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Photoinactivation of *F. nucleatum* and *P. gingivalis* using the ruthenium-based RD3 sensitizer and a conventional halogen lamp

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ABSTRACT

Objective: The photodynamic therapy (PDT) is an alternative method to suppress oral pathogens by the activation of a photosensitizer with laser light. The aim of this study was to investigate the phototoxic effect of three ruthenium-based photosensitizers on *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.

Methods: In this in vitro study *F. nucleatum* and *P. gingivalis* were incubated with three photosensitizers: (i) a hydrophobic tris-(4,7-diphenyl-1,10-phenanthroline)-ruthenium(II)-dication (RD3), (ii) a hydrophilic tris-[(1,10-phenanthroline-4,7-diyl)-bis-(benzenesulfonato)]-ruthenate tetra-anion (RSD3) and (iii) a lower hydrophilic tris-(2,2′-bipyridine)-ruthenium(II) dication (RBY). The subsequent irradiation was done with blue-band halogen light (450–485 nm) for 20 s using a conventional polymerizer. Control samples consisted of bacterial cell suspension irradiated and non-irradiated in the absence of photosensitizer or incubated with the photosensitizer without irradiation. Bacterial inactivation was determined by the numbers of colony-forming units (cfu/ml) after anaerobic cultivation.

Results: The RD3 photosensitizer reduced the viability of *F. nucleatum* by 4-log 10 and of *P. gingivalis* completely after irradiation for 20 s. The viability loss correlated significantly with the concentration of the RD3 photosensitizer and reached a peak at a concentration of 12.5 μM ($p < 0.05$). The RSD3 and RBY photosensitizers had distinctly lower phototoxic effects in comparison to RD3.

Conclusion: The RD3 photosensitizer showed a phototoxic effect on *F. nucleatum* and *P. gingivalis*. The results suggest that the application of the RD3 photosensitizer under visible light may be helpful as an adjunct treatment approach to the inactivation of periodontopathogenic bacteria.

1. Introduction

Periodontitis is one of the most widespread oral diseases, characterized by gingival bleeding, periodontal pocket formation, attachment loss, and alveolar bone loss.[1] The development of these effects is due to special bacteria in association with the immunological response of the host. The complex microbial communities that exist on oral surfaces develop by the way of temporally distinct patterns of microbial colonization.[2,3]

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Especially the Gram-negative periodontopathogenic species *Fusobacterium nucleatum* adheres to different Gram-positive early plaque colonizers and is often detectable in the subgingival biofilm, even in early and more localized stages of periodontitis.[4,5] Other periodontopathogenic bacteria form coaggregations with *F. nucleatum* including the Gram-negative species *Porphyromonas gingivalis*.[3,5] Both bacteria induce apoptosis in gingival epithelial cells and polymorphonuclear blood cells and suppress immunological defense mechanisms.[6–8] For that reason the inhibition of these bacteria is one of the crucial steps in the various procedures of treating periodontitis.[9]

The conventional periodontitis therapy includes the mechanical scaling and root planning methods and, in severe cases, adjunctively the application of antibiotics.[10,11] Not seldom, though, the use of antibiotics induces undesirable side effects, such as allergic reactions, local irritations, and the development of resistant bacteria.[12,13] Therefore, current research is focused on the investigation of alternative antibacterial strategies.[14,15] In this context, the photodynamic therapy (PDT) is a new approach that could be a future alternative adjunct to the application of antibiotics.[16] Recently, our group reported that the PDT effectively improved the clinical signs of periodontitis by reducing periodontopathogenic bacteria.[4,17,18] The underlying mechanism of PDT is the targeting and elimination of bacteria via photosensitizers. These substances bind to bacterial cell wall structures and produce reactive oxidative species (ROS) in response to interaction with a specific wavelength emitted by a suitable light source. ROS interact rapidly with bio-molecules such as enzymes and nucleic acids and with lipid membranes and cell walls. This interaction suppresses the bacteria's protection measures, which leads to cell death.[19–21]

Meanwhile, a wide variety of photosensitizers have been investigated for their action against Gram-positive and Gram-negative bacteria, namely cationic azines, cyanines, macrocyclic photosensitizers, naturally occurring substances and antibody-linked photosensitizers.[19–24] Recently, Villén et al.[22] reported about the reduction of bacterial survival for *Enterococcus faecalis* (Gram +) and *Escherichia coli* (Gram −) by means of ruthenium-based photosensitizers. The photoexcitation of these photosensitizers is highest in the blue region of the spectrum, which matches that of the light-curing units. No studies are available so far which report on testing ruthenium-based photosensitizers in a PDT approach against periodontopathogenic bacteria.

Most PDT applications are associated with laser light. However, some studies indicate that the usage of visible light is also effective, whereas it does not induce any thermal or photochemical damage to the retina.[25,26] For that reason, the application of visible light with a hand-held photopolymerizer in PDT offers some advantages over laser light because neither the patient nor the dentist are exposed to high doses of UV-A and UV-B.

The aim of the present study was to investigate the effect of ruthenium-based PDT on the two periodontal species *F. nucleatum* and *P. gingivalis*. A commercially available halogen lamp emitting in the blue spectral region, commonly used for photopolymerization in dental practices, was used as a light source.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bactericidal effect of PDT was tested on *F. nucleatum* (DSMZ 20482) and *P. gingivalis* (DSMZ 20709) acquired from the *German Collection of Microorganisms and Cell Cultures*. The strains grew anaerobically (10% CO_2, 10% H_2O, 80% N_2) at 36.5–37.0 °C for 24 h in Schaedler anaerobe broth (Oxiod, Hampshire, England) supplemented with 10 μg/ml menadione (Roche, Grenzach-Wyhlen, Germany). When cultures reached the stationary phase of growth, the bacterial cells were harvested by centrifugation and resuspended in a phosphate-buffered solution (PBS) at an optical density (OD) of 0.6 at 465 nm, which corresponds to approximately 10^7 cells/ml.

2.2. Photosensitizers

The three ruthenium-based photosensitizers involved in the study, i.e. tris-(4,7-diphenyl-1,10-phenanthroline)-ruthenium(II)-dichloride (RD3), tris-(2,2′-bipyridine)-ruthenium(II)-dichloride (RBY), and tetrasodium-tris-[(1,10-phenanthroline-4,7-diyl)-bis-(benzenesulfonato)]-ruthenate (RSD3) were dissolved under sterile conditions in a solution of ethanol/distilled water (7/100, v/v) to a concentration of 2 mM and stored at 4 °C until use.

2.3. Photosensitization and irradiation procedure

A total of about 10^7 bacterial cells/ml were incubated in the dark for 15 min with different concentrations (0 μM, 12.5 μM, 25 μM) of each of the three photosensitizers. After this period, the bacteria were classified into two groups: the first one with bacteria unwashed after the incubation period, and the second one with bacteria washed by centrifuging the suspension at 3500 × *g* for 5 min, re-suspended in PBS, and then placed in wells of black 96-well microtitration plates (160 μl/well). Control samples consisted of bacterial cell suspension irradiated and non-irradiated in the absence of photosensitizer, or incubated with the photosensitizer without irradiation.

Irradiation of the bacterial solutions followed, with the well rim supporting the tip of the light source applicator. To ensure a constant distance to the solution, the halogen light source used (Polofil Lux, Voco, Cuxhaven, Germany) was equipped with a light guide (cross-sectional area 0.5 cm^2) for irradiation in the 350–500 nm range, with an emission maximum at 490 nm (Fig. 1). The irradiance applied was 700 mW/cm^2 (output of light source 350 mW), and the corresponding fluence rate for 20 s was 7 J/cm^2.

2.4. Determination of bacterial inactivation

Six successive dilutions (1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$) were prepared. Then, 100 μl of the bacteria suspension in each case were spatulated on Schaedler agar plates (Oxoid, Hampshire, England) supplemented with 10 μg/ml menadione (Roche, Grenzach-Wyhlen, Germany). Incubation proceeded at 37 °C under anaerobic conditions for 4 and 8 days, respectively. After this period, the colony-forming units (cfu/ml) were counted.