possible cumulative effect of LLLT and also of incorporation of the methodology used in more complex models, such as infected wounds *in vivo*, using animal models in the first instance.

The interest in the study of *in vivo* observation of the potential of LLLT is theoretically based on the evaluation of pro-inflammatory cytokines released after cell exposure to laser therapy and assessment of monocytes, macrophages and lymphocytes functions in wounds, conditions that the blood agar mean is not capable of providing[27].

Unlike what we found in the literature, for prevention and precaution LLLT was initiated 24 hours after the surgical procedure. In the *in vitro* study, this fact is justified because this is the period required for the seeded and incubated plates suffer bacterial growth visible to the naked eye. In the *in vivo* study, in addition to maintaining the standardization of parameters, the fact can also be justified by the laser therapy not being indicated during bleeding, which causes increased blood flow.

Laser therapy with the parameters used was not able to cause any biostimulating effect on the bacteria *in vitro*. However, it is noteworthy that the mode of application (single dose over the isolated colony, without contact of the probe with the culture plate), may have been responsible for the absence of effects on bacterial growth, since this type of application can cause significant energy loss[27], for

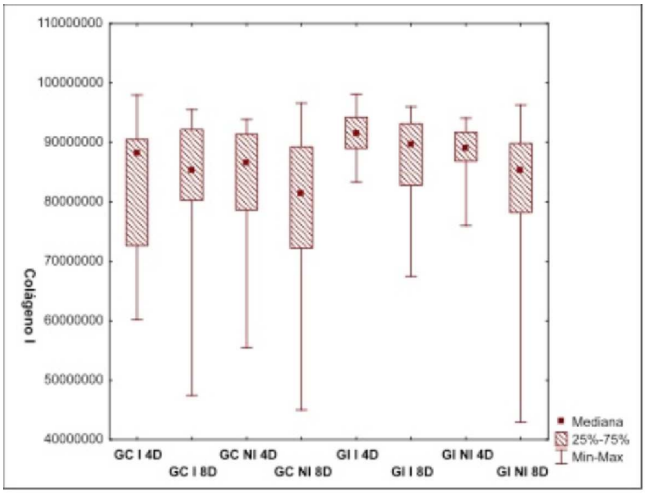

Figure 4 - *Collagen type I.*

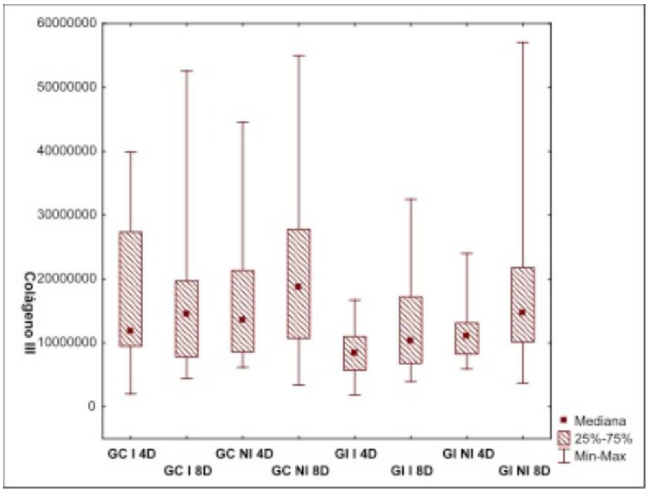

Figure 5 - *Type III collagen.*

in this case irradiation decreases due to the inverse square law (the intensity of the incident radiation is inversely proportional to the square of the distance between the source and the surface) and to the increase in reflection. The use of relatively low doses and a single exposure to radiation are also factors that may explain the lack of effects on *in vitro* bacterial growth.

According to our findings, it seems that the culture condition is vital for the growth of micro-organisms. Although not fully understood, after irradiation with LLLT, the participation of hematopoietic cells seems to be essential in the defense reaction of the host. The blood agar mean seems not to reflect the conditions *in vivo*, despite the presence of high concentration of hemoglobin. The theory based on observation of pro-inflammatory cytokines released after cell exposure to LLLT reported by Moore[27] seems to be the best line of continuity of studies in this area.

New studies evaluating the *in vivo* effects of LLLT on the inflammatory infiltrate when applied for prolonged periods in infected wounds are extremely important in order

to get more answers about the existence or not of interference in the wound healing process.

Since this work is original, we face the difficulty to compare the results with those previously reported. A suggestion for further work would be to investigate the use of LLLT in a controlled study on animal wounds and, subsequently, on human ones, to determine the effects of treatment with LLLT on the growth of bacteria and also on the extent of healing of the host tissue.

The results of this study indicate that the effects of GaAs LLLT irradiation with a wavelength of 904nm, dose 3J/cm^2 *in vitro* did not alter bacterial growth of S. aureus in experimental and control groups, neither on the source nor on the derived plates, until the eighth day of evaluation. The *in vivo* experiment showed a significant increase in the deposition of type I and III collagen in the scar in groups of infected and irradiated animals, when assessed on the fourth day of the experiment.

R E S U M O

Objetivo: *comparar os efeitos da terapia a laser de baixa intensidade no crescimento bacteriano* in vitro *e em feridas infectadas* in vivo*, e analisar a efetividade da tecnologia Laser AsGa, em feridas infectadas* in vivo. ***Métodos:*** in vitro*: cepas de* Staphylococcus aureus *foram incubadas em placas de agar-sangue e irradiadas com laser de 904nm de comprimento de onda e dose de 3J/cm^2, diariamente durante sete dias.* In vivo*: 32 ratos machos Wistar foram distribuídos em Grupo Controle (Não Infectado) e Grupo Experimental (Infectados). Metade dos ratos tiveram suas feridas irradiadas e a outra metade não irradiada, como realizado no estudo* in vitro. ***Resultados:*** in vitro*: não houve variação estatística significativa entre os grupos experimentais, considerando as placas matrizes e derivadas (p>0,05).* In vivo*: houve aumento significativo na deposição de colágeno tipo I e III na cicatriz do grupo dos animais infectados e irradiados, quando avaliados no quarto dia de experimento (p=0,034).* ***Conclusão:*** *a* Low-Intensity Laser Therapy *aplicada com comprimento de onda de 904nm e dose de 3J/cm^2,* in vitro*: não alterou o crescimento de* S. aureus *nos grupos experimentais.* In vivo*: mostrou aumento significativo na deposição de colágeno tipo I e III na cicatriz no grupo dos animais infectados e irradiados no quarto dia de experimento.*

Descritores: *Infecção. Terapia a laser. Terapia a laser de baixa intensidade. Cicatrização. In vitro.*

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