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In vivo killing of *Staphylococcus aureus* by toluidine blue-mediated photosensitization in an animal model wounds

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Abstract

The aims of this study were to determine whether *S. aureus* could be killed by toluidine blue-mediated photosensitization *in vivo* in an animal model. Twelve Wistar rats divided into three groups (n = 12): Group I: Control Group, the wounds were made and submitted to the application of the laser without the drug photosensitizing; Group II: The implementation of wounds received the toluidine blue, without application of laser; Group III: it was used toluidine blue, and application of laser-Indio phosphide Gallium-Aluminum (InGaAIP) λ 660nm power and density of 20 Joules/cm^2. Statistical analysis of CFU by analysis of variance Kruskal-Wallis test shows significant intraoperative difference, photodynamic therapy group ($p < 0, 05$), the Dunns post hoc test shows significant difference between Group I when compared to Group II treated with LLLT ($p < 0001$). The results of this study show that photodynamic therapy with toluidine blue has reduced the number of *Staphylococcus aureus in vivo*.

Key words: Photodynamic therapy; Toluidine blue; Wound infection.

Introduction

Wound infection is a major concern among healthcare practitioners, not only in terms of aggravation of trauma to the patient but also in view of its burden on financial resources and the increasing requirement for cost-effective management within the healthcare system[1].

Whilst the role of microorganisms in chronic wounds (CH) and the antibiotic treatment of CH have been discussed, their relationship with antibiotic resistance is an important public health issue which has yet to be fully investigated[2,3].

The combination of increasing numbers of the population, which is at risk of developing CH, together with the prevalence of antibiotic resistance, makes this a highly pertinent issue. The polymicrobial nature of CH is likely to provide an appropriate environment for genetic exchange between bacteria. Indeed, the first two cases of vancomycin-resistant *S. aureus* in the United States were both isolated from CH patients[3,4].

It has been known since the first days of photodynamic therapy (PDT) early in the 1900's that certain microorganisms can be killed by the appropriate combination of dyes and light *in vitro*, but in the last 15 years progress has been made defining the precise molecular features of the PS necessary for binding to and subsequently killing by photodynamic action microbes such as bacteria, fungi, and viruses[5].

One potential alternative approach is PDT, which could provide a means of killing microbes in localized, topical infections[6-9].

The PDT can be defined as eradication of target cells by reactive oxygen species (ROS) produced by means of a photosensitizing compound and light of an appropriate wavelength[10]. These reactive oxygen species can then oxidize many biological molecules, such as proteins, nucleic acids, and lipids, leading to cell death. Photodynamic therapy has advantage over other therapies of dual selectivity: it is not only the PS used for the affected tissue or cell type, but the light can also be accurately delivered to the appropriate area[5].

The toluidine blue is a cationic dye metacromatic, which belongs to family group of thiazine with property to selectively stain acid groups of components that presents affinity with DNA of cell nuclei and the RNA present in the cytoplasm, and fixing the dye stain deeply[11].

Phenothiazinium dyes are small molecules that have an intrinsic molecular cationic charge that allows them to bind generally the anionic outer membranes of bacteria and fungi[12,13].

The advantages of PDT over conventional antimicrobial agents are the rapid death of target organism depending mainly on the light energy dose delivered and therefore the power output of the light source used. Hence, resistance development would be unlikely as death, this is mediated by singlet oxygen and free radicals, and high concentrations of photosensitizer do not need to be maintained in the disease site for more than a few minutes, in contrast with hours or even days, necessary in case of conventional antimicrobial agents. Finally, antimicrobial effects can be confined to the site of the lesion by careful topical application of photosensitizer, and the area of irradiation can be restricted further by using an optical fibre[14].

The aims of this study were to determine whether *Staphylococcus aureus* could be killed by toluidine blue-mediated photosensitization *in vivo* in an animal model.

Materials and methods

Animals for experimentation

We used 12 adult male rats of Wistar strain with body weighing 300 to 350 grams, from Central Vivarium of the Universidade para o Desenvolvimento do Estado e Região do Pantanal - UNIDERP, maintained under controlled light and temperature, six animals per cage with standard food and water *ad libitum*. All experimental procedures were performed in accordance with the standards of the Brazilian College of Animal Experimentation - COBEA

approved by the Research Ethics Committee of the Universidade para o Desenvolvimento do Estado e Região do Pantanal - UNIDERP under Protocol nº L185/2005 / CEP.

Composition of the Sample

The sample was composed of 12 animals divided into three groups (n = 4) that received the following treatment:

- Group I: "Laser group" is composed of animals subjected to injury of dorsal 9cm² by a punch and were subjected to irradiation with laser of low intensity without application of photosensitizing drugs.
- Group II: "TBO group" is composed of animals subjected to injury of dorsal 9cm² by a punch and submitted to the application of toluidine blue, without treatment with laser of low intensity.
- Group III: "PDT group" is composed of animals subjected to injury of dorsal 9cm² by a punch and submitted to application of toluidine blue, and laser treatment in low intensity.

Sampling Organisms

Staphylococcus aureus is the bacteria used in this study. The specific strains of bacteria were *S aureus* (ATCC 25923), as cataloged in the American Type. The strains of *Staphylococcus aureus* (Newprov) were activated lyophilized amid TSB (Acumedia) according to the instructions of the manufacturer.

Manufacture of skin wounds

After weighing, each animal received preanesthetic administration of Butorphanol (Turbogesic, 2mg/kg) associated with Acepromazine (Acepran, 1mg/kg), both in a single dose intramuscularly. After 15 minutes, it was administered Zolazepan and Tiletamine (Zoletil 50, 40mg/kg). After being anesthetized, the animal was placed in prone position, and it was sterilized with alcohol-iodine and trichotomy. A punch was used to achieve the wounds of approximately 8 mm diameter allowing the removal of a circular area of skin, on the middle portion of the median sagittal plane. After its preparation, the wounds were colonized with a standard solution of *Staphylococcus aureus* (ATCC 25923).

Photosensitizing drugs

The photosensitizing drug used was of Toluidine Blue O (Sigma, USA) at a ratio of 100 micrograms/ml, and the same applied through a micro-pipette, across the length of the edge and central portion of the lesion.

Application of low-intensity Laser

Soon after the application of photosensitizing drugs (toluidine blue,) the wounds of PDT and laser groups were irradiated with a single application of laser-Indio phosphide Gallium-Aluminum (InGaAIP) DMC® Brand Photon Laser III model, with power of 100mW (power density of 1.4 W/cm²), the beam area of 3 mm, and λ the wavelength of 660nm. The application has been filling in the form of scanning the entire length of the wound 8 mm for both the density of energy applied was 20 Joules/cm² and time of 8 seconds.

Observation of microbial growth (colony-formin units)

At the 4th postoperative day, exsudate was collected from the wounds for microbiology and for quantitation of bacterial population. The materials were processed and cultured on selective MacConkey's agar, blood agar and salt manitol agar. The agar plates were incubated at 37ºC and examined for growth after 24 and 48 hours. Any growth in the plates of bacteria of the same biotype as cultured in the wounds was considered positive and expressed as colony-forming units

per gram of tissue (CFU/g). All procedures were performed under laminar air flux.

Statistical analysis

After tabulating the data, the Shapiro-Wilk normality test was used due to the small sample size. When samples presented normal distribution (parametric), analysis of variance (ANOVA) was used with the Tukey *post hoc* test in order to perform a comparative analysis between groups. In cases of non-parametric distribution, the Kruskal-Wallis test and Dunn *post hoc* test was used. The BioEstat® 3.0 statistical software was employed for these procedures, with the significance level (p) set at 5%, i.e. ($p \geq 0.05$).

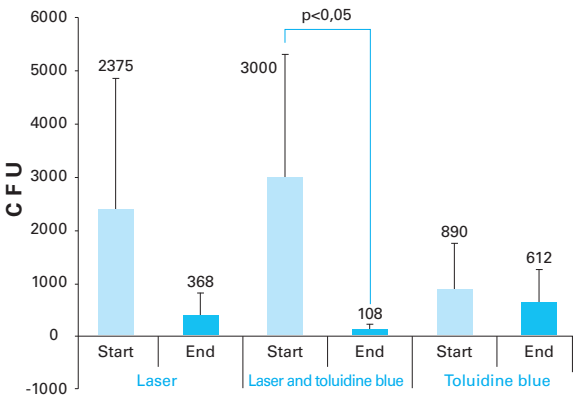

Figure 1: Comparison of the values of CFU between the beginning and end times for each group. Values expressed as mean and standard deviation. Kruskal-Wallis test (p = 0.0559), post-Dunn test (p <0.05)

Results

The values of the variables investigated were expressed as mean and standard deviation was then calculated the percentage of reduction of colony forming units (CFU) for each group investigated.

The statistical observation of reduction was initially tested from the values of colony-forming units in each group, testing the hypothesis of reduction between the moments: Initial and Final, mean ± SD was calculated for each group and compared between times (Table 1).

Statistically significant reduction was observed only in the PDT group with the Kruskal-Wallis (p <0.05). In the two other groups there was no difference due to the wide range of values (Figure 1).

We tested the hypothesis that there is statistical difference between the percentage of reduction between the groups UFCs laser, PDT and TBO, characterized as a control (Table 2).

Comparing the percentage reduction of CFU between groups using the Dunn test, there was a statistically significant difference between the group of laser and toluidine blue and toluidine blue only (p <0.05) (Figure 2).

Table 2: Mean results of initial, final, and percentage reduction in the number of colony-forming units (CFU) between groups

Group	UFC		Reduction %
	Initial	Final	
Laser	2375	367,5	84,5
PDT	3000	108,2	96,4
TBO (control)	890	611,7	31,3

Table 1: Comparison of the values of colony-forming units (CFU) between the start and end times for each group

N	Laser		PDT		TBO	
	Start	End	Start	End	Start	End
1	260	240	5000	134	500	1500
2	5000	120	1000	40	60	57
3	240	110	5000	250	2000	500
4	4000	1000	1000	9	1000	390
Mean	**2375**	**368**	**3000**	**108**	**890**	**612**
SD	**2487,5**	**425,8**	**2309,4**	**108,4**	**833,7**	**621,4**

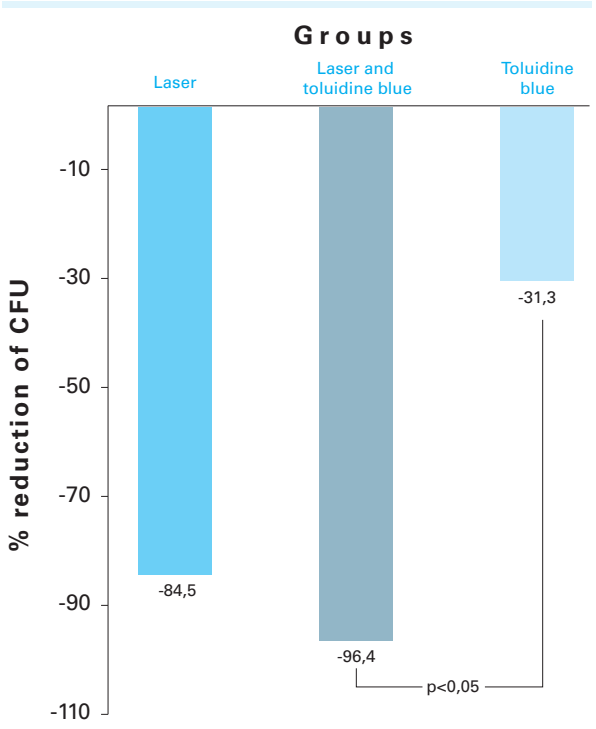

Figure 2: Comparison of percentage of reduction of CFU between evaluated groups. Values expressed in percentage reduction. Kruskal-Wallis test (p = 0.0022) and post-Dunn's test (p <0.05)

Discussion

Studies about the process of tissue repair with the use of low-intensity laser to have awakened in recent years interest of many researchers in different experimental conditions[15,16,17].

However, it is important to note that skin wounds are not always free from bacterial infection.

Currently, treatment of wounds includes irrigating, cleansing, debriding, dressing with wet or dry material, and alleviating pressure[18,19,20]. Other treatments involve the use of ultrasound, electrical stimulation, and ultraviolet and laser irradiation to stimulate tissue healing[10]. Infected skin lesions are typically treated with oral and topical antibacterial agents in addition to other treatments[21,22].

Studies of infected soft tissue lesions have revealed the presence of a variety of microorganisms, including normal skin flora[20,22]. The *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two of the most commonly found bacteria[21,22]. The development of antibiotic resistance in the infected organisms augments the difficulty in disinfecting wounds infected with these bacteria[1,22]. Because bacterial growth interferes with tissue healing, other methods of eliminating microorganisms in the infected wounds would be useful in promoting healing.

The use of lasers with power outputs of ≥ 6 mW in conjunction with photosensitizing agents to induce microbial death has been demonstrated in vitro[23]. Researches related to the bactericidal capabilities of lasers with lower average power output on photosensitized microorganisms are lacking. Such low power lasers (<6 mW) have been used in physical therapy practice[7]. The identification of a bactericidal effect of these lasers could be useful in the treatment of people with infected wounds. This application of laser irradiation could serve as an adjunct to other uses of lasers such as in promoting wound healing[15,24].

Thus, the aims of this study were to determine whether *S. aureus* could be killed by toluidine blue (TBO) *in vivo* mediated photosensitization in an animal model.

The results of this study show that Photodynamic therapy (PDT) was effective in significantly reducing (96.4%) the *Staphylococcus aureus* when compared to the TBO groups (31.3%) and irradiated with laser group (84.5%).

However, we observed that the group which had only the application of the laser also decrease the number of CFU compared to the group TBO, results that are similar to those found in *in vitro* studies in which photodynamic therapy was able to inactivate different types of bacterial cultures[25,26,27,28].

Photosensitization mechanisms are initiated by the absorption of light by a given photosensitizer. After absorption, part of the energy is transferred to the triplet state in the photosensitizer molecule. Either charge (type I reaction) or energy (type II reaction) is transferred

to a substrate or to molecular oxygen to generate reactive oxygen species. In photodynamic action, singlet oxygen (O_2) is considered to execute the most important function. This highly reactive oxygen initiates further oxidative reactions in the proximate environment, such as the bacterial cell wall, lipid membranes, enzymes, or nucleic acids. Therefore, the photodynamic inactivation of bacteria is based on the concept that a photosensitizer can accumulate a significant extent in the cytoplasmic membrane, which subject is the critical target for irreversible damage in bacteria after irradiation[29].

In accordance with the presented results, we can conclude that antibacterial PDT could be an alternative to standard topical antibiotic treatment. However, despite these promising results, only assessment of the technique against a much wider number of clinical *Staphylococcus aureus* isolates can prove the efficacy of PDT for the inactivation of bacteria *in vivo*. PDT could provide an alternative to surgical debridement and topical antimicrobials for these wound and tissue infections, which are otherwise hard to treat.

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