Quantitative Investigation of Efficiency of Ultraviolet and Visible Light in Eradication of *Candida albicans* In Vitro

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Abstract

Objective: The aim of this study was to quantitatively investigate the efficiency of the ultraviolet (UV) and visible light in eradication of *Candida albicans* in vitro; in particular, to determine, for selected wavelengths, the specific eradication coefficients and thresholds in terms of energy density levels required to effect $3.0\log_{10}$ and $4.0\log_{10}$ reduction. **Background data:** Oral candidosis is the most common infection of the oral cavity and is caused by *Candida* species. The widespread use of topical and systemic antifungal agents as conventional treatment for oral candidosis has resulted in the development of resistance in *C. albicans*. Therefore, it has become necessary to develop alternative therapies for the treatment of oral candidosis. **Methods:** *C. albicans* ATCC[®] 90028[™] was irradiated with 254 nm, 365 nm, 406 nm, 420 nm, and broadband Xe spectrum. For each wavelength, a fit of experimental data (survival fraction vs. applied energy density) with an exponential decay function enabled estimation of the specific eradication coefficients and thresholds. **Results:** Based on estimated specific efficiencies (Δ) and eradication thresholds (ET) of the investigated wavelengths, the ranking in eradication efficiency of *C. albicans* (most to least effective) is: 254 nm ($\Delta = 6.1 \text{ mJ/cm}^{-2}$, $\text{ET}_{99.99} = 56 \text{ mJ/cm}^{-2}$), broadband Xe spectrum ($\Delta = 27.7 \text{ mJ/cm}^{-2}$, $\text{ET}_{99.99} = 255 \text{ mJ/cm}^{-2}$), 365 nm ($\Delta = 4.3 \text{ J/cm}^{-2}$, $\text{ET}_{99.99} = 39 \text{ J/cm}^{-2}$), 420 nm ($\Delta = 0.65 \text{ J/cm}^{-2}$, $\text{ET}_{99.99} = 6 \text{ J/cm}^{-2}$), and 406 nm ($\Delta = 11.4 \text{ J/cm}^{-2}$, $\text{ET}_{99.99} = 104 \text{ J/cm}^{-2}$). **Conclusions:** The results provide insight into the wavelength-dependent dynamics of eradication of *C. albicans*. For each investigated wavelength, the eradication coefficient and corresponding eradication threshold were estimated. The observed different eradication efficiencies are consequence of different spectrally dependent inactivation mechanisms. The established methodology enables unambiguous quantitative comparison of eradication efficiencies of optical radiation and selection of most effective wavelengths for clinical and therapeutic use.

Introduction

ORAL CANDIDOSIS IS THE MOST COMMON INFECTION of the oral cavity and is caused by *Candida* species, the commonest being *Candida albicans*.¹ These species may become pathogenic in the presence of predisposing factors, producing infections that range from superficial mucosal lesions to severe and invasive systemic dissemination.² *C. albicans* is the most prevalent pathogen, representing ~60% of all yeasts isolated in clinical samples.³ The widespread use of topical and systemic antifungal agents as conventional treatment for oral candidosis has resulted in the development of resistance in *C. albicans*.^{4,5} Because the conventional antifungal agents exert a more fungistatic than fungicidal

effect, prophylaxis is often inadequate.⁶ Therefore, it becomes necessary to develop alternative therapies for the treatment of oral candidosis. Promising modalities are low-power/level laser irradiation^{7,8} and photodynamic therapy (PDT).⁹ Although PDT is more usually applied for treating cancer, it has been demonstrated that PDT is effective against oral species and may not promote damage to host cells and tissues.¹⁰ Another promising approach, which is potentially less complicated and less harmful than PDT, is use of ultraviolet (UV)^{11–17} or visible (VIS) incoherent light.^{18–20} Although the application of the UV or VIS light in therapy seems promising, and deserves an intensive investigation, far less effort has been put into research following this approach in comparison with PDT. Moreover, the published results of

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such investigations are often more qualitative than quantitative, and, therefore, of limited practical therapeutic value. With regard to damage and/or inactivation of *C. albicans* by optical radiation, possible involved mechanisms could be photochemical, affecting/inhibiting formation of new DNA chains in the process of cell replication, related to single-strand and double-strand DNA breaks and DNA-to-protein cross-links, or related to generation of various reactive oxygen species in cells.^{21–24}

Therefore, the primary objective of our study was to quantitatively evaluate the fungicidal effects of optical radiation on *C. albicans* *in vitro*, and thus provide a sound basis and methodology for comparison and selection of the most effective wavelengths for clinical and therapeutic use, and also, possibly shed some light on the involved damage mechanisms. In this study, we have used optical radiation comprising selected lines and narrow bands from UV and VIS spectral ranges and a full spectrum (UV to near infrared [NIR]) of an incoherent pulsed xenon light source.

Materials and Methods

Inoculums preparation

The inoculum preparation followed standard procedures and recommendations,²⁵ as will be described. *C. albicans*

ATCC® 90028™ was subcultured from vial stock onto Sabouraud dextrose agar with addition of antibiotics for inhibition of bacterial growth. From 18 h *C. albicans* culture, a suspension in 0.85% NaCl 5 McFarland (10^8 colony-forming units [CFU]/mL) was prepared. With 10-fold dilution, an inoculum of 10^7 CFU/mL was obtained, and following another 10-fold dilution, the final inoculum of 10^6 CFU/mL was produced.

Sabouraud agar (Becton Dickinson and Co, Cockeysville, USA) prepared according to manufacturer's instructions, autoclaved at 121°C for 15 min, was hot-poured into Petri dishes, and after 24 h, used in solid form as the substrate for culturing. In each Petri dish (Fig. 1), along the circumference, eight circular areas (diameter 1.5 cm) were marked on the Sabouraud agar, and into each of these areas 10 μ L of *C. albicans* inoculum was evenly spread with a calibrated microbial loop. This configuration was conceived based on the purely geometrical considerations of placing several samples of adequate surface area to be easily irradiated with a beam of circular cross-section. In the experiment, six of these areas with *C. albicans* cultures were subsequently irradiated with a selected optical radiation of known-measured power density, while the remaining two were not irradiated, and served as control areas as described in the section on Experimental design. The power density was

FIG. 1. The arrangement of samples for the irradiation experiment and schematic of the sample image processing. The white *Candida albicans* colonies in various stages of eradication can be seen in the upper photograph. The original color image of *C. albicans* colonies (A) is converted to a gray scale image (B) and subsequently binarized by application of appropriate threshold level (C).

measured in the sample plane as described in the section Experimental design.

Sources of optical radiation and spectral data

The investigation included UV spectral lines 254 nm (UV-C) and 365 nm (UV-A), VIS 406 nm (violet), a narrow band of violet/blue light centered at 420 nm, and a full spectrum of incoherent pulsed xenon light source comprising UV – VIS – NIR radiation. The inclusion of the UV-C spectral range was motivated by results of recent investigations, indicating that UV-C radiation is an efficient antimicrobial agent,^{26,27} and yet, is significantly less harmful for biological systems²⁸ than UV-B radiation, which is accepted as a main cause of skin cancer.²⁹ UV-B radiation was also excluded from the investigation, based on recent findings indicating enhanced sensitivity of oral tissues to UV-B.³⁰ The UV-A band was included, as it is supposedly less carcinogenic than UV-B because of its lesser photon energy, and yet exhibiting considerable photochemical effects.^{31,32} The VIS violet light was selected because of a demonstrated bactericidal effect on a wide range of medically important

bacteria, and increased human safety because of its lower photon energy.³³ The 420 nm band was selected because of its overlap with the maximum of *C. albicans* absorption spectrum.^{18,34}

In the experiments, we have used three different light sources. For the investigation in the UV spectral range, an UVLS-24 EL (Ultra-Violet Products Ltd., Cambridge, UK) was used as the dual wavelength UV light source (selectable single lines at 254 or 365 nm). The full widths at half maximum (FWHM) of the spectral lines were 2.5 and 16 nm for 254 and 365 nm, respectively. A laser diode model: HK-E03508 (Laserpointerus, USA) emitting 406 nm radiation (FWHM 2.5 nm) was used as a source of VIS radiation in the violet range of the spectrum. Pulsed xenon light source PX2 (Ocean Optics, USA) was used as the source of UV-VIS-NIR spectrum with range of 220–950 nm. The narrow band (FWHM 26 nm) of visible violet/blue light (centered at 420 nm) was obtained from the PX2 spectrum using adjustable band pass filter LVF-UV-HL (Ocean Optics). The broadband PX2 xenon light source spectrum is depicted in Fig. 2 together with other spectral lines used in the measurements (shown in the inset).

FIG. 2. Broadband spectral output of the pulsed xenon light source PX2 and spectral irradiances of selected isolated narrow spectral lines at 254 nm, 365 nm, 406 nm, and a narrow band centered at 420 nm used in the measurements (inset).

Experimental design

A Petri dish with samples (eight circular areas with *C. albicans*) distributed along its rim (Fig. 1) was placed in a fixture eccentrically below the light source, so that the light source was centered above the single sample area at the rim of the Petri dish. This setup ensured that simple rotation of the Petri dish for 45 degrees brought another sample under the fixed light source. The samples were irradiated one at a time from a light source with selected wavelength placed above the sample plane at a predetermined fixed distance (2 cm), ensuring uniform irradiation of the whole single sample area. The power density at the sample plane (i.e., at distance 2 cm from the source) was measured. As the distance of the source from the sample plane was kept constant during the irradiation experiment so the power density P (W/cm^{-2}) was also constant. Hence, the total energy density E (J/cm^{-2}) delivered to the sample was controlled by the duration of the irradiation t (s). The measured parameters for each wavelength were the power density in the sample plane and irradiation (exposure) time. Then, the delivered energy density at given wavelength was calculated from $E = Pt$. In an experiment, six out of eight samples within a Petri dish were irradiated, and two were not, and served as the control samples. For a selected wavelength, all samples were irradiated with the same power density at a sample plane, and only the irradiation time varied in a controlled way, thus controlling the amount of delivered energy density. When the irradiation of single sample was finished, the Petri dish was rotated so as to bring the next sample under the light source. The first two samples were irradiated one by one for a selected period of time, and then the other two samples were irradiated for a prolonged time. This procedure was repeated until all samples were irradiated. After all six samples in a Petri dish were irradiated, another Petri dish was put into the fixture, and the procedure was repeated for the next six samples, with ever prolonged irradiation times. The procedure was repeated with increasing exposure times until the eradication of >99.99% of colonies was achieved. In this way, for each investigated spectral line or band, four to six Petri dishes (24–36 samples) were required to accomplish the whole experiment. When the series of experiments with a selected wavelength were accomplished, then another light source with a different wavelength was put in place and whole procedure was repeated.

In experiments with the UV and violet light (406 nm), samples were exposed to direct radiation from the light sources, whereas by using the PX2 xenon light source, the samples were irradiated using a UV grade fiberoptic light guide. Spectral output of sources was measured using ILT900-R Wideband Spectroradiometer (International Light). Power density and energy of optical radiation were measured using power/energy meter 13 PEM 001 (Melles Griot).

Sample and image processing

Following the irradiation of samples the irradiated and control (not irradiated) samples were incubated in air atmosphere for 48 h at $35 \pm 1^\circ\text{C}$. After that the samples were photographed and images (3072×2304 px) of irradiated and control samples were stored in jpg format for further processing. The images were converted to gray scale images in bitmap format with intensity levels ranging from 0 (black) to

255 (white). In the next step, the images were binarized, that is, converted to black and white images by applying the appropriate threshold level. The schematic of image processing is shown in Fig. 1.

The binary (black and white) images of irradiated and control samples are stored and processed to infer the fraction of surviving colonies. In the image, the surviving colonies are white on a black background. The fraction (0–100%) of considered area covered by surviving colonies is determined with respect to the control (not irradiated) sample using Fractal3e software.³⁵

Calculation of the eradication coefficient for a given wavelength

The average value of survival fraction was calculated from six samples that were irradiated with the same energy density at a selected wavelength. The measured data showing the average fraction of surviving colonies of *C. albicans* versus applied energy density at a selected wavelength were then fitted with an exponential decay function:

$$U(\lambda) = Ae^{-E/\Delta} \quad (1)$$

Here, $U(\lambda)$ is the average survival fraction of *C. albicans* colonies for irradiation with wavelength λ , A is a constant related to the control sample ($A \approx 1$), e is the base of the natural logarithm ($e = 2.718$), $\Delta(\lambda)$ is the specific eradication coefficient, and E is the total energy density (exposition) delivered to the sample. The value of specific eradication coefficient corresponds to the energy density required to reduce survival fraction to the value $1/e$ (≈ 0.37). A small specific (i.e., wavelength dependent) eradication coefficient indicates that a relatively small energy is required to eradicate colonies of *C. albicans*, and vice versa: a large eradication coefficient indicates that higher energy densities are required to attain the same degree of eradication.

The specific eradication coefficient Δ and constant A are then inferred from the best fit to experimental data.

Calculation of irradiation effects for a given wavelength

Once the specific eradication coefficient Δ has been determined, insertion of A and Δ obtained for a particular wavelength into Equation 1 permits calculations of: (1) irradiation effect, that is, degree of eradication attained with a known (applied) energy density, or (2) estimation of eradication thresholds (ET), that is, levels of energy density required to reduce the initial *C. albicans* colonies to a certain fraction U . The latter is obtained by solving Eq. 1 for E using assumed/desired $U(\lambda)$.

Description of statistical tools used for the evaluation of results

Statistical processing of results, comprehending calculation of mean values, median, standard deviations, analysis of variance (ANOVA), and correlation, was accomplished using Analysis Tool Pack within Microsoft Excel software. The normality of data was checked by the Shapiro–Wilk normality test (an improvement on the more general Kolmogorov–Smirnov test).³⁶ Grubbs' test³⁷ was applied for detection of possible outliers (results that are sufficiently

FIG. 3. The effect of 254 nm irradiation on colonies of *Candida albicans* (white areas) for different exposure times (energy densities). (a) Control sample; (b) 5 sec ($E=3.8 \text{ mJ/cm}^2$); (c) 10 sec (7.6 mJ/cm^2); and (d) 40 sec (30.4 mJ/cm^2).

different from all other results to warrant further investigation). There were no outliers in the measurement results.

Results

Irradiation with UV radiation with $\lambda=254 \text{ nm}$ and $\lambda=365 \text{ nm}$

The samples of *C. albicans* were irradiated with the UV-C spectral line with wavelength $\lambda=254 \text{ nm}$ and the UV-A spectral line with wavelength $\lambda=365 \text{ nm}$ at power densities of 0.76 and 1.67 mW/cm^2 , respectively. For wavelength $\lambda=254 \text{ nm}$ the exposure times varied from 5 sec to 60 sec, therefore resulting in energy densities in the range of 3.8 – 45.6 mJ/cm^2 . The effects of irradiation on colonies of *C. albicans* for different exposure times, that is, energy densities are depicted in Fig. 3.

The measured data were fitted with the exponential function (1) and the values of parameters inferred from the best fit. The quality of fit was assessed based on the value of the corresponding determination coefficient (square of the correlation coefficient R). The measured survival fraction of *C. albicans* colonies versus applied energy density for $\lambda=254 \text{ nm}$ and the UV-A spectral line with wavelength $\lambda=365 \text{ nm}$ are depicted in Fig. 4 together with the corresponding best fits with Equation 1.

Visible light in violet-blue spectral range and Xe spectrum (220–920 nm)

The survival fraction of *C. albicans* colonies versus applied energy density for irradiation with continuous monochromatic violet radiation ($\lambda=406 \text{ nm}$) and with pulsed (100 Hz) narrow band (FWHM 2 nm) violet/blue visible light centered at $\lambda=420 \text{ nm}$ together with the results obtained with pulsed (100 Hz) broadband (UV-VIS-NIR) Xe spectrum are shown in Fig. 5.

Eradication coefficients and eradication efficiencies

The parameters entering Equation 1 (the specific eradication coefficient Δ and the constant A) were inferred from the best fit to experimental data as described in the section Calculation of the eradication coefficient. Their values, listed in Table 1, were further used to estimate the eradication thresholds as described in the section Calculation of irradiation effects. The estimated values of eradication thresholds $ET_{99.9}(\lambda)$ and $ET_{99.99}$, that is, the energy densities

effecting $3.0 \log_{10}$ and $4.0 \log_{10}$ reduction of *C. albicans* colonies, respectively, are listed in Table 1.

Discussion

The efficiency of *C. albicans* eradication for a selected wavelength decreases as the specific eradication coefficient (Δ) increases (Table 1). The eradication thresholds for a particular wavelength are directly related to the magnitude of the specific eradication coefficient. Even for closely positioned spectral lines/bands, the variations in Δ can be greater than one order of magnitude (cf. $\lambda=406 \text{ nm}$ and $\lambda=420 \text{ nm}$). These variations in Δ can be related to variations in the spectral absorption coefficient of *C. albicans*, resulting in correspondingly great differences in eradication thresholds.

The results of our study show that the UV-C 254 nm is by far the most effective wavelength for eradication of *C. albicans*, because of its extremely small specific eradication coefficient. As a consequence, the resulting eradication thresholds are very low, and the required exposure times are

FIG. 4. The survival fraction of colonies of *Candida albicans* versus delivered energy density (a log-log plot) for the investigated ultraviolet (UV) wavelengths $\lambda=254 \text{ nm}$ ($\square$) and $\lambda=365 \text{ nm}$ ($\circ$). Symbols represent average values of measured data and line the corresponding exponential fit with Equation 1. Error bars represent standard deviation.

FIG. 5. The survival fraction of *Candida albicans* versus delivered energy density for irradiation with wavelengths: 406 nm ($\square$), 420 nm ($\circ$), and broadband Xenon spectrum (Δ). Symbols represent average values of measured data and line the corresponding exponential fit. Error bars represent the corresponding standard deviations.

very short. With regard to possible damage mechanisms, this high efficiency can be attributed to the strong absorption of pyrimidine (cytosine, thymine) and purine (adenine, guanine) bases in the UV-C spectral range,³⁸ resulting in the maximum of DNA absorption at ~ 260 nm. Therefore, the inactivation mechanism would be primarily photochemical because of the formation of predominantly pyrimidine dimers inhibiting the formation of new DNA chains in the process of cell replication, and resulting in clonogenic death.²²

With respect to the UV-C line 254 nm, irradiation with the UV-A spectral line with wavelength $\lambda = 365$ nm, despite higher power density of 1.67 mW/cm^2 , required a few orders of magnitude longer exposure times (hours vs. seconds) to achieve visible effects in eradication of *C. albicans* colonies. The estimated specific eradication coefficient and eradication thresholds are three orders of magnitude higher than those corresponding to irradiation with 254 nm (Table 1). Such high values render the use of the 365 nm UV-A radiation impractical for eradication of *C. albicans* because of unacceptably long irradiation times.

The estimated eradication threshold values for 406 nm are significantly higher than those for 365 nm, also rendering the 406 nm radiation impractical for an efficient application.

This relative inefficiency can be explained by considering the inactivation mechanisms for the UV-A region, and for the neighboring 406 nm line. These are completely different from the photochemical damage caused by UV-C or UV-B photons. Although detectable, the yields of pyrimidine photoproducts induced by UV-A photons are low by comparison, especially at longer wavelength photons, such as 365 nm. At 405 nm, pyrimidine dimers are not detectable.²³ The UV-A photons produce DNA lesions more characteristic of ionizing radiation: single-strand and double-strand DNA breaks and DNA-to-protein cross-links. If not repaired, such cross-links block the DNA replication.³⁹ This supports the conclusion that eradication with 365 or 405 nm light is caused by non-dimer damage, possibly DNA-to-protein cross-links. The UV-A photons are weakly absorbed by DNA, but rather excite other endogenous chromophores, generating various reactive oxygen species in cells.²⁴ Therefore, the indirect DNA damage caused by oxidation may also play an important role. Furthermore, the low eradication efficiency can be related to the relatively fast DNA repair mechanisms that are so efficient that all single strand breaks produced by 365 nm photons would be concomitantly repaired within short times.⁴⁰

More promising results were obtained with blue/violet radiation, for which the specific eradication coefficient is relatively small. The small value of Δ (420 nm) can be explained by the correspondence of this line with the maximum of the absorption spectrum of *C. albicans* occurring at 414 nm.^{18,34}

Very low values for Δ and, consequently, eradication thresholds, were obtained for the Xe spectrum. Based on our results obtained for the UV spectral range, we assumed that these low threshold values were predominantly caused by the presence of prominent spectral lines in the UV-C range ($\lambda < 320$ nm) and ~ 420 nm in the Xe spectrum (Fig. 2), coinciding with the maximum of absorption of *C. albicans* colonies occurring at 414 nm.^{18,34} This could also explain the relatively low efficiency in eradication of *C. albicans* with simulated sunlight (i.e., also a broadband spectrum, but with significantly suppressed UV-C component) reported recently.¹⁹

Conclusions

We have extended the investigation of eradication of *C. albicans* by optical radiation to new spectral lines and

TABLE 1. SPECIFIC ERADICATION COEFFICIENTS AND ERADICATION THRESHOLDS FOR THE INVESTIGATED WAVELENGTHS AND XENON SPECTRUM

Spectral range wavelength	A	$\Delta(\lambda) (\text{J/cm}^{-2})$	$ET_{99.9}(\lambda) (\text{reduction: } 3.0 \log_{10}) (\text{J/cm}^{-2})$	$ET_{99.99}(\lambda) (\text{reduction: } 4.0 \log_{10}) (\text{J/cm}^{-2})$
UV-C 254 nm	0.999 ± 0.133	$(6.11 \pm 0.95) \times 10^{-3}$	$(42 \pm 7) \times 10^{-3}$	$(56 \pm 10) \times 10^{-3}$
UV-A 365 nm	1.001 ± 0.085	4.26 ± 0.33	29 ± 3	39 ± 3
Violet 406 nm	0.999 ± 0.030	11.35 ± 0.87	78 ± 6	104 ± 8
Violet/blue 420 nm	1.001 ± 0.021	0.65 ± 0.05	4.5 ± 0.3	6.0 ± 0.5
Xe (220–950 nm)	0.999 ± 0.070	$(27.72 \pm 2.33) \times 10^{-3}$	$(191 \pm 18) \times 10^{-3}$	$(255 \pm 23) \times 10^{-3}$

bands, and determined the corresponding specific eradication coefficients and the eradication thresholds.

The established methodology enables determination and unambiguous quantitative comparison of eradication efficiencies of applied optical radiation and, therefore, selection of the most effective wavelengths for clinical and therapeutic use.

Based on estimated specific efficiencies and eradication thresholds of the investigated wavelengths, the ranking in eradication efficiency of *Candida albicans* (most to least effective) is: 254 nm, broadband Xe spectrum, 365 nm, 420 nm, and 406 nm.

The observed different eradication efficiencies are a consequence of different spectrally dependent inactivation mechanisms.

The results of our study show that the UV-C 254 nm is by far the most effective wavelength for eradication of *C. albicans*. Broadband Xe spectrum and, to a degree, violet/blue visible light at $\sim \lambda = 420$ nm, provide effective alternative solutions.

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Author Disclosure Statement

No competing financial interests exist.

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