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Original Article

EPR Spectroscopy Investigation of Oxygen Radical Production by Methylene Blue and Indocyanine Green in Aqueous Solutions under Laser Irradiation in the Context of Antibacterial Photodynamic Therapy

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Abstract

Introduction: Antibacterial photodynamic therapy is a promising treatment modality in the anti-infective therapy of numerous oral diseases. It involves photo activation of a reactive substance (dye), thus releasing reactive oxygen species (ROS-radicals) which are highly destructive to the bacterial cell. However, thorough investigation of radical production properties of different dyes is not common in literature.

Aim: The aim of this study was to investigate and evaluate oxygen radical-producing potential of two commonly used photoactive dyes in the context of antibacterial photodynamic therapy.

Materials and methods: The radical-producing properties of two commonly used dyes for photodynamic therapy in oral medicine, methylene blue and indocyanine green, irradiated under laser irradiation are investigated using electron paramagnetic resonance (EPR) spectroscopy. The detection of reactive oxygen species is performed with "spin-trapping" technique.

Results: The selected photoactive dyes showed promising yields of reactive oxygen species (ROS) in aqueous solutions. The comparative analysis of the results deemed methylene blue as the more productive photoactive agent.

Conclusions: By employing the spin-trapping technique, this study indicates EPR-spectroscopy as a promising method of relative quantification of reactive oxygen species released by the photodynamic reaction in aqueous solutions.

Keywords

antibacterial photodynamic therapy, EPR spin trapping, Indocyanine green, methylene blue, oxygen radicals

INTRODUCTION

Photodynamic therapy is a form of phototherapy involving a light source and a photosensitising chemical agent used in conjunction with molecular oxygen to induce cell death.[1] The targeted cells can vary from cancerous to bacterial.[2] In the therapeutic context of dental medicine, especially periodontology, photodynamic therapy is a promising tool for targeting pathogenic bacteria in periodontal pockets.[3] However, the basic principle of this process relies on the production of free oxygen radicals (ROS) which are short-lived and highly toxic to the bacterial cell wall. In most instances, the efficacy of this process is measured indirectly through clinical or microbiological evaluation of the antibacterial effect.[4] Hence, it is rather difficult to study the radical production properties of available dyes (photosensitizers) as well as developing criteria or standards for new and alternative dyes.

AIM

The aim of this study was to evaluate the oxygen-releasing potential and ROS forming properties of two of the most common photoactive substances used in antibacterial photodynamic therapy in dentistry.

MATERIALS AND METHODS

The experimental design involved preparing 0.33% aqueous solutions of methylene blue (Valerus™, Bulgaria) and indocyanine green (Frontier Scientific™, USA) from their solid states. The selected concentrations are commonly used in clinical photodynamic therapy. The BMPO spin-trap was prepared into a 20-mM aqueous solution. Each dye solution (160 μl) was mixed with 40 μl spin-trap solution and the mixture was transferred to a quartz capillary tube 1 mm in internal diameter **(Fig. 1)**. The sample tube was then positioned into a TE_{011} cylindrical resonator of JEOL JES FA 100 ESR spectrometer (JEOL, Japan), at 9.5 GHz frequency. All spectres were registered at room temperature. Each solution sample was irradiated for 20 seconds at peak absorption wavelength of 660 nm and 810 nm for methylene blue and indocyanine green, respectively. The working laser parameters were as follows: indocyanine green was irradiated with 810 nm laser light (D-touch™, Syneron Lasers, Israel), at 500 mW, 134 J/cm^2. The used laser tip was 400 micron fibre. Methylene blue was irradiated with 662 nm laser light (SIX Laser TSC™, Atlantis Lasers, Bulgaria), at 100 mW, 15 J/cm^2. The used tip produced a beam diameter of 3.5 mm by 4.5 mm. The irradiation of the solution samples was performed through a special aperture of the EPR spectrometer, at a 10-cm distance from the laser tip to the sample **(Figs 2, 3)**.

Figure 1. Quartz tube filled with sample solution.

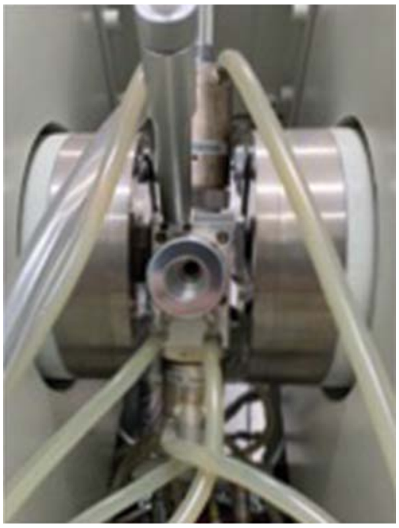

Figure 2. Irradiation aperture of the EPR-spectrometer.

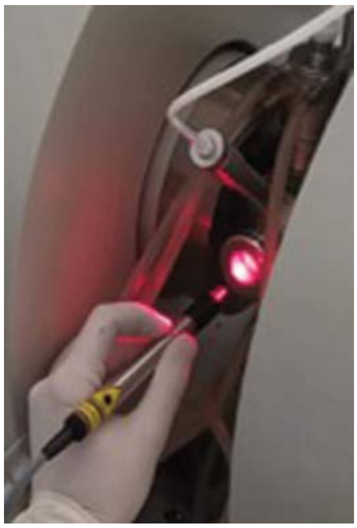

Figure 3. Irradiation of the sample solutions.

RESULTS

The registered EPR spectres of both dye solutions are presented in **Fig. 4**. The registered spectres are complex and show superposition of EPR signals of two or more radicals. All samples show a narrow signal band with a g factor of 2.008 due to the presence of unpaired electron radical. This registered band is evident in all samples. One possible explanation of the observed signal is a spin-orbital interaction. In any case, a value below 2.0023 does not interfere with the results. The aqueous solution of BMPO before irradiation does not produce EPR-signal. However, after irradiation with 662 nm laser light, some slight signal bands are detected **(Fig. 4.1)**. This can be explained with the oxygen radicals ($O_2^{\bullet\bullet}$) water molecules form under irradiation, consecutively detected with BMPO. The registered EPR-signal bands of all dye samples are similar **(Figs 4.2, 4.3)**, but there are differences in the intensity of the signal bands (the distance from peak to peak). The rest of the registered EPR parameters are shown in **Table 1**.

Analysis of the available literature shows that the laser irradiated EPR spectre of methylene blue contains the spectre of BMPO-OOH adduct showing the presence of superoxide oxygen radical ($O_2^{\bullet-}$). The laser irradiated EPR spectre of indocyanine green contains weak signal bands of BMPO-OOH adduct of particularly lesser intensity. These differences can be accounted for by the different distance of the unpaired electron relative to the nitrous and hydrogen atoms in the molecules of the dyes.

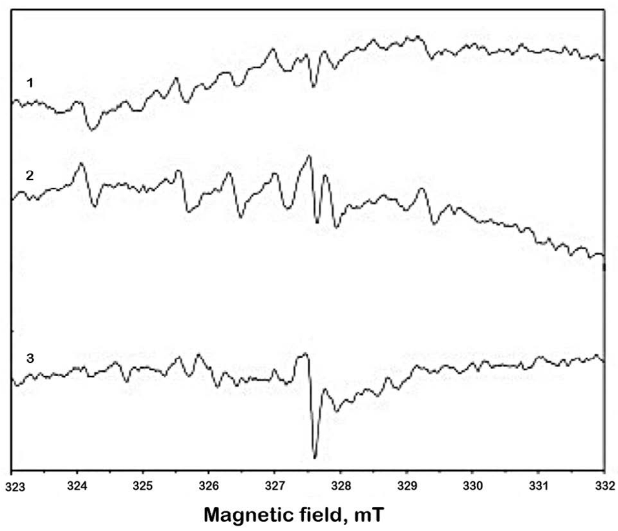

Figure 4. EPR spectre of: **1**) BMPO irradiated with 662 nm laser light; **2**) Methylene blue solution irradiated with 662 nm laser light; **3**) Indocyanine green solution irradiated with 810 nm laser light.

DISCUSSION

Electron paramagnetic resonance spectroscopy (EPR) is a technique used to study chemical species with unpaired electrons. EPR spectroscopy plays an important role in the understanding of inorganic and especially organic radicals, as well as some biomolecules. In this experimental study, EPR was used to investigate the comparative production of oxygen radicals of two different photoactive dyes irradiated with laser light, commonly used in the antimicrobial photodynamic therapy. However, the said radicals are short-lived, so the use of a “spin-trapping” technique was implemented. Spin-trapping technique involves the adding nitrone or nitroxyl derivatives (BMPO, DMPO and others). These spin-trapping substances allow registering some of the short-lived radicals of oxygen – hydroperoxyl radical (OH), superoxide radical (O_2^-), hydroperoxyl radical (OOH). The molecule of the spin-trapping substance reacts with the released oxygen radical, thus creating spin adducts which have longer half-life and can be detected by EPR spectroscopy. The half-life time of BMPO-formed spin adducts is 23 minutes.

According to the comparative analysis of available literature data[5], the laser irradiated EPR spectre of methylene blue contains the spectre of BMPO-OOH adduct, which shows the presence of superoxide oxygen radical ($O_2^{\bullet-}$). The laser irradiated EPR spectre of indocyanine green contains weak signal bands of BMPO-OOH adduct of notably lesser intensity. These differences can be explained with the different distance of the unpaired electron relative to the nitrous and hydrogen atoms in the molecules of the dyes. In the performed experimental conditions, this pilot study deems methylene blue as the more potent photoactive substance in comparison to indocyanine green. Oxygen reactive species production is the core mechanism of action behind the efficacy of photodynamic therapy; hence, the spin-trapping EPR-spectroscopy presents a novel opportunity in relative quantitative testing of the oxygen radical production in photoactive dyes.

Table 1. Registered EPR parameters as of **Figure 4**

Sample	EPR parameters			
	A_N, mT	A_H, mT	g	H_{pp}, mT
BMPO	1.47; 1.46	-	2.0132; 1.999	0.2
Methylene blue	1.47; 1.47	-	2.0128; 1.999	0.2
Indocyanine green	1.44	-	2.0127	0.2

CONCLUSIONS

The conducted EPR spectroscopy investigation of methylene blue and indocyanine green aqueous solutions under laser light irradiation shows generation of reactive oxygen radicals registered by the spin-trapping technique. Using the intensity of the EPR signal bands as a value for quantity of released radicals, the following row of decrease of radical production can be stated: methylene blue > indocyanine green. This pilot study confirms the potential of EPR-spectroscopy to examine photoactive dyes and their radical production properties. However, further research is needed to expand the possibilities of physico-chemical analysis of radical releasing agents.

Acknowledgments

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ЭПР-спектроскопическое исследование образования радикалов кислорода метиленовым синим и индоцианиновым зелёным в водных растворах при лазерном облучении в контексте антибактериальной фотодинамической терапии

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Резюме

Введение: Антибактериальная фотодинамическая терапия – перспективный терапевтический вариант противоинфекционной терапии многих заболеваний полости рта. Он включает световую активацию реактивного вещества (красителя), высвобождая таким образом активные формы кислорода (радикалы АФК), которые очень разрушительны для бактериальных клеток. Однако углублённое изучение свойств образования радикалов из разных красителей в литературе встречается нечасто.

Цель: Целью этого исследования было изучить и оценить потенциал производства кислородных радикалов двумя широко используемыми фотоактивными красителями в контексте антибактериальной фотодинамической терапии.

Материалы и методы: С помощью спектроскопии электронного парамагнитного резонанса (ЭПР) исследованы радикально-образующие свойства двух красителей, обычно используемых для фотодинамической терапии в оральной медицине, метиленового синего и индоцианинового зелёного, облучения лазерным излучением. Обнаружение активных форм кислорода осуществляется методом спиновой ловушки.

Результаты: Выбранные фотоактивные красители показали многообещающее образование активных форм кислорода (АФК) в водном растворе.

Заключение: Это исследование с использованием метода спинового захвата доказывает, что спектроскопия ЭПР является многообещающим методом для относительного количественного измерения активных форм кислорода, выделяемых во время фотодинамической реакции в водных растворах.

Ключевые слова

антибактериальная фотодинамическая терапия, спиновое улавливание ЭПР, индоцианин зелёный, метиленовый синий, кислородные радикалы