Photoinactivation of *Escherichia coli* using xanthene dyes and light-emitting diodes

Fotoinativação de Escherichia coli utilizando corantes xantenos e Diodo Emissor de Luz

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Abstract

The development of antibiotic resistance by pathogenic bacteria is currently one of the major problems in medicine. Therefore, the study of new treatment modalities such as photodynamic therapy is important. The aim was to evaluate the effects of the Rose Bengal and erythrosine dye combined with a light-emitting diode (LED) on *Escherichia coli*. An *E. coli* suspension was prepared from a clinical strain and subjected to the following treatments: LED and Rose Bengal, LED and erythrosin, LED and physiological solution, and physiological solution only as control, and exposure to light for 60, 120 and 180 seconds. After incubation at 37°C for 24 h, the number of colony-forming units (CFU) was calculated and submitted to analysis of variance (ANOVA). Photodynamic therapy using Rose Bengal resulted in a reduction of 5.58 $\log_{10}$ in the number of CFU/mL after light exposure for 60 s and complete elimination after 180 s. However, photodynamic therapy using erythrosin only caused a slight reduction in the number of CFU/ml (0.30 $\log_{10}$) compared to the control group. The use of the LED alone had no toxic effect on the strain tested. In conclusion, Rose Bengal was more effective than erythrosin in photodynamic therapy against *E. coli*.

Uniterms

Photochemotherapy; *Escherichia coli*; microbiology.

Introduction

The first experience with photodynamic therapy (PDT) dates back approximately 100 years when Raab (1900) observed that exposure to the acridine dye and light is lethal to *Paramecia*. What Raab reported was the principle of PDT, nowadays used for the treatment of cancer and infectious diseases[13].

PDT is a clinical treatment that uses light and a photosensitizer (dye). Its mechanism of action consists of the absorption of photons from the light source by the photosensitizer whose electrons jump

to an excited state. In the presence of a substrate such as oxygen, the photosensitizer, when returning to its ground state, transfers energy to the substrate, forming highly reactive and short-lived anions such as singlet oxygen and peroxide radicals, which cause damage to the cell by irreversible oxidation of its components and consequent cell death[8,12,19].

According to Wainwright [22] (1998), the combination of a dye and a light source for antimicrobial treatment should be called photodynamic antimicrobial chemotherapy (PACT). This therapy represents a highly effective alternative for the treatment of localized microbial infections, such as chronic ulcers, acne lesions and a variety of oral infections. PACT seems to be effective against antibiotic-sensitive and resistant microorganisms. In addition, repeated applications do not result in the selection of bacteria[23].

The synthesis and development of photosensitizer, the key element in effective PDT, has drawn tremendous academic and industrial interest in recent years. For antimicrobial applications, a good photosensitizer should ideally possess such features as: (1) high quantum yield of generating singlet oxygen; (2) minimal or no dark toxicity, and (3) good specificity or selectivity towards the target(s)[10].

The use of dyes as topical drugs has been discussed since the beginning of the century. An ideal photosensitizer should not be toxic *per se* but should only exert toxicity after being activated by irradiation[14]. The dyes most frequently used in PDT are phenothiazines (methylene blue and toluidine blue), phthalocyanines (azulene and other phytotherapeutic agents), chlorins (polylysines), porphyrins (hematoporphyrins HCl) and xanthenes (erythrosin, eosin, Rose Bengal)[22]. Banks et al.[1] (1985) inactivated the following microorganisms using Rose Bengal: *Brochothrix thermosphacta*, *Deinococcus radiodurans*, *Streptococcus*, *Micrococcus*, *Staphylococcus*, *Bacillus*, *Arthrobacter*, *Kurthia*, *Pseudomonas*, and Enterobacteriaceae. Dahl et al. [6] (1988) compared the response of different bacteria to PDT using Rose Bengal. Photoinactivation of Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Streptococcus salivarius*) was 200-fold faster than inactivation of Gram-negative bacteria (*Salmonella typhimurium*). This finding was attributed to the fact that the cell wall of Gram-negative bacteria contains lipopolysaccharides and positive charges that are opposite to the cationic nature of dyes. Schäfer et al.[20] (2000) were also successful in inactivating *Escherichia coli* using Rose Bengal as the photosensitizer.

Xanthene dyes such as erythrosin and Rose Bengal show strong absorption of light in the spectral range of 500 to 550 nm, a range corresponding to that emitted by light-emitting diodes (LEDs; blue and green light). LEDs represent an alternative light source for PDT because of their low cost, low thermal component and monochromatic light with a bandwidth in the order of 40 nm[13].

LEDs are power efficient, light in weight, produce less heat, and have a longer lifetime. These unique characteristics of LEDs make them very optimal light sources for phototherapy devices. As LED devices could generate significantly higher light irradiance levels compared to all currently available conventional phototherapy units, much higher irradiance levels are likely to be used clinically in the future[4]. Blue LEDs are widely used in dentistry for tooth whitening and light-curing of composite resins. In an attempt to offer multifunctional devices to professionals, some companies are marketing devices that combine blue light sources or hybrid sources with low-intensity diode laser emitting visible (red) and infrared light for biomodulation[3,12].

Microorganisms of the Enterobacteriaceae family may act as opportunist pathogens and colonize the human oral cavity, especially in patients with debilitating diseases who are submitted to prolonged treatments with antibiotics or cytotoxic medications. Previous studies reported the presence of this group of microorganisms in the oral cavity[5]. A clinical study conducted by Goldberg et al.[9] evaluated the prevalence of Enterobacteriaceae in four different populations and they detected Enterobacteriaceae in 48% of patients with complete dentures, 27.1% of patients with halitosis, 16.4% of controls and 13% of orthodontic patients. During chemotherapy, Napeñas et al.[16] reported that the most frequent Gram-negative species isolated were from the Enterobacteriaceae family, *Pseudomonas* spp. and *E. coli.*

Escherichia coli, a Gram-negative and non-sporulating facultative anaerobe, is found in the gut microbiota, which consists of more than 500 species of bacteria that total $10^{10} – 10^{11}$ cells per gram of large-intestinal content. Although the anaerobic bacteria in the bowel outnumber *E. coli* by 100/1 to 10,000/1, this bacterium is the predominant aerobic organism in the gastrointestinal tract[21]. This is the bacterial species more isolated in the clinical laboratories and already it was associated the infectious illnesses involving all virtually the human fabrics and organic systems. *E. coli* strains that contain enterotoxins and/

or other virulence factors, cause diarrheal disease. This bacteria is also a major cause of urinary tract infections and nosocomial infections including septicemia and meningitis[11].

Nowadays the levels of antibiotic resistance in *Escherichia coli* have reached a point where they pose severe clinical challenges to humans. *E. coli* is frequently used for studies of resistance status and development, and displays a relatively rapid transfer of resistance between strains, particularly if plasmid-borne. It is also a major player in the spread of the recently emerged resistance to β-lactam antibiotics through the action of CTX-M β-lactamases, enzymes that have their origin in chromosomally located genes of other members of the Enterobacteriaceae family, i.e. *Kluyvera* sp. and possibly others[2].

The objective of the present study was to evaluate the effects of PDT on *E. coli* isolated from the human oral cavity.

Material and Methods

Preparation of the *E. coli* suspension

A suspension containing 10^6 cells/mL was prepared from a clinical *E. coli* strain isolated from the human oral cavity. For this purpose, the strain was seeded onto MacConkey agar (Difco, Detroit, USA) and incubated for 48 h at 37°C. Next, the microorganism was cultured in brain-heart infusion broth (Difco) for 18 h at 37°C. The culture was then centrifuged at 1300 x*g* for 10 min and the supernatant was discarded. The sediment was resuspended in 5 mL sterile physiological solution (0.85% NaCl). This procedure was repeated and the number of cells in the suspension was determined by spectrophotometry (B582, Micronal, São Paulo, Brazil) at a wavelength of 590 nm.

Photosensitizer and LED

Rose Bengal and erythrosin (both from Sigma, São Paulo, Brazil) at a concentration of 50 μM were used as photosensitizers for the sensitization of *E. coli*. Each dye solution was prepared in 0.85% NaCl and sterilized by membrane filtration (pore diameter of 0.22-μm; MFS, Dublin, USA).

A blue LED (Twin Flex II Multifuncional System, MM Optics, São Carlos, Brazil) with a peak wavelength at 460 nm, corresponding to the region of maximal absorption of the photosensitizers, was used as the light source (power of 200 mW). Exposure times of 60, 120 and 180 s were tested. The LED illuminated an area of 0.38 cm^2.

In vitro photosensitization

An aliquot (0.1 mL) of the *E. coli* suspension and 0.1 mL of the photosensitizer or physiological solution were added to a sterile flat-bottom 96-well microtiter plate. The plate containing the samples was shaken for 5 min in an orbital shaker (Solab, Piracicaba, Brazil). After this period, the content of each well was irradiated according to the following groups: LED and Rose Bengal (L+RB+), LED and erythrosin (L+E+), LED and physiological solution (L+P-), and physiological solution only as control (L-P-). Five assays were carried out per experimental group using three LED irradiation times, for a total of 60 assays.

The samples were irradiated under aseptic conditions in a laminar flow hood. The experiment was carried out in the dark and an opaque black shield with an orifice whose diameter corresponded to that of the well opening was used to prevent the spreading of light.

After irradiation, serial dilutions were prepared from each sample and 0.1 mL aliquots of the dilutions were seeded, in duplicate, onto brain-heart infusion agar (Difco). After incubation for 48 h at 37°C, the number of colony-forming units (CFU/ml) was determined.

Statistical analysis

The CFU/mL results were log transformed and analyzed by analysis of variance (ANOVA) and the Tukey test. Statistical analysis was performed using the Minitab program (Minitab Inc, PA, EUA) with the level of significance set at 5%.

Results

PDT with Rose Bengal (L+RB+) resulted in a reduction of the number of CFU/mL when compared to the control group (L-P-) for all exposure times studied. There was a reduction of 5.58 $\log_{10}$ when the LED was applied for 60 s, 6.60 $\log_{10}$ when applied for 120 s, and complete elimination of the microorganism was observed when the LED was applied for 180 s (Figure 1). The differences observed between the L+RB+ and L-P- groups were statistically significant for all exposure times (Tables 1, 2 and 3).

Table 1 - Comparison between the experimental groups (*p* value) after exposure to the LED for 60 s (Tukey test)

	L+RB+	L+E+	L+P-	L-P-
L+RB+	-	-	-	-
L+E+	0.000*	-	-	-
L+P-	0.000*	0.999	-	-
L-P-	0.000*	0.220	0.197	-

*Statistically significant difference

L+RB+: rose bengal and LED, L+E+: erythrosine and LED, L+P-: physiologic solution and LED, L-P-: physiologic solution alone

Table 2 - Comparison between the experimental groups (*p* value) after exposure to the LED for 120 s (Tukey test)

	L+RB+	L+E+	L+P-	L-P-
L+RB+	-	-	-	-
L+E+	0.000*	-	-	-
L+P-	0.000*	0.262	-	-
L-P-	0.000*	0.540	0.947	-

*Statistically significant difference

L+RB+: rose bengal and LED, L+E+: erythrosine and LED, L+P-: physiologic solution and LED, L-P-: physiologic solution alone

Table 3 - Comparison between the experimental groups (*p* value) after exposure to the LED for 180 s (Tukey test)

	L+RB+	L+E+	L+P-	L-P-
L+RB+	-	-	-	-
L+E+	0.000*	-	-	-
L+P-	0.000*	0.002*	-	-
L-P-	0.000*	0.005*	0.977	-

*Statistically significant difference

L+RB+: rose bengal and LED, L+E+: erythrosine and LED, L+P-: physiologic solution and LED, L-P-: physiologic solution alone

PDT with erythrosine (L+E+) resulted in slightly lower mean number of CFU/mL when compared to the control group L-P- for all exposure times. A reduction of 0.3 $\log_{10}$ was observed when the LED was applied for 60 s, of 0.32 $\log_{10}$ when applied for 120 s, and of 0.37 $\log_{10}$ when applied for 180 s (Figure 1). A significant difference between the L+E+ and L-P- groups was only observed for the exposure time of 180 s (Tables 1, 2 and 3).

The L+P- group presented results similar to those of the control group (L-P-) at the three times studied, indicating that the use of the LED alone did not cause microbial reduction (Figure 1).

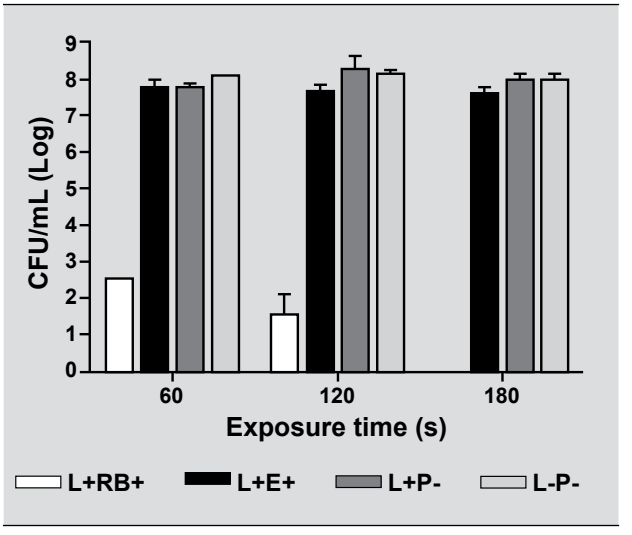

Figure 1 - Mean and standard deviation of CFU/ml of *Escherichia coli* in the groups submitted to treatment with an LED and Rose Bengal (L+RB+), LED and erythrosin (L+E+), LED and saline (L+P-), and saline only (L-P-).

Discussion

Xanthene dyes such as Rose Bengal, eosin, fluorescein and erythrosin are cyclic compounds that contain three aromatic rings in a linear arrangement and an oxygen atom in the center of the ring, which

absorbs light in the visible range. These dyes do not bind to the cell membrane and are found in the cytoplasm of the cell. The use of xanthenes as photosensitizers in PDT has been reported by investigators such as Paulino et al.[17] (2005), Demidova et al.[7] (2005) and Wood et al.[24] (2006).

The present results showed that *E. coli* can be inactivated by the combination of Rose Bengal as the photosensitizer and a blue LED with a peak wavelength at 460 nm after 60, 120 and 180 s of irradiation. These results agree with previous studies showing the antimicrobial effects of PDT using an LED[18] and Rose Bengal [7] against *E. coli*. Peloi et al.[18] (2008), testing a red LED combined with methylene blue as the photosensitizer, observed a reduction of 93.05%, 93.70% and 93.33% in *Staphylococcus areus*, *E. coli* and *Candida albicans* counts, respectively.

Demidova et al.[7] (2005) compared the efficacy of Rose Bengal and toluidine blue activated by visible light against *S. aureus*, *E. coli* and *C. albicans* and observed that toluidine blue was less effective than Rose Bengal in all cases. In that study, PDT using Rose Bengal as a photosensitizer resulted in a reduction of *E. coli* counts of 2, 3 and 4 $\log_{10}$ in experiments containing 10^9, 10^8 and 10^7 cells/ml, respectively. Similar results were obtained in the present study using an *E. coli* suspension containing 10^6 cells/ml, with a reduction of 5.58 and 6.60 $\log_{10}$ after exposure for 60 and 120 s and complete elimination of the microorganism after exposure for 180 s.

Paulino et al.[17] (2005) also investigated the photodynamic action of Rose Bengal against *Streptococcus mutans*. The authors tested different concentrations of the dye ranging from 0 to 50 μM and observed 100% death of S. *mutans* when Rose Bengal concentrations higher than 0.5 μM were used.

In the present study, the use of erythrosin and LED (L+E+) resulted in a slight reduction of the number of CFU/ml of *E. coli* compared to the control group (L-P), with the difference being significant for the exposure time of 180 s. However, some studies have reported a greater antimicrobial action of photosensitization with erythrosin activated by a light source. Wood et al.[24] (2006) investigated the efficacy of PDT against biofilms of *Streptococcus mutans* using erythrosin and light of a wavelength in the range of 500-650 nm. The authors observed reductions of 2.2 and 3.0 $\log_{10}$ in CFU for biofilms aged 48 and 28 h, and concluded that erythrosin is an excellent photosensitizer for the treatment of oral biofilms. Metcalf et al.[15] (2006) also tested the photodynamic action of erythrosin against *Streptococcus mutans*. The authors observed a reduction in bacterial counts of 2 $\log_{10}$ with 5 min of continuous irradiation and of 3.7 $\log_{10}$ when the light was fractionated into time intervals of 10 to 30 s.

Compared to other light sources, LEDs seem to be an excellent option for PDT because of their high efficiency and low cost. In addition, xanthene dyes are promising because of their low toxicity. Rose Bengal, a halogen derivative of fluorescein, has been used in ophthalmology as a dye for the diagnosis of various external diseases of the eye. Erythrosin is used for staining of the dental biofilm and has been approved for use in the oral cavity[19]. The present results show that the photosensitizer Rose Bengal seems to be a good option for use in PDT against *E. coli*, whereas further studies are necessary to evaluate the efficacy of erythrosin.

Conclusion

Rose Bengal was effective as a photosensitizer in PDT against *E. coli*. PDT using erythrosin only resulted in a slight reduction of bacterial counts compared to the control group. The use of the LED alone had no bactericidal effect on the microorganism studied.

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Resumo

O desenvolvimento de resistência aos antibióticos por bactérias patogênicas é um dos maiores problemas da medicina atual. Assim, torna-se importante o estudo de novas modalidades de tratamento, como a terapia fotodinâmica. O objetivo deste estudo foi avaliar os efeitos dos fotossensibilizadores rosa bengal e eritrosina associados a um diodo emissor de luz (LED) sobre *Escherichia coli*. Foi preparada uma suspensão padronizada de *E. coli* (10^6 células/mL) a partir de uma cepa clínica isolada da cavidade bucal humana. Essa cepa foi submetida aos seguintes tratamentos: laser e rosa bengal (L+RB+), laser e eritrosina (L+E+), laser e solução fisiológica (L+F-) e apenas solução fisiológica como controle (L-F-) nos tempos de 60, 120 e 180 segundos de exposição à luz. Foi utilizado LED emissor de luz azul (460 nm), rosa bengal e eritrosina na concentração de 50 μM. A seguir, foram realizadas culturas em ágar Infuso Cérebro-Coração para a contagem de unidades formadoras de colônias (UFC/mL) e os dados submetidos à análise de variância. A terapia fotodinâmica utilizando rosa bengal foi capaz de reduzir o número de UFC/mL de 5,58 $\log_{10}$ no tempo de exposição do LED de 60 seg até eliminação completa do microrganismo no tempo de 180 seg. Entretanto, a terapia fotodinâmica com eritrosina apresentou discreta redução do número de UFC/mL (0,30 $\log_{10}$) quando comparada ao grupo controle. O uso isolado do LED não apresentou toxicidade para as cepas testadas. Concluiu-se que o Rosa Bengal foi mais eficaz do que a eritrosina como fotossensibilizador na terapia fotodinâmica sobre *Escherichia coli*.

Unitermos

Fotoquimioterapia; *Escherichia coli*; microbiologia.

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