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Original Article

Microscopic Study of Cultural and Diffusion Kinetics of the Effects of a Prototype Extracellular Matrix on the Photodynamic Therapy of Periopathogenic Bacteria – A Pilot Study

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Abstract

Introduction: In dental medicine, photodynamic therapy is a promising treatment for bacterial infections. Oral biofilms, on the other hand, can produce an extracellular matrix that provides protection and stability against external forces.

Materials and methods: In this pilot study, we investigate the relationship between a prototype extracellular matrix and the efficacy of photodynamic therapy with three different photosensitizers. To assess the efficacy of the photodynamic therapy, we use culture analysis of the colony-forming units (CFU) and diffusion kinetics microscopy using fluorescence recovery after photobleaching (FRAP).

Results: Our results show evidence of decreased efficacy of the photodynamic process in the presence of extracellular matrix, as observed in experimental culture models and in direct FRAP observation. Additionally, the mathematically modeled diffusion coefficients of the used photosensitizers suggest low diffusion of these molecules in the prototype extracellular matrix.

Conclusions: The presence of an extracellular matrix in oral biofilms may reduce the efficacy of photodynamic therapy in dentistry. These findings highlight the need for further investigation into the development of photosensitizers with better diffusion properties in extracellular matrix. Overall, this study provides valuable insights into the potential limitations of photodynamic therapy in dental medicine.

Keywords

extracellular matrix, FRAP microscopy, periopathogenic bacteria, photodynamic therapy

INTRODUCTION

Photodynamic therapy is a novel therapeutic approach in dental medicine encompassing a broad spectrum of applications – from diagnostics to cytotoxic effects. This therapy relies on a well-known photochemical phenomenon: certain photoactive substances (called photosensitizers) react with light of a specific wavelength and intensity, thus generating short-lived oxygen radicals known as reactive oxygen species (ROS).[1,2] These radicals further interact with tissue or bacterial components leading to potential cytotoxicity. In dental medicine, photodynamic therapy is often seen as an antibacterial treatment due to the above mentioned interactions. However, most of the bacteria in the oral cavity exists in highly organized communities called biofilms which possess certain protective structures. One such structure is the extracellular matrix produced by most of the oral bacteria. Composed of various carbohydrates, glycoproteins, and lipids, it acts as a semi-conductive barrier against the environmental changes in the oral cavity.[3] In the context of periodontology, organized biofilms are one of the main factors at play in the complex pathogenetic process of periodontal disease.[4]

Periodontal pathogens such as *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* play a critical role in the development and progression of periodontal disease. These pathogens are found in high numbers in periodontal pockets and are associated with the destruction of periodontal tissues. *Prevotella intermedia* and *Porphyromonas gingivalis* are known to produce proteases and toxins that can degrade host tissue, leading to bone resorption and tooth loss.[5] *Fusobacterium nucleatum* can also contribute to periodontal pathogenesis by promoting the adhesion and invasion of other bacteria into host cells.[6]

AIM

The presented pilot study employs culture analysis of CFUs and fluorescence recovery after photobleaching (FRAP) microscopy experimental designs to investigate the relationship between the efficacy of photodynamic therapy in treating periopathogenic flora and a prototype of bacterial extracellular matrix.

MATERIALS AND METHODS

The experimental design of the presented study is organized in 3 phases. Phase 1 is a preparatory phase which consists of the preparation of a prototype extracellular matrix and an original method of simultaneous cultivation of three periopathogenic bacterial species. Phase 2 consists of photodynamic therapy with 3 commonly used photosensitizers with and without application of the prototype extracellular matrix and consequent CFU-analysis of the results. Phase 3 consists of FRAP-microscopy of the prepared prototype extracellular matrix and photosensitizer solution, followed by mathematical model of the lateral diffusion coefficients.

Phase 1

Simultaneous cultivation of periopathogenic flora

The cultivation process included strains of *Porphyromonas gingivalis* (2561, ATCC USA), *Prevotella intermedia* (VPI 4197, ATCC USA), and *Fusobacterium nucleatum* (VPI4355, ATCC USA). The selected bacterial strains were activated and prepared for cultivation. For the cultivation, two Hellendal jars (100 mm^3, Chimtex Ltd., Bulgaria) were filled with a mixture of BHI-medium (Nutri-Select Plus, Sigma-Aldrich, USA) and defibrinated sheep blood (Ridacom Ltd., Bulgaria). Microscope slides were used for fixture of a solid substrate for the cultivation process – standardized specimens cut from extracted and sterilized human teeth with an approximate size of 5×7×2 mm. The prepared and fixed teeth specimens were mounted on the microscope slides and submerged in the cultivation medium. Subsequently, the cultivation baths were filled with medium and inoculated with a mixture of the activated bacterial strains and placed in anaerobic conditions for 7 days.

Preparation of prototype extracellular matrix

The prototype extracellular matrix consisted of a 1:1 ratio of mixed dextran with a molecular weight of at least 70 KDa (DEXTRAN 70, Sigma-Aldrich, USA) and xantan with a molecular weight of at least 50 KDa (Sigma-Aldrich, USA), mixed with distilled water. The prepared mixture is heated to 37.0°C for 15 minutes until complete homogenization of the components. Next, the prototype matrix is transferred to a 96-well plate (300 microliters per well), filling 15 wells with 200 microliters per well and left to cool down at room temperature (20°C) until complete polymerization of the matrix.

Phase 2

In this phase, both Hellendal jars prepared in phase 1 were subjected to photodynamic therapy with 0.33% aqueous solutions of three different photosensitizers – methylene blue (Valerus Ltd. Bulgaria), indocyanine green (Frontier Scientific, USA), and chlorin E6 (Frontier Scientific, USA). One of the jar served as a control group, while the specimens in the other were covered with a prototype extracellular matrix prior to photodynamic therapy. The methylene blue and chlorin e6 solutions were activated with a 662 nm laser light (SIX Laser TSC, Atlantis Lasers, Bulgaria), while indocyanine green was activated with 810 nm laser light (D-Touch, Syneron Lasers, Israel). All laser activation was done for 1 minute at a distance of 10 mm in a continuous wave at 0.171 mW of total energy. Each jar contained 4 specimen glasses – one for every photosensitizer solution

and one non-activated control glass. After the photodynamic process, the specimens from both groups (jars) were subjected to CFU-analysis to determine the viable forms left after the intervention.

Phase 3

In Phase 3, 100 microliters of 0.33% aqueous solutions of the three photosensitizers conjugated with a fluorescent probe solution were filled into fifteen of the sixteen wells of the transferred prototype extracellular matrix. Each photoactive substance was placed in 5 wells **(Fig. 1)**. The 16th well with prototype matrix was left without photosensitizer to serve as a positive control. Next, the prepared plate was subjected to FRAP microscopy (Leica WF FRAP, Leica Germany) at five time intervals per photosensitizer. The results were normalized with LAS X software (Leica, Germany) and subjected to mathematical modeling to determine the diffusion coefficients of the studied structures.

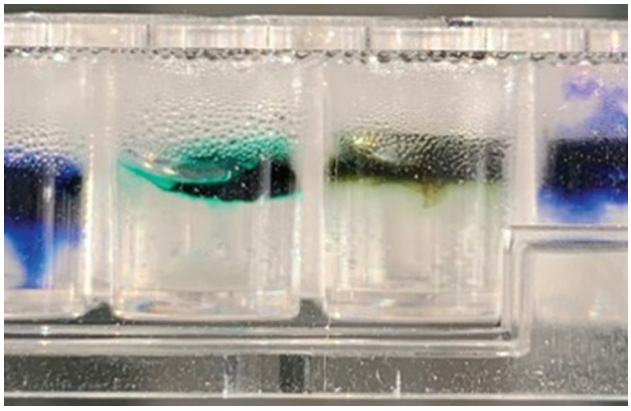

Figure 1. Well plate with transferred prototype extracellular matrix and probe-dubbed photosensitizers.

RESULTS

Phase 2

After the photodynamic therapy, all four of the test specimens of both jars were removed and subjected to CFU-analysis to determine the relative number of viable bacterial forms left after the intervention. The method of decreasing dilutions was employed for both groups (jars).

Specimens without prototype extracellular matrix (control group)

The results from the CFU counting revealed a reduction in the viable forms by a mean of 2 logarithmic units which correlates to 99% reduction of the viable forms after photodynamic therapy across all used photosensitizers. This value was determined for the control group **(Fig. 2)**. The CFU analysis is performed by a series of sequential smears taken from the cultivated mediums and substrates and then subsequently diluted in a separate sterile medium. The obtained number of viable forms is then multiplied by the dilution factor to provide the CFU/ml value. In this work, the Pour Plate method is utilized. The reduction of colony-forming units in the diluted sample is expressed through a logarithmic unit reduction (log). The number of log units expresses the percentage after the decimal mark. In this particular case a log reduction of 2 units corresponds to 99% reduction.

Specimens with prototype extracellular matrix (test group)

The results from the CFU counting revealed a mean reduction in the viable bacterial forms of 1 logarithmic unit which correlates to 90% reduction after application of the prototype extracellular matrix on the specimens and consequent photodynamic therapy. These results correlate to the

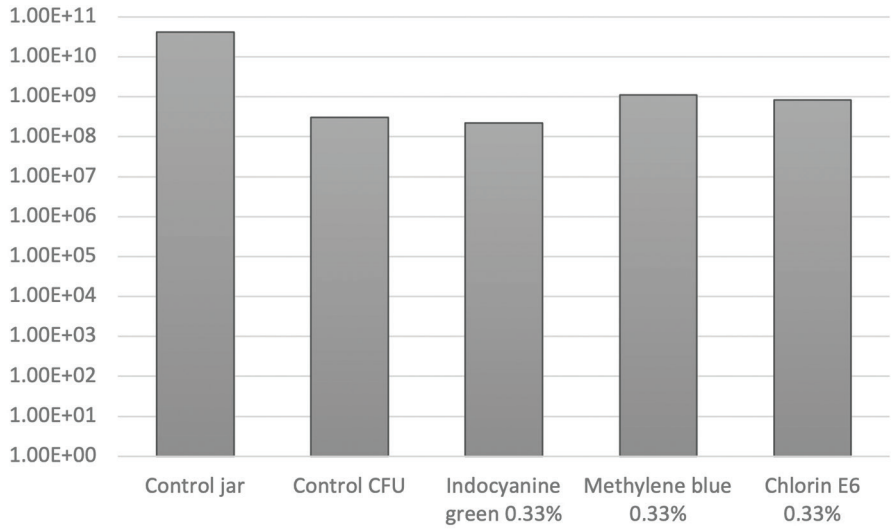

Figure 2. Mean logarithmic CFU reductions after photodynamic therapy in control group.

protective function of the extracellular matrix in biofilms against external changes (e.g., photodynamic therapy). The results are shown in **Fig. 3**.

Phase 3

FRAP microscopy

The aqueous solutions of the used photosensitizers act as natural fluorophores in the context of the presented study, and thus do not require further binding to a fluorescent probe. The test specimens from the well plate are scanned before and after FRAP irradiation (455 nm, argon laser) with a 205-ms time interval. A negative control containing only prototype matrix is FRAP-captured as well. The time for observation of the specimens is between 0 sec and 300 sec after the FRAP irradiation. In total, the quantitative examination of the obtained FRAP curves shows fluorescent stabilization above 95% of the entry value in the observed time interval. In the control specimen, the same processes were observed but with a slight decrease of the captured fluorescence; however, the final value exceeded 95% of the entry value.

Mathematical analysis of the intensity profile and determination of the local diffusion coefficient

The FRAP curves obtained on direct FRAP microscopy were subjected to a mathematical analysis modified from Braga et al.[7] Firstly, all curves of fluorescence recovery are extracted from the image series and then are normalized to 1 for the prebleaching phase **(Fig. 4)**. This allows direct comparison of every obtained FRAP curve and artefact check. For example, curves with pronounced intensity decrease at the end of the recovery phase show irregular photobleaching of the specimen **(Fig. 4c, arrow)**. In other curves, irregular peaks with higher intensity than the entry value show fluctuations of the laser beam of the argon laser **(Fig. 4b, arrow)**. In both instances, these curves are removed from the analysis. The observation interval is reduced to the time of the prebleaching phase (70 seconds).

The mathematical analysis continues with determination of the local diffusion coefficients of all three photosensitizers against the diffusion coefficient of the prototype matrix. This analysis is two dimensional and shows very close values to the direct profile – 10 to 11 $\mu m^2 \cdot s^{-1}$. The normalized curves of all tested photosensitizers show very low dispersion **(Fig. 4b)** in the prototype matrix as the obtained values are very close to the values of the control – 10 to 13 $\mu m^2 \cdot s^{-1}$ **(Fig. 4b, inset)**. Furthermore, **Fig. 5** shows a summarized view of the mean normalized FRAP values of the three photoactive substances tested. As seen on the figure, the relative difference between the fluorescence emission spectra in test (with matrix) and control (without matrix) samples is measured in arbitrary units (AU). The difference in emitted spectra between almost all test and control samples has no statistical value, with the exception of methylene blue at the 250- and 300-sec marks. This can be partly explained with the unstable chemical structure of methylene blue when exposed to any kind of lightwaves and its highly oxidative nature. However, the 250- and 300- sec marks are very difficult to achieve in clinical settings as the needed time for potential penetration introduces many variable factors in oral environment which can alter the efficacy of photodynamic therapy – these include increased salivary flow, increased gingival-crevicular fluid flow within the pocket, penetration of the photosensitizer into exposed/necrotic cementum etc.

The summarized statistical results of the FRAP microscopy against a positive control is shown in **Fig. 5**.

DISCUSSION

As previously stated in literature[8-10], the periopathogenic microorganisms of different species and strains tend to

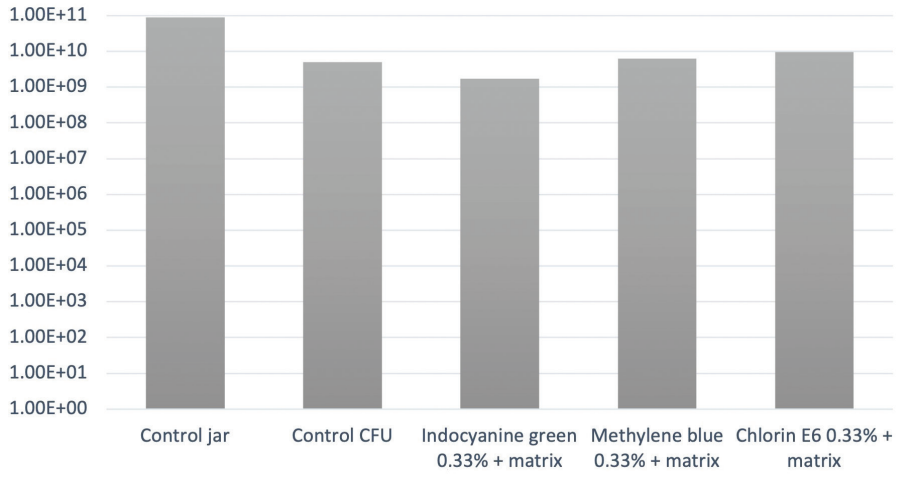

Figure 3. Mean logarithmic CFU reductions after photodynamic therapy in test group.

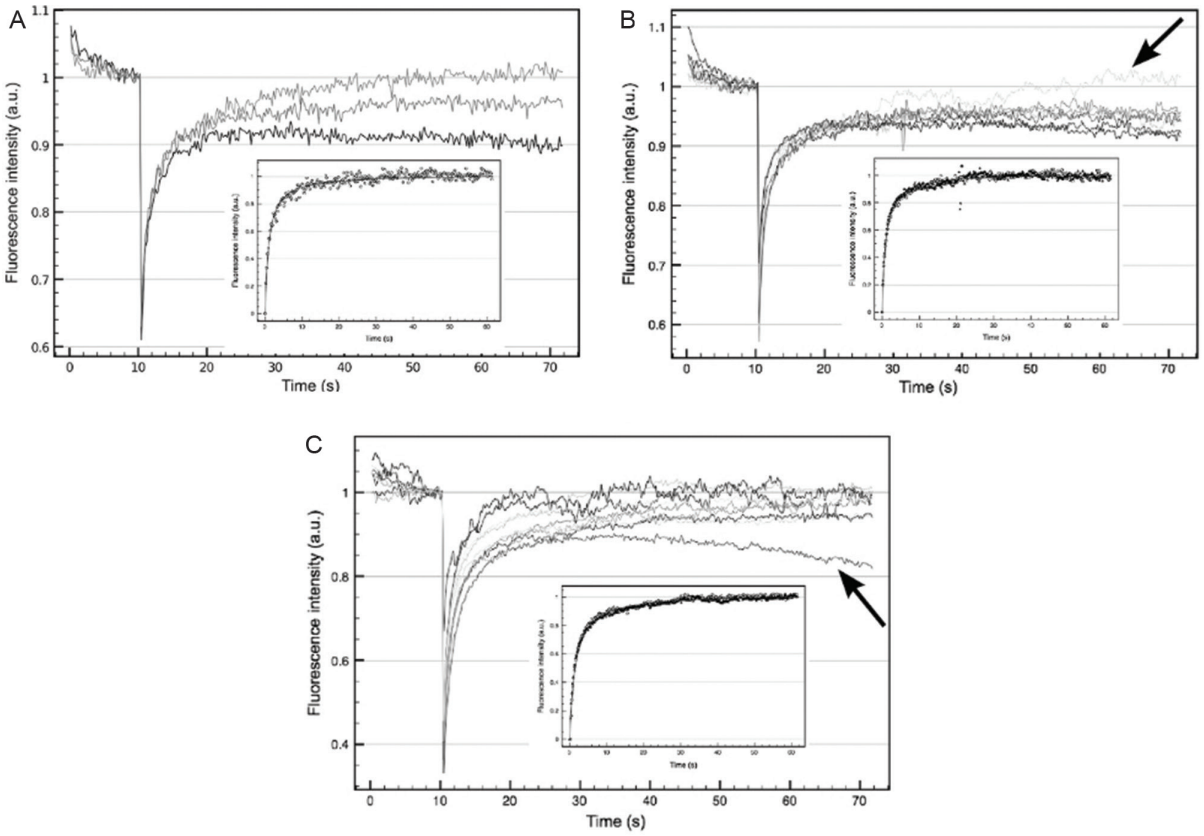

Figure 4. Analytical models of diffusion coefficient. Summarized curves of fluorescence recovery for indocyanine green **(a)**, chlorin e6 **(b)**, methylene blue **(c)**. Insets show mathematical summary.

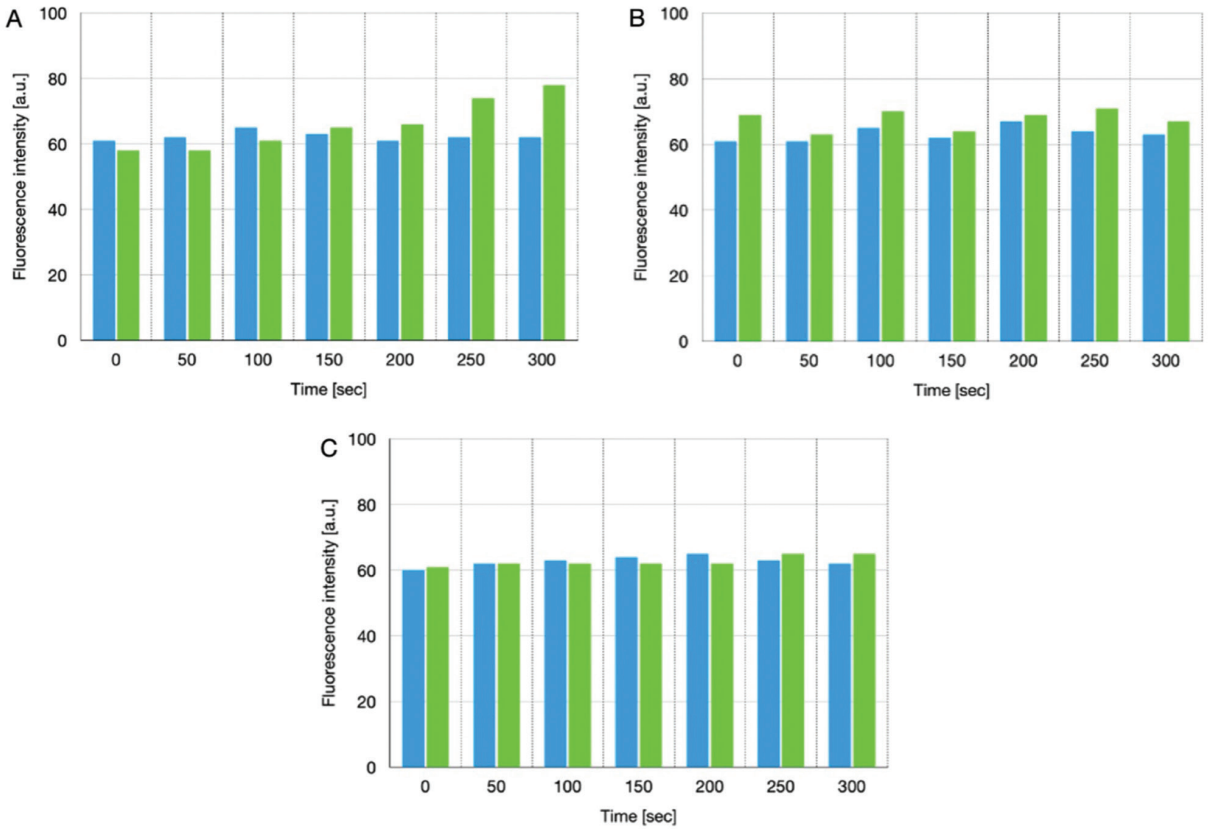

Figure 5. Normalized FRAP values for methylene blue (**A**), chlorin e6 (**B**), and indocyanine green (**C**). Blue columns show the control values; green columns show the test values.

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congregate forming highly organized communities called biofilms. These structures provide mechanical, 3-dimensional stability of the bacteria as well as protection against external forces in the form of extracellular matrix.[11] The extracellular matrix tends to act as a semi-conductive barrier allowing the transfer and diffusion of nutrients, genes, receptors and other components of the quorum-sensing phenomenon.[12] We believe these facts have a substantial role on the photodynamic process in the context of non-surgical periodontal treatment.

The presented findings and observations of this pilot study led us to hypothesize that without mechanical removal of the extracellular matrix of the biofilm in the subgingival space, for example, photodynamic therapy would lose efficacy due to the low diffusion coefficient of photosensitizer solutions in carbohydrates of high molecular weight, which are a basic component of the extracellular matrix. The findings in Phase 2 of this study lead to the assumption that mature, complex subgingival periopathogenic flora is more resistant to external forces and, respectively, less sensitive to photodynamic therapy. Based on these findings, we concluded that the immovable fraction of the test photosensitizer molecules against the control is negligible (no interaction between the clusters of prototype matrix and the test solutions was detected). These results are consistent with several other studies suggesting the inhibitive role of biofilm on the mechanisms of efficacy of periodontal photodynamic therapy in its antibacterial context.[13-16]

The limitations of the presented pilot study consist of the rather simple composition of the prototype extracellular matrix. In nature, bacterial extracellular matrix is much more complex and variable depending on the type and composition of its biofilm.[17] Further research into in vitro modeling of extracellular bacterial matrix is needed.

The clinical significance of the presented results is related to the place of photodynamic therapy in periodontal treatment – mechanical debridement of the subgingival space is still an essential prerequisite for successful photodynamic therapy or, in other words, photodynamic therapy is more effective as a sequential, adjunctive treatment after classic scaling and root planing.

CONCLUSIONS

Extracellular bacterial matrix is a key component in the resistance of oral biofilms to external forces. Being a topical, non-invasive treatment modality, photodynamic therapy can decrease its efficacy on subgingival or oral biofilms if the extracellular matrix is not managed.

Acknowledgements

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Author contributions

M.T.: development and execution of the experimental protocol, gathering and primary analysis of the results. I.D.: development and execution of the experimental protocol, complete analysis of the results, consultative advice and editing.

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Микроскопическое исследование культуральной и диффузионной кинетики воздействия прототипа внеклеточного матрикса на фотодинамическую терапию периопатогенных бактерий – пилотное исследование

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Резюме

Введение: В стоматологии фотодинамическая терапия является перспективным методом лечения бактериальных инфекций. С другой стороны, биоплёнки полости рта могут образовывать внеклеточный матрикс, обеспечивающий защиту и стабильность против внешних сил.

Материалы и методы: В этом пилотном исследовании мы изучаем взаимосвязь между прототипом внеклеточного матрикса и эффективностью фотодинамической терапии с использованием трёх различных фотосенсибилизаторов. Для оценки эффективности фотодинамической терапии мы используем культуральный анализ колониеобразующих единиц (CFU) и диффузионно-кинетическую микроскопию с использованием восстановления флуоресценции после фотообесцвечивания (FRAP).

Результаты: Наши результаты свидетельствуют о снижении эффективности фотодинамического процесса в присутствии внеклеточного матрикса, что наблюдается на экспериментальных культуральных моделях и при прямом наблюдении FRAP. Кроме того, математически смоделированные коэффициенты диффузии использованных фотосенсибилизаторов позволяют предположить низкую диффузию этих молекул в прототипе внеклеточного матрикса.

Заключение: Наличие внеклеточного матрикса в биоплёнках полости рта может снизить эффективность фотодинамической терапии в стоматологии. Эти результаты подчёркивают необходимость дальнейших исследований в области разработки фотосенсибилизаторов с лучшими диффузионными свойствами во внеклеточном матриксе. В целом, это исследование даёт ценную информацию о потенциальных ограничениях фотодинамической терапии в стоматологии.

Ключевые слова

внеклеточный матрикс, FRAP-микроскопия, периопатогенные бактерии, фотодинамическая терапия