Evaluation of the effect of photoactivated disinfection with Radachlorin® against *Streptococcus mutans* (an in vitro study)

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**KEYWORDS**
Photodynamic therapy; Photoactivated disinfection; Radachlorin®; *Streptococcus mutans*

**Summary**

**Background:** The use of photoactivated disinfection has had a significant medical and technological effect in bacterial inactivation, as an alternative to conventional antimicrobial methods. The main goal of this study was to investigate the effect of photoactivated disinfection on *Streptococcus mutans*, when Radachlorin® was used as a photosensitizer.

**Methods:** *Streptococcus mutans* samples of two different initial concentrations were treated with Radachlorin® gel (0.1%), irradiated by the light of a He—Ne laser (633 nm), with energy density of 6 J/cm^2^, and cell viability was evaluated after culturing.

**Results:** It was observed that the combination of Radachlorin® and laser was more effective than Radachlorin® or laser alone (*p* < 0.05), in reduction of *S. mutans* and Radachlorin® was cytotoxic, in the dark, only for the lower concentration of bacteria. Lower concentration of *S. mutans* resulted in higher amount of killing, in the case of using Radachlorin® with or without laser.

**Conclusions:** The photoactivation of Radachlorin® using a He—Ne laser could inactivate *S. mutans* to a significant level. In addition Radachlorin® might be cytotoxic in the dark, for the lower concentration of bacteria.

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**Introduction**

Dental caries is a chronic contagious disease, involving demineralization of the tooth, followed by the destruction of the organic phase of the dentin [1]. The purpose of caries control would better focus on diagnosis and elimination of the main contributing factors, not only the treatment of the lesion itself [2] because as it is always mentioned prevention of
disease is more desirable from financial point of view and is less time consuming. *Streptococcus mutans* has been implicated as the primary etiologic factor in caries formation, because of its relatively high number in plaque prior to the appearance of caries lesion. The bacterium has the ability to degrade carbohydrates, produce abundant acid and induce a tolerance to low pH environment [3]. The ability of acid production lowers the pH to around 4.5 on the tooth surface, resulting in enamel and dentin demineralization [4,5].

Current treatment protocol for caries includes mechanical removal of the infected structure and maintenance of good oral hygiene [5], which can be the subject of poor patient compliance [6]. An alternative strategy to treat this lesion would be killing the causative microorganism in situ [3]. Several methods have been developed to control bacterial proliferation in the mouth, consisting of vaccination, fluoride therapy and antibiotic therapy [7–9]. However these protocols usually confront the following problems:

1. Difficulty in maintaining therapeutic concentration of the agent in the mouth environment in which there is a high flow of saliva and gingival sulcular fluid.
2. Development of multidrug resistance and superinfection via effect on normal friendly flora, due to widespread systemic use of antibiotics [10].

Since caries lesion is a localized infection it would be well suited to photodynamic therapy (PDT) [6], which has been routinely used in tumor cells, bacterial and yeast inactivation [11–14]. PDT is a treatment modality which employs the interaction between a nontoxic photosensitizer (PS), light source of a specific wavelength and molecular oxygen, usually present in the tissue [3,4,15,16]. It is based on the principle that a PS is activated by exposure to a suitable light source in the presence of oxygen. The transfer of energy from the activated PS to available oxygen results in the formation of toxic oxygen species such as singlet oxygen and free radicals, which are extremely lethal to certain cells and bacteria [17–21]. A branch of PDT, called photoactivated disinfection (PAD), has been specifically employed for microbial elimination. The bactericidal effect of PAD might be due to two different mechanisms: (I) DNA injuries and (II) cytoplasmic membrane damage leading to events such as inactivation of membrane transport system, inhibition of plasma membrane enzymes, lipid peroxidation and others [17,22]. The advantages of photoactivated disinfection over conventional antimicrobial approaches include simple delivery at the exact target area, little likelihood to develop microbial resistance, non-invasive nature, repeatability and high selectivity [18].

The first water soluble chlorine e6 drug agent was applied as PDT drug in 2000–2001 and its improvement led to formation of Radachlorin® [23]. Radachlorin® represents an aqueous solution of three chlorins, including sodium chlorine e6 (90–95%) as the major ingredient, sodium chlorine p6 (5–7%) and a third chlorin which was not disclosed by the manufacturers [24,25]. Radachlorin®, with a short clearance period, has been efficiently successful in destroying tumors. It has a high maximum absorption wavelength (662 nm) so that the treatment can be carried out at a greater depth [20,24].

Although Radachlorin® has proven to be effective in photodynamic inactivation of tumor cell [24,26,27] and other chlorine e6 derivatives have been able to induce death in different microorganisms [28–30], there have been a few studies on the effect of PAD with Radachlorin® against microorganisms. Therefore in this study we evaluated the effect of photoactivated disinfection with Radachlorin® on *S. mutans* in a bacterial suspension model.

**Materials and methods**

**Streptococcus mutans** growth condition

The ATCC 35668 strains of *S. mutans* were obtained from Remel (Dartford, Kent, UK) and checked for purity before being grown in broth. The liquid medium, used to cultivate and check the cell viability was trypic soy broth (TSB) (Merck, Germany) and mitis salivarius agar (MSA) (Que-lab, Canada) was used as solid medium to count bacterial colonies. Both media were prepared according to the manufacturer’s instructions and autoclaved at 121 °C for 15 min prior to use. Cultivations were carried out in a candle jar in order to obtain a micro-aerophilic atmosphere after lighting a candle inside the jar to burn part of oxygen. For microbial assay, the initial turbidity of the bacterial culture was adjusted to $A_{600 \text{nm}} = 0.5$ (1.5 $\times$ 10⁸ cells/ml), using a spectrophotometer (Milton, Roy, Spectronic, Genesys2). Then 15 μl of the stock was inoculated in “falcon tubes” (15 ml) containing 4 ml of previously autoclaved TSB medium. The initial number of colony forming units per milliliter (CFU/ml) was estimated by measuring the suspension turbidity with a spectrophotometer and verified using CFU/ml counts on MSA after anaerobic growth at 37 °C for 36 h. The solutions were then serially diluted in broth to produce 7.5 $\times$ 10⁷ CFU/ml (higher concentration) and 3.75 $\times$ 10⁷ CFU/ml (lower concentration) and then distributed (1 ml) in assay tubes.

**Photosensitizer and light source**

Radachlorin® gel (0.1%) (Rada-farma Ltd, Russia) was purchased and stored at 0–8 °C in the dark. He–Ne laser (Plasma, Russia) (633 nm) with continuous wave irradiation mode and power of 60 mW was used as the light source. The distance between the laser tip and the tube was adjusted to 5 mm by means of a fabricated holder in order to illuminate the whole suspension from lateral direction.

**Experimental groups**

Each bacterial concentration sample was divided into four different groups ($n = 20$):

- **Ps** group to evaluate Radachlorin® toxicity in the dark.
- **L** group to evaluate laser toxicity without PS.
- **C** group to determine laser toxicity without PS or laser.
- **PS/L** group to indicate the effect of Radachlorin® under laser exposure.
The bacterial suspensions alone or with Radachlorin® were mixed well by a shaker for 2 min to obtain homogenous suspension prior to experiment. 120 s of exposure was performed, using the He–Ne laser to produce energy density of 6 J/cm². In PS and PS/L groups the cell suspensions were incubated with Radachlorin® for 10 min in the dark and at room temperature.

After overnight incubation of all groups, microorganisms were grown by dropping and spreading 50 µl of cell suspension directly on to MSA and incubating it (protected from light) in a candle jar for 36 h at 37°C. The MSA plates were sectioned into 8 equal parts and the number of colonies was then calculated and multiplied to 8 to obtain the colony forming units of every dish. The index numbers were 10³ and 10⁵ according to Kuruvilla scale [31] so that CFU/ml of less than 10³ was taken into account as an ideal result, CFU/ml < 10³ Ideal outcome

10³ ≤ CFU/ml < 10⁵ Acceptable outcome

10⁵ ≤ CFU/ml Unacceptable outcome

The results are summarized in Table 1.

Table 1  Index for the colony count interpretation.

<table>
<thead>
<tr>
<th>Colony count</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU/ml &lt; 10³</td>
<td>Ideal outcome</td>
</tr>
<tr>
<td>10³ ≤ CFU/ml &lt; 10⁵</td>
<td>Acceptable outcome</td>
</tr>
<tr>
<td>10⁵ ≤ CFU/ml</td>
<td>Unacceptable outcome</td>
</tr>
</tbody>
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Discussion

The use of photoactivated disinfection as a method to inactivate microorganisms has a considerable medical effect in addition to being a complementary and alternative technique to the classical antibiotic therapy for infection control [4]. Dental caries may be a disease, well suited to PAD. Caries is often a localized infection and so the sensitizer could be applied to the lesion by means of a syringe and the light could then be delivered via an optical fiber [32]. Therefore use of photoactivated disinfection in caries prevention by inactivation of S. mutans, as the primary cause of the infection, has been the subject of many studies.

Radachlorin® possesses ideal spectral and pharmacokinetic properties to be used in PDT, including water solubility, high quantum yield of singlet oxygen (75%), low toxicity in the dark, rapid clearance, fluorescence at 668 nm and intensive absorption band in the medium red part of the spectrum in the biological medium, where tissues are transparent to a considerable extent [25]. Despite having attractive characteristic, Radachlorin® has been the subject of a few studies, evaluating its PAD effect on microorganisms [33]. However photodynamic therapy for treatment of cancerous lesion has been performed, using Radachlorin® as the photosensitizer and it has proved to be a successful approach [24,26,27]. Furthermore many investigations have suggested PAD with chlorin e6, the major component of Radachlorin®, to be an effective modality in bacterial reduction [28–30] and some data exist which support that Radachlorin possesses quicker kinetics in comparison with chlorin e6 alone [26,27]. To the best of our knowledge, this article might be the first one that determines the effect of photoactivated disinfection with Radachlorin® to eliminate cariogenic bacteria.

This investigation showed that Radachlorin® caused significant reduction in viable colonies of S. mutans irrespective of bacterial concentration, however its interaction with...
He–Ne laser demonstrated to be more effective and the difference was statistically significant. This was in consistency with Risovannaia study which stated that Radachlorin® resulted in death of 100% of Streptococcus pyogenes cells in vitro when irradiated with either continuous or super-pulse laser light [33]. This can be illustrated according to photodynamic principles and the structure of Gram positive bacteria. When Radachlorin® molecules meet bilayer membrane of Gram positive S. mutans, due to their amphiphilic characteristic; they pass through the membrane into the cytosol and then are moved into the other organelles. Following absorption of the laser light, Radachlorin® molecules save light’s energy in an excited electron energy level and then transfer it to oxygen or other biologic molecules. Active oxygen species and free radicals generation contribute to cytotoxic activities inside the bacterial cells. Although it might seem to be an appropriate explanation, the exact mechanism of PAD with Radachlorin® still needs to be more investigated.

Radachlorin® showed toxicity in the dark, which could have been due to the high concentration used in this study. According to the high free radical production of Radachlorin®, in the presence of laser, employing lower concentrations may result in elimination of dark toxicity. Demidova et al. [29] and Hamblin et al. [30] also reported dark toxicity for different derivatives of chlorin e6 (major component of Radachlorin®) against Staphylococcus aureus in low concentration. However this toxicity did not occur in the case of simple chlorin e6. In contrast, Hope and Wilson showed no bactericidal effect of SnCe6 on S. pyogenes [28]. Although no toxicity of Radachlorin® has been reported on S. mutans while using more suitable laser such as diode laser, employing lower concentration. However it is appropriate to suggest applying PAD with Radachlorin® still needs to be more investigated on Radachlorin® toxicity on microorganisms and human cells.

Zanin et al. incubated S. mutans biofilms of different ages with toluidine blue O in the dark for 5 min and irradiated them with He–Ne laser or LED light for 5, 15 or 30 min [3]. Although these parameters showed to be effective, shorter incubation and irradiation periods, as applied in our study would be more clinically acceptable. 2 min irradiation with He–Ne laser alone did not show any significant effect on the bacteria in either concentration, just like what was discussed by Zanin et al. [3].

Although He–Ne laser (663 nm) was not compatible with the maximum absorption wavelength of Radachlorin®; their interaction was effective in the inactivation of S. mutans. This can be explained by considering the wide absorption spectrum of Radachlorin®, previously mentioned. However it is appropriate to suggest applying PAD with Radachlorin®, while using more suitable laser such as diode (662 nm) to obtain much more optimum results. Moreover it should be mentioned that there seems to be a lack of investigation in regard to the evaluation of PAD effect of Radachlorin® on S. mutans biofilm and to specifically limit its action to the microorganism itself, therefore they might be considered as the subjects for future investigation.

In conclusion, under the conditions of this study, photoinactivation of Radachlorin® with He–Ne laser could inactivate S. mutans to a significant level. In addition Radachlorin® might be cytotoxic in the dark for lower concentration of the bacteria.

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References


