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Photodynamic therapy effect on cell growth inhibition induced by Radachlorin and toluidine blue O on *Staphylococcus aureus* and *Escherichia coli*: An in vitro study

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ABSTRACT

Introduction: Photodynamic therapy is an innovative treatment modality, which is appropriate for tumor detection and for the treatment of cancer as well as nontumoral diseases, such as psoriasis (2), bacterial and viral eradication.
Material and method: Effect of two photosensitizer (toluidine blue O (TBO) and Radachlorin was investigated on *Staphylococcus Aureus* ATCC 25923 (American Type Culture Collection) and *Escherichia coli* (ATCC 25922).
Results: PDI by TBO caused *S. aureus* 5.83 log10 killing (P.Value < 0.0001) and reduce 0.08 log 10 in *E. coli* (P.Value = 0.321). PDI by Radachlorin® reduce 0.17 log 10 in *E. coli* (P.Value < 0.0001) and *S. aureus* showed 6.1 log 10 colony count reduction.
Conclusion: Within the limitation of this in vitro study, we can conclude that both PS have the same effect on *S. aureus* and *E. coli* with good inhibition effect on *S. aureus* and partial inhibition effect *E. coli*.

1. Introduction

Photodynamic therapy is an innovative treatment modality, which is appropriate for tumor detection and for the treatment of cancer [1] as well as nontumoral diseases, such as psoriasis [2], bacterial and viral eradication [3,4]. Antimicrobial Photodynamic Inhibition (PDI) is based on the concept that a photosensitizer is favorably taken up by bacteria and subsequently activated by light of the appropriate physical parameter (mainly light wavelength) in the presence of oxygen to generate cytotoxic reactive oxygen species, which are toxic to bacterial species [5,6]. Photosensitizer (PS) is a main factor that determines the effectiveness of PDI. Ideally, this molecule should have some properties, including high binding affinity to microorganisms; high concentrations of the activated drug in target area; high singlet oxygen quantum yield; and a broad scale of action [7,8]. Radachlorin is a hydrosoluble chlorin and composed of set of sodium salts of chlorin e6, chlorin p6, and purpurine-5 [9]. Chemo-physical properties of Radachlorin including water solubility, high quantum yield of singlet oxygen (75%), low toxicity in the dark, intensive absorption band in the middle-red part of the spectrum, quicker kinetics in comparison with chlorin e6 alone and high phototoxicity make it a good agent as a photosensitizer for PDI [10–15]. At the present time, phenothiazinium salts such as toluidine blue O (TBO) is used clinically for antimicrobial treatments [16]. The slight toxicity of these dyes to human cells, plus their ability to produce high quantum yields of singlet oxygen, has produced a great interest in testing these PSs as photo-activated antimicrobial agents [17]. These compounds are cationic at physiological pH (7.4), which enables them to target bacterial membranes of both Gram-positive and Gram-negative bacteria [18].TBO is effective PSs against a broad range of microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, streptococci, Listeria monocytogenes [19–22].

Common wound infecting bacteria are *Staphylococcus intermedius*, *Streptococcus canis*, *Pseudomonas aeruginosa*, and

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Table 1
Mean value log 10 *E. coli* and *S. aureus* colony counts for *TBO*, (C1): no laser, no photosensitize,(PS1): treated only with photosensitizer, (L1): treated only with laser and (LPS1): treated with laser and photosensitizer; photodynamic therapy group.

	Control (C1)	PS only (PS1)	Laser only (L1)	Laser + PS (LPS1)	P Value
S. aureus	7.9875	7.9310	7.9265	2.1535	<0.01
E. coli	7.9475	7.9065	7.9000	7.8605	0.321

Escherichia coli [23] *Staphylococcus Aureus* is an important human pathogen capable of causing health care associated as well as community acquired infections. It may cause local infections, such as postoperative or injury wound infections and osteomyelitis [24–27]. This bacterium is also responsible for infections related to prosthesis, catheters and other biomaterials [25,27,28]. Also *Staphylococcus aureus* causing generalized infections some of them may be life-threatening, especially in the case of immunocompromised patients, causing bacteremia, endocarditis, and sepsis or toxic-shock syndrome (TSS).

The main purpose of this study was to explore the PDI effect of Radachlorin and TBO on *S. aureus* and *E. coli*.

2. Material and method

2.1. Bacteria

Two strains of bacteria used in this study were *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection) and *Escherichia coli* (ATCC 25922).

These bacteria were maintained by weekly subculture on nutrient agar (Merck). These bacteria were grown in brain-heart infusion broth in an orbital shaker at 37 °C for 24 h. An aliquot of this suspension was then added to nutrient broth and grown to mid-log phase (OD600 = 0.6, 10^8 cells/mL).

2.2. Photosensitizers

Radachlorin® gel (0.1%, 25 g) was obtained from RADA-FARMA Ltd, Russia and stored at 0–8 °C in the dark.

Toluidine blue O (TBO) (Sigma, St. Louis, MO, USA) solutions were prepared fresh for each experiment in dissolved in sterile saline firstly to reach the final concentration of 0.1% and then subsequently kept in the dark.

2.3. Laser sources and photodynamic therapy

For Radachlorin® gel, the laser sources were used a diode laser (Milon-LAHTA, Russia) with a fiber optic diameter of 800 μm, a maximum output of 2.5 W and a predominant wavelength of 662 nm.

For TBO, the laser sources were used a diode laser (Mustang 2000, Moscow, Russia) with a hand held probe of KLO4 and a maximum power output of 32 mW at a wavelength of 633 nm for TBO.

The laser parameters used in this study for bacterial suspension were 213 mW/cm^2 (power density) and 6 J/cm^2 (energy density) and continuous mode for Radachlorin® and 32 mW/cm^2, 6 J/cm^2 and continuous mode for toluidine blue O.

The intensity of the light spot was evaluated as follows. A power meter (model DMM 199 with 201 standard head, Coherent, Santa Clara, CA) is used to measure the irradiance (power density in mW/cm2).

Preparation of suspension of microbial cells, Prepare liquid media (brain-heart infusion broth, BHI, for bacteria) and autoclave. Prepare solid media by addition of 1.5% microbiological agar to above broth and pour into 10 × 10 cm square petri dishes. The concentration of 0.2 ml of each Photosensitizers were applied on 0.2 ml of the bacterial suspensions and were placed in a 48-well microtiter plate. The distance between the laser tips and the illuminated area was adjusted to create a spot light of 1 cm in diameter with a fixed power density for each photosensitizer.

The following groups were used; *(C)*: no laser, no photosensitizer, *(PS)*: treated only with photosensitizer, *(L)*: treated only with laser, *(LPS)*: treated with laser and photosensitizer; photodynamic therapy group. Finally we have 16 groups, eight groups for Radachlorin® and eight groups for TBO.

Each aliquot of microbial cell suspension is individually subjected to five ten-fold serial dilutions in sterile PBS. This will provide tubes with dilutions of 1×, 10×, 100×, 1000×, 10,000×, and 100,000×. 10 μL of each is dilution is horizontally streaked on square agar plates according to the method of Jett et al. [29].

After 24-h incubation the plates are counted. Ideally two or three rows can be counted on each plate, the results multiplied by the appropriate power of ten and averaged to give the number of CFU/ml in each aliquot of cell suspension.

2.4. Statistical analysis

Values were expressed as log 10 means ± standard deviation. Comparisons between means of groups And between Radachlorin® versus TBO and *S. aureus* vesus *E. coli* response for PDI were used the univariate analaysis of variance and Post $P < 0.05$ was considered statistically significant.

2.5. Results

This study showed photodynamic therapy by TBO caused *S. aureus* 5.83 log10 killing (LPS1) and There are highly significant differences in Photodynamic therapy group (LPS1) (P.Value < 0.0001) and other groups (Table 1). Other groups of *S. aureus* (PS1 and L1) showed no significant differences in compared to the control group (C1). Photodynamic therapy by TBO reduce 0.08 log 10 in *E. coli* (LPS1) and There are not significant differences between LPS1 group and other groups was obtained (P.Value = 0.321). Profile pilot diagram of *E. coli* and *S. aureus* show colony count means value in each groups (Fig. 1). For analysis of difference between *E. coli* and *S. aureus* in response to photodynamic therapy by TBO Independent *t*-test was done. This test show colony count of *S. aureus* compared to *E. coli* significantly was reduced (P.Value < 0.0001).

PDI mediated by Radachlorin® reduce 0.17 log 10 in *E. coli* (LPS2) and There are significant differences with control group (C2) (P.Value < 0.01) (Table 2) but no differences in other groups and control group (C2) was obtained. Photodynamic therapy by Radachlorin® in *S. aureus* showed 6.1 log 10 colony count reduction (LPS2) and There are highly significant differences in Photodynamic therapy group (P.Value < 0.0001) and other groups (Table 2). Other groups of *S. aureus* (L2 and PS2) showed no significant differences in compared to the control group (C2). Profile pilot diagram of *S. aureus* and *E. coli* show colony count means value in each groups (Fig. 2). For analysis of difference between *E. coli* and *S. aureus* in response to photodynamic therapy by Radachlorin® Independent *t*-test was done. This test show colony count of *S. aureus* compared to *E. coli* significantly was reduced (P.Value < 0.0001).

Table 2
Mean value log 10 *E. coli* and *S. aureus* colony counts for *Radachlorin*®, (C2): no laser, no photosensitizer, (PS2): treated only with photosensitizer, (L2): treated only with laser and (LPS2): treated with laser and photosensitizer; photodynamic therapy group.

	Control (C2)	PS only (PS2)	Laser only (L2)	Laser + PS (LPS2)	P Value
S. aureus	7.9185	7.9380	7.8910	1.8150	<0.01
E. coli	7.9480	7.8930	7.8545	7.7705	<0.01

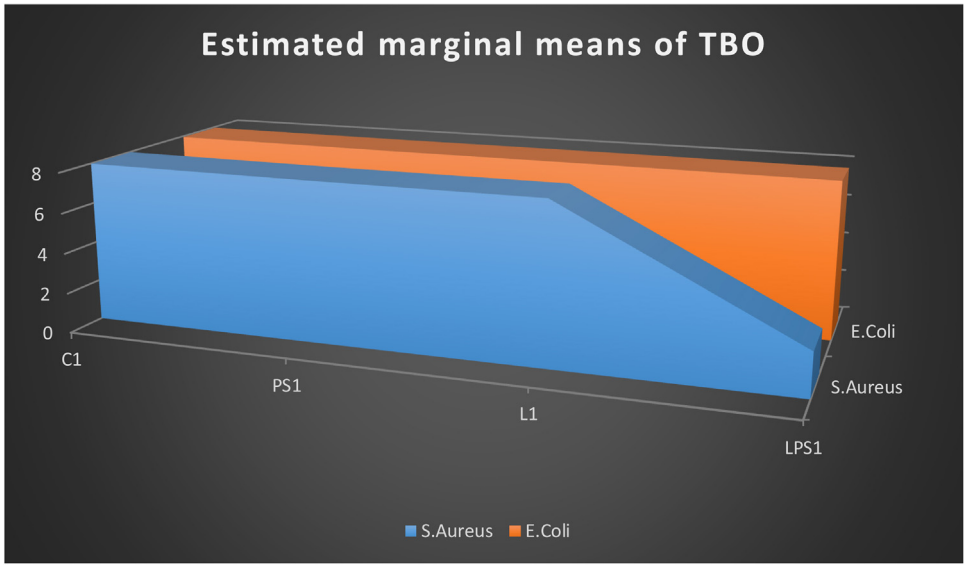

Fig. 1. Estimated marginal means *E. coli* and *S. aureus* colony counts for *TBO*, (C1): no laser, no photosensitizer, (PS1): treated only with photosensitizer, (L1): treated only with laser and (LPS1): treated with laser and photosensitizer; photodynamic therapy group.

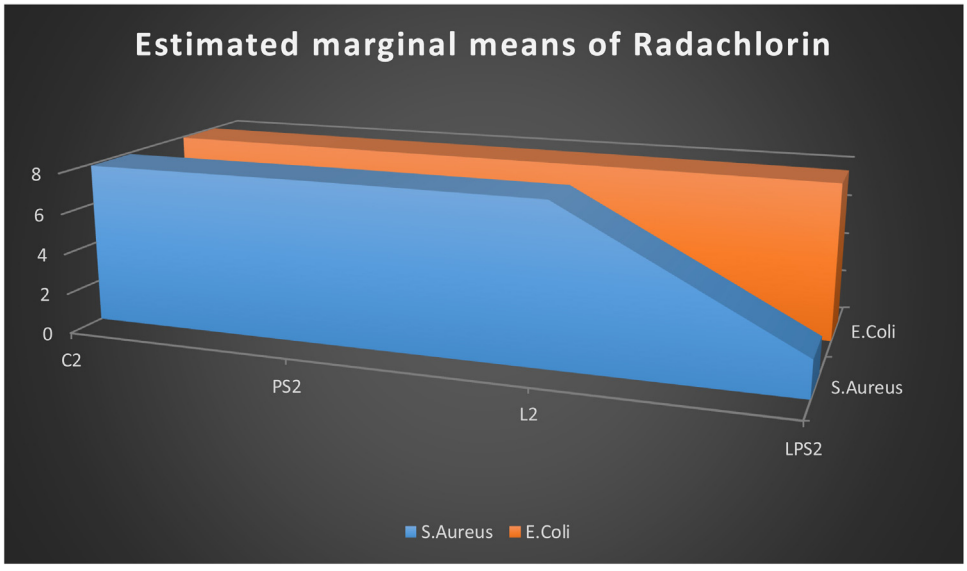

Fig. 2. Estimated marginal means *E. coli* and *S. aureus* colony counts for *Radachlorin*®, (C2): no laser, no photosensitizer, (PS2): treated only with photosensitizer, (L2): treated only with laser and (LPS2): treated with laser and photosensitizer; photodynamic therapy group.

2.6. Discussion

The ability of Radachlorin® to act as a photosensitizer after irradiation with laser photons has been demonstrated in a few studies, but been several studies on chlorin e6, which is a major component of Radachlorin® [30–34] this study examined the antimicrobial effect of Radachlorin® and TBO mediated PDI against *S. aureus*, *E. coli*. Statistical analysis showed that Radachlorin® and TBO mediated PDI are very effective in inhibiting the growth of *S. aureus* (6.1 log 1o versus 5.83 log 10) but PDI against *E. coli* by Radachlorin® and TBO showed less effectiveness (0.17 log versus 0.04). The efficacy of antimicrobial PDT for different pathogens depends on the type and concentration of the photosensitizer. Furthermore, reduced susceptibility to PDT has been demonstrated when microorganisms are organized in biofilms [35].

It was observed that there was a fundamental difference in susceptibility to PDI between Gram-positive and Gram-negative bacteria. It was found that in general neutral or anionic PS

Table 3
Mean value log 10 *E. coli* colony counts for *TBO and Radachlorin®*, (C1): no laser, no photosensitize,(PS1): treated only with photosensitizer, (L1): treated only with laser and (LPS1): treated with laser and photosensitizer; photodynamic therapy group.

	Control (C1)	PS only (PS1)	Laser only (L1)	Laser + PS (LPS1)
TBO	7.9475	7.9065	7.9000	7.8605
Radachlorin®	7.9480	7.8930	7.8545	7.7705
P Value	0.345	0.311	0.287	0.307

Table 4
Mean value log 10 *S. aureus* colony counts for *TBO and Radachlorin®*, (C2): no laser, no photosensitizer, (PS2): treated only with photosensitizer, (L2): treated only with laser and (LPS2): treated with laser and photosensitizer; photodynamic therapy group.

	Control (C1)	PS only (PS1)	Laser only (L1)	Laser + PS (LPS1)
TBO	7.9875	7.9310	7.9265	2.1535
Radachlorin®	7.9185	7.9380	7.8910	1.8150
P Value	0.215	0.314	0.301	<0.01

molecules are efficiently bound to and inactivate Gram-positive bacterial and fungal cells, whereas Gram-negative bacterial cells are relatively resistant to these compounds [36]. The high susceptibility of Gram-positive bacteria and fungi was clarified by their physiology as their cytoplasmic membrane is surrounded by a relatively spongy layer of peptidoglycan and lipoteichoic acid, or beta-glucan and chitin, respectively, and both these structures allow non-cationic PSs to cross [37].

Bactericidal activity of one antimicrobial agents means >3 log10 reduction of bacterial counts and Bacteristatic activity of one antimicrobial agents means <3 log10 reduction of bacterial counts [38]. According to this study, Radachlorin® have Bactericidal effect on *S. aureus* (6.1 log 10) and Bacteristatic effect on *E. coli* (0.17 log 10). Several studies showed that Gram negative bacteria are largely resistant to antimicrobial photodynamic therapy due to their special cell wall structure [39,40]. Park et al. reported pure chlorin e6 mediated PDT also nearly inhibited the colony formation of *S. aureus* and *P. aeruginosa*, and partially inhibited that of *E. coli* and *S. typhimurium* [41]. Fomichev et al. reported the effectiveness of *E. coli* photo-inactivation in the presence of chlorines was 100–200 times lower as compared with that of B. subtilis [42]. Hope et al. reported The SnCe6/Phi11 conjugate achieved a statistically significant reduction in the number of viable bacteria of both 8325-4 and EMRSA-16 strains by 2.31 log(10) and 2.63 log(10), respectively. The conjugate could not however instigate lethal photosensitization of *Escherichia coli* [43]. According to above studies, almost PDI efficacy of the Radachlorin® was same as the chlorin e6 and both have good effect on *S. aureus* and partially inhibited *E. coli* Table 3.

The ability of TBO to act as a photosensitizer demonstrated in several studies, but the results of PDI are different. According to Tang et al. study, resulted in 3-log killing of MRSA and 2-log killing of the corresponding *S. aureus* strain that irradiation at 30 J/cm2 in the presence of TBO at 80 M (approximately 25 g/ml) however, for TBO-mediated PDI (400 M TBO, 30 J/cm2) against the *E. coli* strains, the degree of killing (95%) observed for ESBL producing *E. coli* was identical to that observed for *E. coli* (ATCC 25922) [34]. Hajim et al. reported a total of 34 isolates of *S. aureus* were exposed for 5, 10 and 15 min to a HeNe laser at a 7.5 mW output power in the presence of 50 g/ml toluidine blue O photosensitizer. The viable count was substantially decreased for the three exposure times, with 100% killing with the 15-min exposure time [44]. In the present study showed PDI by TBO 5.83 log killing was obtained for *S. aureus* (ATCC 25923), while 0.07 log10 of *E. coli* (ATCC 25922).

This study's results depended to physical properties of laser, bacterial strain and PS dose. Maybe by change some parameters may get different results.

Nowadays the cost of PS and laser equipment effect on finally cost of PDI treatment. Hence according to limitation of this study may use of TBO are cost to benefit.

The advantages of antimicrobial photodynamic therapy over usual antimicrobial agents are non-invasive nature, good selectivity, repeatability, no resistance to drugs, rapid killing of target microorganisms in a few minutes depending on the energy densities delivered and antimicrobial effects of PS may be confined to the site of the lesion [45].

Although Radachlorin® and TBO mediated antimicrobial photodynamic therapy is very effective in inhibiting Gram-positive bacteria such as *S. aureus*, it is further necessary to design suitable strategies enhancing the permeability of the outer wall for PS in Gram negative bacteria such as *E. coli* and evaluate bacteria on biofilm or animal wound Table 4.

3. Conclusion

Within the limitation of this in vitro study, we can concluded that both PS have same effect on *S. aureus* and *E. coli* that good inhibition effect on *S. aureus* and partially inhibition effect *E. coli*.

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