ОРИГИНАЛЬНЫЕ СТАТЬИ

БАКТЕРИЦИДНЫЙ ЭФФЕКТ HE-NE ЛАЗЕРА (632,8 НМ) НА КОЛОНИИ STAPHYLOCOCCUS AUREUS

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Резюме

Нами был изучен бактерицидный эффект низкочастотного лазера с длиной волны 632,8 нм с целью определения эффективной мощности и времени воздействия лазера на бактерии *Staphylococcus aureus*, участвующие в патогенезе ряда дерматологических заболеваний. Ранее проведены многочисленные исследования количественной оценки эффективных параметров лазера: световой дозы, плотности мощности и времени воздействия. В настоящем исследовании на колонии бактерий *Staphylococcus aureus* воздействовали лазерным излучением мощностью 1 и 3 мВт при разном времени воздействия (от 3 до 30 мин). Колонии бактерий были выделены у больного с воспаленными ранами. Воздействие лазером уменьшило количество бактериальных колоний во всех экспериментах. Результаты выявили значительное дозозависимое бактерицидное воздействие гелий-неонового лазера на *Staphylococcus aureus.* При мощности 3 мВт при воздействии в течение 30 мин количество бактерий снизилось до уровня менее 2% от его первоначального количества. Результаты показали уменьшение количества колоний в зависимости от времени воздействия. Лазерное излучение на длине волны 632,8 нм обладает бактерицидным действием в отношении *Staphylococcus aureus*.

Ключевые слова: *Staphylococcus aureus*, низкоинтенсивная лазерная терапия, воздействие лазером.

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THE BACTERICIDAL EFFECTS OF 632.8 NM HE-NE LASER ON STAPHYLOCOCCUS AUREUS COLONIES

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Abstract

The bactericidal effect of 632.8 nm low level laser has been studied in order to point out both the effective power and laser exposure time on *Staphylococcus aureus*, which is reported to be involved in several dermatology problems. Low level laser has been reported to be useful for infected wounds, tissue necrosis, nerve injury, osteoarthritis or other chronic pain syndromes. Numerous studies have been conducted to quantify the effective laser parameters, i.e. dose, power, and exposure time, which ultimately leads toward clinical implementation. *Staphylococcus aureus* bacteria colonies were exposed to laser doses with powers of both 1 and 3 mW at different exposure times varies between 3 to 30 minutes. The bacterial colonies were isolated from a patient with inflamed wounds. Two sets of bacterial colonies were prepared to be exposed to laser beam. Next, the bacterial colonies were compared before and after exposing them to laser doses. The results showed that laser sessions have reduced the number of the bacterial colonies for both doses; 1 and 3 mw at the different exposure times and concentrations. The results revealed significant dose dependent bactericidal effects of He-Ne laser on *Staphylococcus aureus* at 3 mW for 30 minutes, which was found to be more effective in reducing the amount of bacteria to the less than 2% of its initial count. The results exhibited the reduction of the number of colonies as a function of exposure time. Appropriate doses of 632.8 nm can kill *Staphylococcus aureus*, suggesting that a similar effect may be used in clinical cases of bacterial infection.

Keywords: *Staphylococcus aureus*, low level laser therapy, laser exposure.

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Introduction

Light therapy has been suggested as a potentially effective medical treatment approach for a variety of human conditions. Suggested amenable conditions range from sleep disorders, photo-aged facial skin, depression in the elderly, and treatment of acne *vulgaris* to a variety of neuron-musculoskeletal conditions such as peripheral neuropathy, second degree ankle sprains, and osteoarthritis of the knee and cervical spin [1,2,3,4]. Therefore, the use of low level laser therapy (LLLT) has become wide-spread in medicine [5,6,7].

LLLT is, mostly, used in clinical practice for the promotion of tissue healing and pain control. Specific applications of laser are diverse, in part, because each of the involved mechanisms can be applied to a number of body systems. Examples include management of indolent or infected wounds, tissue necrosis due to envenomation, nerve injury, osteoarthritis or other chronic pain syndromes, fracture healing, tendinous or ligamentous injury, and post-surgical incision care [1,5,8]. Nevertheless, laser treatments that are intended to stimulate protein synthesis in wounds may also reduce the bacterial growth, which would further stimulate the wound healing [9]. It's known that the bacterial infection is the most common problems underlying chronic wounds, therefore, there are limited options for the management of infected wounds and bacterial colonies, and some commonly used methods have undesired side effects. For example, typical disinfectants, such as hydrogen peroxide, that could eliminate certain bacteria as well as being toxic to new granulation tissue. The development of antibiotic resistant strains of bacteria is a limiting factor in the prophylactic use of broad spectrum antibiotics. Low-intensity laser therapy (LILT) has been investigated as a bio-stimulatory modality for the treatment of killing bacteria [5,10].

In the literature, three are several reports claimed that LLLT/LILT can facilitate the healing processes of many disorders. However, there is still significant debate regarding the efficiency of laser in producing the desired clinical response [8]. Among such studies, Ribeiro et al. (2004) have investigated the influence of low intensity polarized visible laser radiation on the acceleration of skin wound healing. Their histological analysis showed that the healing of irradiated wounds was faster than that of non-irradiated wounds. Moreover, it was observed that skin wound repair is dependent on polarization orientation with respect to a referential axis [11].

Another field in which LLLT was used is the bactericidal effects of laser. Nussbaum et al. (2002) have studied the effects of 630, 660, 810, and 905 nm laser irradiation at delivering radiant exposure of 1-50 J/cm^2 on three species of bacteria (*S. aureus* (ATCC 29213), *E. coli* (ATCC 25299), and *P. aeruginosa* (ATCC 27853)). They found that the applied LLLT to wounds, with radiant exposures in the range of 1-20 J/cm^2, could produce changes in bacterial growth of considerable importance for wound healing. A wavelength of 632.8 nm appeared to be the most commonly associated with bacterial inhibition. Their findings might be useful as a basis for selecting LLLT for infected wounds [5]. In addition, Guffey & Wilborn (2006) reported some bactericidal effects of 405 and 470 nm light on two bacteria: *S. aureus* and *P. aeruginosa*. Their results indicated that, *in vitro*, 405 and 470 nm blue light produce dose dependent bactericidal effects *S. aureus* and *P. aeruginosa* but not on *P. acnes* [1].

Moreover, LLLT finds its way to dentistry applications. In this concern, Folwaczny et al. (2002) have studied the antimicrobial effects of Er: YAG laser radiation on teeth root surfaces. Depending on the number of laser pulses, the bacterial load in the *E. coli* group has been reduced by the Er: YAG laser radiation after exposure to 105 laser pulses to 5.5% of the initial count, while the *S. aureus* group was reduced to 15.1% of the initial count. Beside the selective removal of plaque and calculus, the Er: YAG laser radiation causes reduction in bacteria on root surfaces [12]. However, the *S. aureus* has been reported to be killed via He-Ne laser pulses, even for the methicillin-resistant *S. aureus* (MRSA) [12,13].

On the other hand, Avram & Rogers (2009) have tried to solve hair health problems, such as hair growth through LLLT. Their results indicated that, on average, patients had a decrease in the number of vellums hairs, an increase in the number of terminal hairs, and an increase in shaft diameter when they were exposed to laser pulses. However, their results showed some limitations, since some of their findings were not statistically significant [14]. In addition, Avaci et al. (2014) have used LLLT to stimulate the hair growth in mice, which were subjected to chemotherapy induced alopecia and also in alopecia aerate. Among various mechanisms, the main mechanism is hypothesized to be stimulation of epidermal stem cells in the hair follicle bulge and shifting the follicles into anlagen phase [15].

In regards to the laser doses, the treatment dose is calculated as the amount of energy (Watts) delivered over a period of time or a specific tissue area. However, doses are often listed as Watts (Joules per second) per square centimetre or Joules (intensity of the energy in Watts multiplied by the treatment time) per square centimetre. Thus, the energy emitted per unit of time, total energy administered, the size of the area being treated and the treatment time are all important and interrelated variables in determining the desired laser doses [8]. Nevertheless, laser light is emitted either in a continuous wave (CW) or pulsed form. When laser light is emitted in pulses, the pulse frequency may impact the effectiveness of the treatment. Other variables that impact the effectiveness of the treatment include the distance from the laser source to the tissue surface

and the target tissue, the speed of movement over the treatment area, and the number and frequency of treatments [8]. In conclusion, the physiologic effects of laser are reported to include stimulation of mitochondrial activity, increased cell turnover, recruitment and proliferation, modulation of the cellular metabolites involved in the inflammatory response, vasodilatation, involved in the inflammatory response, vasodilatation, release of exogenous endorphins, and increased oxygen availability in the tissues [10,16,17].

From the previously mentioned studies, the He-Ne laser seems to have antimicrobial properties, with the ability to kill a wide range of bacteria including *E coli, P. aeruginosa* and *S. aureus*, which is reported to be involved in several dermatology problems [18,19,20].

Therefore, one could propose that LLLT presents a great opportunity in treating bacteria related diseases. In this context, the current study is conducted to point out the possibility of using laser therapy to reduce the *S. aureus* count, as well as reporting the effective laser parameters to achieve that.

Materials and methods

For this study, a bacterium sample has been collected from a patient with an injury with inflammation, and then cultured in media to get a pure culture for a specific type of bacteria. The samples collection and processing were conducted according to the local ethical committee of Al Neelian University, the Sudan, and were in accordance to the International Guiding Principles for Research Involving Animals and Human Beings. The media for Mannitol Salt Agar (MSA) have been taken in a flask and dissolved in distilled water; the type of this medium was used as selective media for the desired bacteria, *S. aureus*. The solution was transferred to autoclave in order to make it sterilized. The autoclave was used with a pressure of 5 Pascal, under temperature of 121°C for 15 minutes. Next, the media have been put on sterilized plates, and then the bacteria were cultured by a sterile loop in plates and incubated for 24 hours at 37 °C. After the growth of the bacteria, they were a subject for further tests to be classified as *S. aureus* using H_2O_2 test. Samples from colonies have been taken and placed in a tube then the bacteria suspension was prepared. In order to do that, 1.5 g of Peptone water, which was measured by sensitive balance, were added to 0.2 g of distilled water. The mixture was subjected to sterilization by autoclave. Next, 10 ml was drained in a tube which contains Peptone water. Bacteria have been taken by loop and cultured in tube contained Peptone water.

Seven Eppendorf tubes in separate step were sterilized. Each one of the tubes contained 1 ml from the bacterial suspension. The next step was to expose the tubes to He-Ne 632.8 nm laser with a power of 1 mw for several time intervals: 3, 6, 9, 12, 15 and 18 minutes. After exposure to the laser beam, directly the samples drained and cultured in 6 plates contain MSA media. The flame used as sterilizer during the process culture. However, one tube was used as a control, thus it has never been subject to laser exposure. All of the plates involved in this study were incubated for 24 hours, including the control plate. After incubation, the growth colonies on each plate were counted. The experiment was repeated to check for the concentration of the suspension, which was an important issue to estimate the bacterial growth after exposing to laser doses.

In the dilution process, 6 empty tubes were used to make a serial dilution from bacterial suspension. 0.1 ml has been taken from the suspension by micro pipette and drained in the first tube. The sample was shaken and added to 0.9 ml of distilled water. Then, 0.1 ml of this mixture was taken from the tube (numbered as tube number one) using micro pipettes and placed in another tube, labelled as the tube number two that contained 0.9 ml distilled water. This step was repeated for four times in order to prepare six tubes. From each one of the previous tubes, 0.1 ml of the solution was taken and cultured in MSA media. After incubation for 24 hours, the bacteria were grown on the plates and created colonies.

Colony counter was used to count the number of colonies on each plate. The tube with bacterial

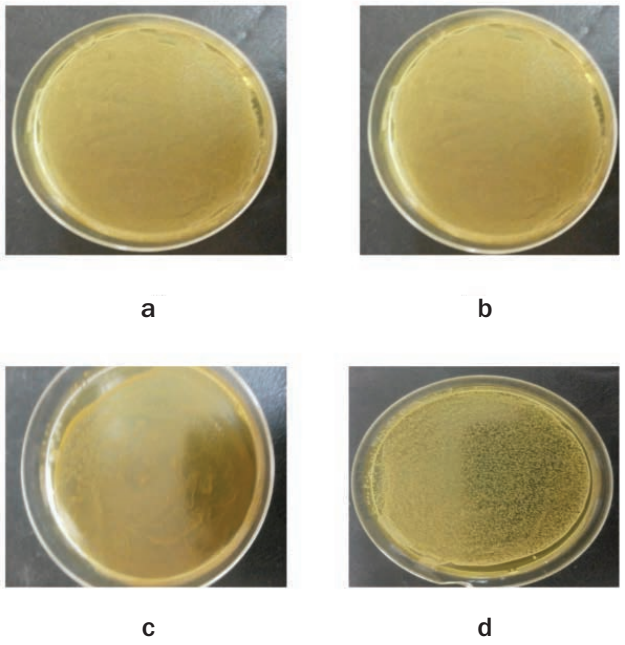

Рис. 1. Колонии *Staphylococcus aureus*, проба №A1:
a – контрольный образец; b – колонии после воздействия лазером в течение 3 мин; c – колонии после воздействия лазером в течение 6 мин; d – колонии после облучения лазером в течение 18 мин.
Fig. 1. Snapshots of the *Staphylococcus aureus* plates of the sample #A1: a – control sample; b – colonies after exposing to laser for 3 minutes; c –colonies after exposing to laser for 6 minutes; d – colonies after irradiation to laser for 18 minutes.

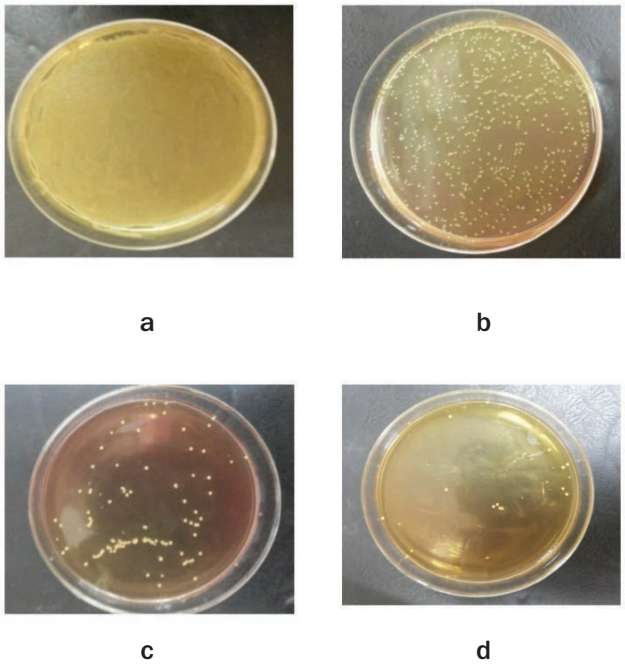

Рис. 2. Колонии *Staphylococcus aureus*, проба №A2:
a – контрольный образец; b – колонии после воздействия лазером в течение 5 мин; c – колонии после воздействия лазером в течение 15 мин; d – колонии после облучения лазером в течение 30 мин.
Fig. 2. Snapshots of the *Staphylococcus aureus* plates of the sample #A2: a – control sample; b – colonies after exposing to laser for 5 minutes; c –colonies after exposing to laser for 15 minutes; d – colonies after irradiation to laser for 30 minutes.

suspension of higher concentration have been selected for further study, of which the tubes were exposed to He-Ne 632.8 nm laser, using KZ-350-LB setup, at output power of 3 mW with different exposure times (5, 10, 15, 20, 25 and 30 minutes). The number of colonies after exposing to laser in each time interval was counted in order to check for the decrease in the bacteria as a function of exposing to laser.

Results and discussion

The numbers of colonies of the *S. aureus* bacteria in the Eppendorf tubes have been counted both before and after irradiating the bacteria to the laser. This way, one sees whether they are affected by laser irradiation, and the effective exposure time that helps to reduce the bacteria to the minimum. Fig. 1a shows the colonies in the control sample while Fig. 1d shows the bacteria after laser exposure for 18 minutes. The same for sample №A2 is presented in Fig. 2. Fig. 2a shows the control sample while Fig. 2d shows the colonies after 30 minutes of laser irradiation.

Table represents the number of the bacterial colonies that counted for sample №A2 every 5 minutes of exposing to the laser, till almost bacteria are reduced to the minimum. A plot of those results is presented in Fig. 3.

Таблица
Количество колоний бактерий при разном времени экспозиции для образца №A2
Table
The bacteria colonies number as a function of exposure time for sample #A2

Время экспозиции (мин) Exposure time (minutes)	Число колоний Number of colonies
0	500 ± 25
5	400 ± 22
10	220 ± 14
15	175 ± 8
20	97 ± 5
25	25 ± 3
30	10 ± 2

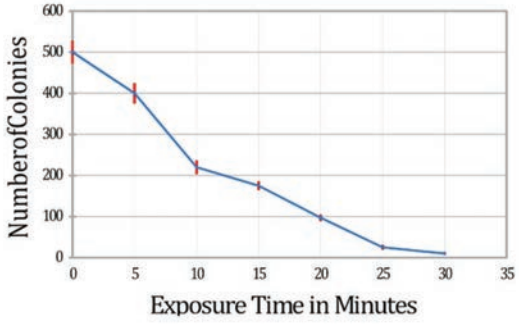

Рис. 3. Зависимость количества колоний бактерий от времени лазерного воздействия.
Fig. 3. The number of bacterial colonies as a function of the laser exposure time.

In this study, two *S. aureus* bacteria samples were exposed to a He-Ne laser source with a wavelength of 632.8 nm laser irradiation. The first one has been exposed to a power of 1 mW for different exposure times; 3, 6, 9, 12, 15 and 18 minutes. However, for the second sample, 3 mW was used at exposure times of 5, 10, 15, 20, 25 and 30 minutes. This way, one can see the effective laser parameters that could be used to reduce the bacteria to the minimum.

From the experiments, one notices that the number of bacterium colonies is decreased gradually as a function of exposure time, mainly at a laser power of 3 mW. The reduction in the colonies number occurs due to some effects and changes that laser is involved in. These effects were reported to include making holes or pores on the bacterial cells wall when they were subject to the laser beam and released the contents of bacteria cells on media [16,18].

Another reason for such a decrease is the thermal effect on the bacteria, which is produced by laser beam. This heating makes vacuoles inside the cell that leads to the killing of bacteria. Furthermore, laser is able to produce some changes at both photochemical and photobiological levels in bacterial cells, thus functions to reduce the number of bacterial colonies [10,17].

Fig. 1a shows that the control sample contains uncountable number of bacteria colonies, which means that it is more than 500 colonies. When the sample was exposing to laser, the number of colonies started to be counted and then get reduced as a function of exposure time. However, the colonies were not affected as happens to the sample №A2 due to the difference in both exposure time and laser power, which is reduced dramatically from the control, Fig. 2a to exposure of 30 minutes as in Fig. 2d. The results show that using He-Ne with a wavelength of 632.8 nm at 3 mW for 30 minutes seems more effective to reduce the amount of bacteria to the less than 2% of the initial count. This facilitates and speeds up healing from the *S. aureus* bacteria related diseases. Nevertheless, it is well-known that this bacterium is involved in so many skin infections, thus it can be easily treated using laser as skin can be exposed directly to the laser and doesn't require sophisticated precautions.

The bactericidal effect of He-Ne laser was reported in some studies to occur due to the production of reactive oxygen species (ROS) in the cells of the bacteria, which leads to cell death. Furthermore, the bactericidal effect of He-Ne lasers appears to be dependent on the intensity and duration of the laser treatment, with higher intensities and longer durations generally resulting in greater bactericidal activity. Some studies have suggested that the bactericidal effect of He-Ne lasers may be enhanced by the use of photosensitizers, which can increase the production of ROS in bacteria when exposed to light [21].

The current results allowed concluding that the laser at a wavelength of 632.8 nm has a bactericidal effect on *S. aureus*, which is involved in several dermatology problems. Using He-Ne with a wavelength of 632.8 nm at 3 mW for 30 minutes seems to be effective to reduce the amount of bacteria to less than 2% of the initial count.

Conclusion

The bactericidal effect of 632.8 nm low level laser on *S. aureus* was examined through exposing several bacterial colonies to He-Ne laser at different parameters. The results showed that more than 98% of the bacteria are killed when a wavelength of 632.8 nm at 3 mW for 30 minutes is used. This can be attributed to the production of reactive oxygen species in the cells of the bacteria which is reported in the literature to be associated with the laser intensity, power and exposure time. The research on the bactericidal effect of 632.8 nm low level laser at certain power and exposure time could be elaborated to find its way to clinical application since *S. aureus* is reported to be involved in many dermatological diseases.

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