# Clarification of the sterilization mechanism of antimicrobial photodynamic therapy for *Candida albicans*

（*Candida albicans* に対する抗菌的光線力学的療法の殺菌メカニズムの解明）

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# Title: Clarification of the sterilization mechanism of antimicrobial photodynamic therapy for *Candida albicans*

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**<Abstract>**

　**Background**：There has been a continuing increase in dental fungal infections in recent years, and according to studies of the frequency of occurrence of fungal infections that look at the frequency of different causative fungi, there has been a particular increase in infections caused by *Candida* and *Aspergillus* species. The conventionally used antifungal drugs can not reach the depth of the biofilm, and as a result, they have the disadvantage of causing resistant bacteria.

Therefore, we focused on antimicrobial photodynamic therapy (a-PDT), which is receiving attention because of the absence of such side effects, and examined its fungicidal mechanism against *C. albicans*.

　**Objective**：The aims of the present study were two-fold: (1) to investigate the relationship between the amount of $^1O_2$ generated using the electron spin resonance (ESR) spin-trapping technique and the fungicidal effects on *C. albicans*; and (2) to observe the destruction of the cell wall after a-PDT by scanning electron microscopy (SEM). Thus, two experiments were performed.

**Materials and Methods**：The first experiment used a 0.01% aqueous solution of

methylene blue (MB) and ultrapure water as a control. Both were irradiated with a diode

laser, and the amount of $^1O_2$ generated was measured using ESR spectroscopy. Then, the

number of colony-forming units per milliliter (CFU/mL) of *C. albicans* present after

incubation under different sets of conditions was determined, and the experimental groups

were defined as follows: with laser-irradiation, L(+); without laser-irradiation, L(-);

containing MB, M(+); and not containing MB, M(-). These were then combined to form

four groups: L(+)M(+); L(+)M(-); L(-)M(+); and L(-)M(-). The second experiment

followed the first with observation of the cell wall of *C. albicans* by SEM.

**Results**：The irradiation of MB with a 660 nm diode laser was caused an irradiation

time-dependent increase in the generation of $^1O_2$, and that the *C. albicans* sterilization

rate increased proportionally. Observation of SEM images of *C. albicans* exposed to $^1O_2$

showed that the surface of the fungal cells fused, and the normal morphology of single,

independent cells was lost in an irradiation time-dependent fashion, meaning that fusion

was dependent on the amount of $^1O_2$ generated, and images of cells beginning to fuse together and of irregular, bumpy shapes were observed.

**Conclusion:** The present findings clarified the relationship between $^1O_2$ generation via excited MB and the fungicidal effect on *C. albicans*. Moreover, it was considered that *C. albicans* might be sterilized by $^1O_2$ attacked to surface layer.

<＜和文対訳＞

近年、真菌症は増加傾向にあり、歯科領域においてカンジダおよびアスペルギルス属が起因となる感染症の増加が特に見られる。従来使用されている抗真菌薬はバイオフィルムの深さまで到達することができず、結果として耐性菌を生じさせてしまうなどの欠点があった。そこで我々はそのような副作用のない事で脚光を浴びている抗菌的光線力学的療法（a-PDT）に着目し、*C. albicans* に対する殺菌メカニズムについて検討を行った。本研究は（１）電子スピン共鳴(ESR) spin-trapping 法を利用し、a-PDT から発生した一重項酸素($^1O_2$)量と *C. albicans* の殺菌効果の関係性について検討、（２）走査型電子顕微鏡（SEM）を用いて a-PDT 後の *C. albicans* の菌体表面を観察することを目的として行った。

本研究では 0.02%のメチレンブルー（ＭＢ）水溶液を用い、純水（PW）を対照として両者に半導体レーザーを照射し、生成された $^1O_2$ の量を ESR 分光法にて測定した。実験群はレーザー照射の有無を L (+)、L (-)、0.02% MB の有無を M(+)、M(-)と定義し、これらを組み合わせて L（+）M（+）; L（+）M（-）; L

(-) M（+）；L(-) M（+）の４つのグループに分け行った。次に４つのグループそれぞれに *C. albicans* を作用させ、インキュベートした後の *C. albicans* の１ミリリットル当たりのコロニー形成単位の数(CFU/mL)を測定した。その後 SEM による *C. albicans* の細胞壁の観察を行った。結果は、MB に 660 nm 半導体レーザーを照射することにより $^1O_2$ は照射時間依存的に発生量が増加し、それに比例して *C. albicans* の殺菌率も増加することを認めた。$^1O_2$ に暴露された *C. albicans* の SEM 画像を観察すると照射時間依存的に真菌細胞の表面が融合し、単一の独立した正常な形態が失われ、融合し始めた像や不規則な凹凸を呈する像が観察された。これらのことから、本研究により、a-PDT により励起された MB から発生した $^1O_2$ が *C. albicans* に対し濃度依存的に殺菌効果を示し、$^1O_2$ により *C. albicans* の表層が侵害されることにより殺菌に至ることが推測された。

**\<Introduction\>**

Dental diseases such as dental caries, periodontitis, endodontic disease and denture candidiasis are mostly infectious diseases caused by oral microorganisms [1-3]. As microorganisms in oral cavity have been shown to be involved in systemic diseases such as endocarditis, aspiration pneumonia and diabetes [4-6], how to control microorganisms is the most important topics in clinical practice.

There has been a continuing increase in fungal infections in recent years, and according to studies of the frequency of occurrence of fungal infection that look at the frequency of different causative fungi, there has been a particular increase in infections caused by *Candida* and *Aspergillus* species.

The background of increasing fungi is improvements in detection techniques and detection sensitivity, at the same time as (1) an increase in the use of broad-spectrum antibiotics and steroid medications that have side effects of oral candidiasis (OC) and oral dryness, (2) an increase in the number of high-risk patients, and (3) an increase in the number of elderly people [7].

OC is a disease encountered with a relatively high frequency in dental treatment. It is

caused by *Candida* species, particularly *Candida albicans* (*C. albicans*), which are part

of the resident oral microbial flora. *C. albicans* is an opportunistic pathogen that normally

has weak pathogenicity and proliferative ability, but which multiplies and demonstrates

pathogenicity, causing an outbreak of opportunistic infection, if the body's immunity is

compromised [7]. *C. albicans* exhibits dimorphism, having a filamentous form comprising

hyphae or pseudohyphae and a yeast form [8,9]. It is usual for such fungi to invade mucous

membranes as hyphae that adhere strongly to the mucous membrane, and with repeated

recurrence, the infection progresses to intractable OC [10]. At present, Japan is rapidly

becoming a super-aging society, and changing disease patterns in dentistry are being seen

as a result. This is leading to a need for dental clinics to take on the responsibility of

treating elderly people who are receiving primary nursing care at home or in facilities.

There is a high rate of OC in such patients, and expertise in OC will therefore be essential

for dentists. In addition, there is an increased risk of fungal pneumonia among elderly

people due to pulmonary aspiration of *Candida* species, and the risk is further increased

if swallowing function is reduced as a result of cerebral infarction or other disease. There

is, therefore, considerable attention being paid to the importance of oral care for elderly

persons [7,10].

Although the conventional treatment of OC applied to topical or systemic antifungal

agents (azole, polyenes), it resulted in the development of resistant *Candida* species [11].

Additionally, the organization of microorganisms in biofilms is a protective shell,

enabling the survival of these pathogens even in unfavorable conditions and providing

high resistance to antifungal agents [12]. Considering the increased incidence of resistant

pathogens to conventional antifungal treatments and drug toxicity, studies have searched

for strategies to control fungal species [13]. In recent years, novel methods of disinfection

for use in treating dental caries, periodontal disease, endodontic disease [14-18] and OC [19-23] have become available. It is well known that the application of photodynamic therapy

(PDT), including antimicrobial PDT (a-PDT), can be used as a disinfection method [24-26].

Many studies have demonstrated that the use of a-PDT for bactericidal and fungicidal

effect requires that many variables be taken into account when developing a-PDT protocol,

including light parameters, photosensitizers, and light delivery techniques [27-29]. Many

investigators have demonstrated that *C. albicans* is effectively sterilized by a-PDT in the

following manner: (1) photosensitizing agents (PS) attach to the cell membrane of *C.*

*albicans*, (2) irradiation with light at a specific wavelength matched to the peak absorption

of PS leads to the generation of singlet oxygen ($^1O_2$), and (3) fungidal death via

destruction of the cell walls is induced by $^1O_2$ [30-33]. However, although the oxidizing

power of $^1O_2$ has been shown to induce fungicidal effects, the details of the relationship

between the amount of generated $^1O_2$ and the degree of fungicidal effects have not yet

been clarified. Moreover, there is no report that visually observed cell wall destroyed by

a-PDT.

$^1O_2$ is a reactive oxygen species (ROS). Nakano et al [34] and Tatsuzawa et al [35] reported

that $^1O_2$ was toxic to prokaryotic cells and is almost completely nontoxic to eukaryotic

cells. In addition, Silva et al [36] reported that moderate angiogenesis, fibrogenesis, or

noninflammatory cells were observed in the animal models treated with PDT. On the

other hand, the methylene blue (MB) used in this study may cause biotoxicity due to cell

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staining. George and Kishen [37] reported that 10 μmol/L MB, when irradiated for 20

minutes with a 30-mW diode laser, killed 30% of fibroblasts. Therefore, to safely apply

a-PDT, the amount of generated $^1O_2$ should be carefully considered.

The aims of the present study were two-fold: (1) to investigate the relationship between

the amount of generated $^1O_2$ using the electron spin resonance (ESR) spin-

trapping technique and the fungicidal effects on *C. albicans* (2) to observe the destruction

of cell wall after a-PDT by using scaning electon microscopy (SEM).

**<Materials and Methods>**

**Reagents and Laser Source**

Pure water (PW: Ultrapure Water for Molecular Biology) was purchased Merck Millipore

(Tokyo, Japan), MB and 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TMP) were purchased

from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MB was used as a

PS in this study. The 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (4-oxo-TEMPO) was

purchased from Sigma Aldrich (St. Louis, MO, USA). All other reagents were analytical

grade. A diode laser ($\lambda$=660 nm, 200 mW in CW) supplied by Osada Electric Co. Ltd.

(Tokyo, Japan) was used as the irradiation source. A diode laser was used in a non-contact

mode, and delivered with distance from the tip ($\phi$ 300 μm) of the quartz fiber to the

surface of bacterial suspension being 3 cm. The laser irradiation time periods were set to

600, 1,200 and 1,800 seconds and the power densities were 106, 212 and 318 W/cm$^2$,

respectively.

**Experiment 1: The relationship between the amount of generated $^1O_2$ and the**

**fungicidal effects on *C. albicans***

The 0.02% MB aqueous solution was used in this study. PW was used as a control. The 0.02% MB and control were irradiated with a diode laser. The amount of generated $^1O_2$ was measured using ESR spectroscopy (JES FA-200, JEOL, Tokyo, Japan). The 0.02% MB and 40 mM 4-oxo-TMP were mixed in test tubes ($\phi$ 12 mm) to make final concentrations of 0.01% MB and 20 mM 4-oxo-TMP. Immediately, the mixtures were irradiated with the diode laser for 600, 1,200 and 1,800 seconds. Subsequently, the mixture was transferred into an ESR flat cell and then was measured using an ESR spectrometer. The ESR measurements were conducted under the following conditions: magnetic field, $335 \pm 5$ mT; modulation width, 0.025 mT; time constant, 0.1 seconds; microwave power, $4.00 \pm 0.05$ mW; sweep width, 5 mT; sweep time, 2 minutes; and amplitude, 100. The signal intensities were normalized to a MnO marker and the concentrations of the stable radical products (4-oxo-TEMPO) were determined using an external standard based on the signal height [38].

Finally, the amount of generated $^1O_2$ was evaluated according to the $^1O_2$ specific oxidation from 4-oxo-TMP to 4-oxo-TEMPO (Figure 1), which is detectable with ESR.

*C. albicans* was obtained from the American Type Culture Collection (ACTT18804). A suspension of *C. albicans* from culture grown on brain heart infusion (Becton, Dickinson, and Co., NJ, USA) at 37°C for 24 hours was prepared in sterile physiological saline. The final concentration was adjusted to $1 \times 10^7$ cells/ml of the suspension and 0.01% MB in 2 mL of saline. Immediately after mixing the test tubes, the mixtures were irradiated with stirring for 600, 1,200 and 1,800 seconds using a diode laser. After the laser irradiation was complete, 10-fold serial dilutions were prepared and 100 µL aliquots of each dilution were seeded in duplicate onto Sabouraud dextrose agar (Difco, MI, USA) plates and incubated for 48 hours at 37°C. Finally, the number of colony-forming units per milliliter (CFU/mL) present after incubation was determined. The experimental groups were defined as follows: with laser-irradiation, L(+); without laser-irradiation, L(-); containing MB, M(+); and not containing MB, M(-). These were combined to form four groups: L(+)M(+); L(+)M(-); L(-)M(+); and L(-)M(-).

**Experiment 2: The observation of cell wall fracture after Experiment 1**

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The each experimental group mixtures, which is involved *C. albicans*, were centrifuged for 10 minutes at 1,300 x g. The cell pellet was fixed in 2.5% glutaraldehyde for 1hour and dehydrated in several ethanol washes (10, 25, 50, 75, and 90% for 20 minutes and 100% for 1hour). Then, the cell pellet was incubated at 37℃ for 24 hours to dry, and transferred to aluminium stubs and covered with Au-Pd for 120 seconds at 40 mA. After metalization, the cell wall of *C. albicans* was examined and photographed by SEM (S-3400N, Hitachi, Japan), operating at 15 kV, at ×10.0 k magnification [39].

**Statistical Analysis**

The results of experiment 1 were analyzed with one way analysis of variance (ANOVA). When appropriate, ANOVA was followed by post-hoc Tukey's test to compensate for multiple comparisons ( $\alpha = 0.05$ ).

**<Results>**

**Experiment 1:** The relationship between TEMPOL and signal intensity ratio based on

MnO marker increased in a concentration-dependent manner (Figure 2). Figure 3 showed

the typical ESR-spectra of 0.01% MB irradiated by the diode laser for 600, 1,200 and

1,800 seconds. The ESR spectra displayed a 1:1:1 triplet signal characteristic of 4-oxo-

TEMPO having a hyperfine splitting constant (aN=1.608 mT) [38]. Figure 4 showed the

amount of $^1O_2$ generated from 0.01% excited MB. The amount of generated $^1O_2$ was

increased by the laser irradiation in a time-dependent manner. A positive correlation was

observed between the amount of generated $^1O_2$ and the laser irradiation time ($R^2$=0.999).

According to the equation of linear relationship between the amount of 4-oxo-TEMPO

and the irradiation time, the amount of $^1O_2$ generated from 0.01% excited MB during 600,

1,200 and 1,800 seconds of irradiation was about 82.7, 159.4 and 245.3 μM, respectively.

Figure 5 showed the numbers of CFU/mL of *C. albicans* in groups L(-)M(-), L(+)M(-),

L(-)M(+) and L(+)M(-). In group L(+)M(+), the number of CFU/mL was significantly

reduced in association with the laser irradiation time compared to the other groups

($p<0.05$). On the other hand, no fungicidal effects were observed in groups, L(-)M(-), L(+)M(-) or L(-)M(+). In brief, the amount of generated $^1O_2$ necessary to kill *C. albicans* (> 99.99%) was at least about 245.3 μM.

**Experiment 2:** In the observation of the surface of the fungal cells after irradiation, in groups L(-)M(-), L(+)M(-), and L(-)M(+), no structural damage was seen, and there were no surface changes, so that the characteristic shape was preserved (Figure 6). In group L(+)M(+), the surface of the fungal cells was fused in an irradiation time-dependent fashion, meaning that fusion was dependent on the amount of $^1O_2$ generated. Cells beginning to fuse together and the formation of irregular bumpy shapes were observed. In the SEM images following 1,800 seconds of irradiation, there were images of cells that had fused and undergone further breakdown in morphology, becoming amorphous lumps of material.

**\<Discussion\>**

*C. albicans* is a dimorphic fungus present in the regular flora of the mouth, skin, and pharynx of healthy people. When it shows pathogenicity, it undergoes a transition from the yeast form to the filamentous form, establishing itself in host tissues and multiplying, causing damage to target tissues. Progression of the infection can lead to fungemia or systemic infection [40,41]. In recent years, it has been thought that this ability to transition to the filamentous form is a major factor in pathogenicity in deep-seated mycosis [42-44].

There is an increasing range of options available for the treatment of fungal infections, but there is nothing currently that can offer dramatic effects. Furthermore, there are numerous problems, such as the side effects of drugs [45].

On the other hands, a-PDT mechanism damages fungal cells when ROS penetrate the cell walls and membranes, the allowing displacement of the PS into the cell. Then, oxidizing species generated by the excitiation of light induce the photodestruction of internal cellular organelles, leading to cell death. Thus, the $^1O_2$ generated by the excitiation of PS is non-specific oxidizing agent against which there is no defence [46,47].

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The present study therefore aimed to use $^1O_2$, which has few side effects, as a safe method to kill *C. albicans* and also to clarify the mechanism by which death occurs.

The results show that irradiation of MB as PS with a 660 nm diode laser caused an irradiation time-dependent increase in the generation of $^1O_2$, and that the *C. albicans* sterilization rate increased proportionally. Observation of SEM images of *C. albicans* exposed to $^1O_2$ showed that the surface of the fungal cells fused, and the normal morphology of single, independent cells was lost in an irradiation time-dependent fashion, meaning that fusion was dependent on the amount of $^1O_2$ generated. Cells beginning to fuse together and the formation of irregular bumpy shapes were observed. This suggests a mechanism whereby the surface layer of *C. albicans* is disrupted by a-PDT, leading to the death of the fungus. At the same time, it has been reported that the half-line of $^1O_2$ in the cell system is just 2 μs, so that even if $^1O_2$ were generated in the extracellular fluid of eukaryotic cells, the $^1O_2$ would be deactivated and transform to its ground state of molecular oxygen before it could enter the cells; it would therefore be unable to reach mitochondrial respiratory chain enzymes in the cells and attack them. In other words, in

eukaryotic cells, which do not have respiratory enzyme systems in the cell membrane, but instead have them in the mitochondria within the cell, as long as the $^1O_2$ cannot reach the respiratory system within the cell, this mechanism of cell destruction cannot operate [34,35].

C. albicans is eukaryotic, and therefore the above finding should be valid for this species. Consequently, there should be no sterilization effect, but a sterilization effect was found in the present study. We considered that there are two reasons. As one reason, the PS used in this study was MB, which is known for its use as a cell staining agent, and MB stains the cell wall of C. albicans [48]. The cell wall of fungi plays a number of roles in the biological activity of the cell. Then, as well as protecting the cell against stress from the physical environment, it is involved in functions such as retaining the morphology of the cell, the intake of nutrients from the outside world, and the exchange of materials with the outside world. Therefore, appears that $^1O_2$ generated from within the cell wall itself disrupts the cell wall so that these functions are lost, leading to the death of the cell. As another reason, although C. albicans has some kinds of antioxidants enzymes, such as catalase, superoxide dismutase and glutathione peroxidase [49-51], because they are

ineffective against $^1O_2$, internal cellular organelles were also injured after surface layer destruction by $^1O_2$, leading to the death of the cell. However, *C. albicans* was not completely sterilized. As the reason, *C. albicans* may have some defense mechanisms, so further studies are needed. Hsieh YH reported that although PDT alone effectively eradicated *C. albicans* biofilms, when combined with fluconazole, PDT significantly inhibited *C. albicans* to greater extent [52]. We also consider that the development of resistant bacteria is the most feared in OC, therefore it is necessary that developing methods of therapeutic system to break down the biofilm with PDT and penetrate the antifungal drug deeply.

In conclusion, the present findings clarified the relationship between $^1O_2$ generation via excited MB and the fungicidal effect on *C. albicans*. Moreover, it was considered that *C. albicans* might be sterilized by $^1O_2$ attacked to surface layer.

**&lt;Acknowledgments&gt;**

**&lt;Conflict of Interest&gt;**

No potential conflicts of interest were disclosed.

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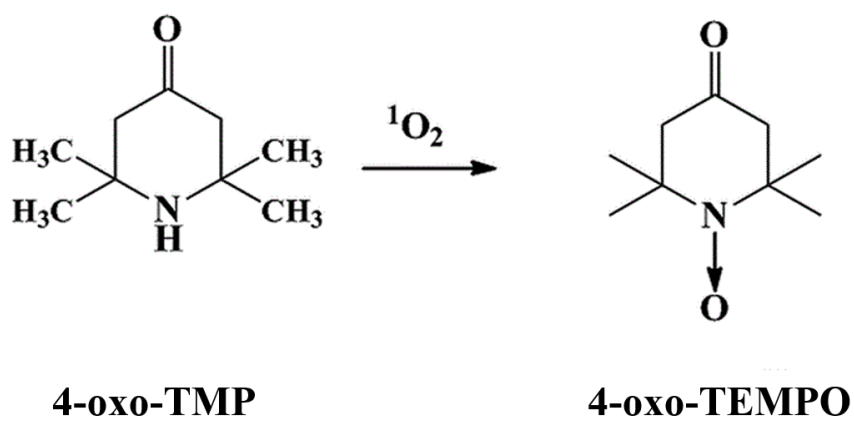
Figure 1    From 4-oxo-TMP to 4-oxo-TEMPO by generated $^1O_2$

A 4-oxo-TMP is a scavenger with high reactivity with $^1O_2$. As a result, stable 4-oxo-

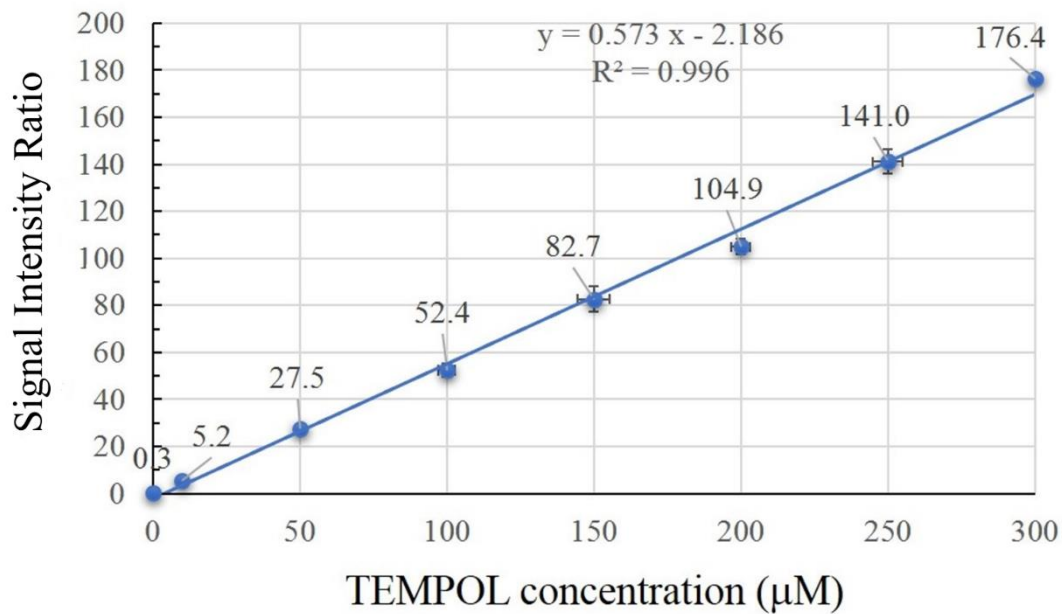TEMPO free radical is produced as a reaction product.

Figure 2    External standard curve of the TEMPOL

A positive correlation was observed between each concentrations of TEMPOL and

signal intensity ($R^2$=0.996). The equation of the line is $y = 0.573x - 2.186$. The data
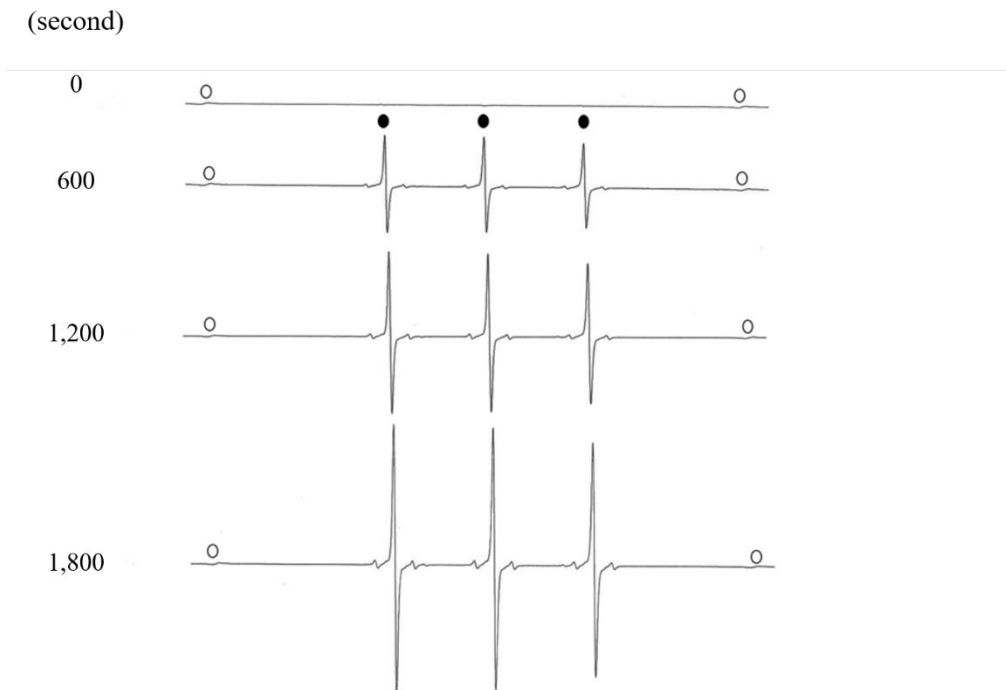
points indicate the mean values (n=6) with standard deviation bars.

(second)



Figure 3    ESR-spectra obtained after laser irradiation

The typical ESR-spectra of the control and 0.01% MB after laser irradiation for 600,

1,200 and 1,800 seconds. The white and black circles indicate the $Mn^{2+}$ marker and the
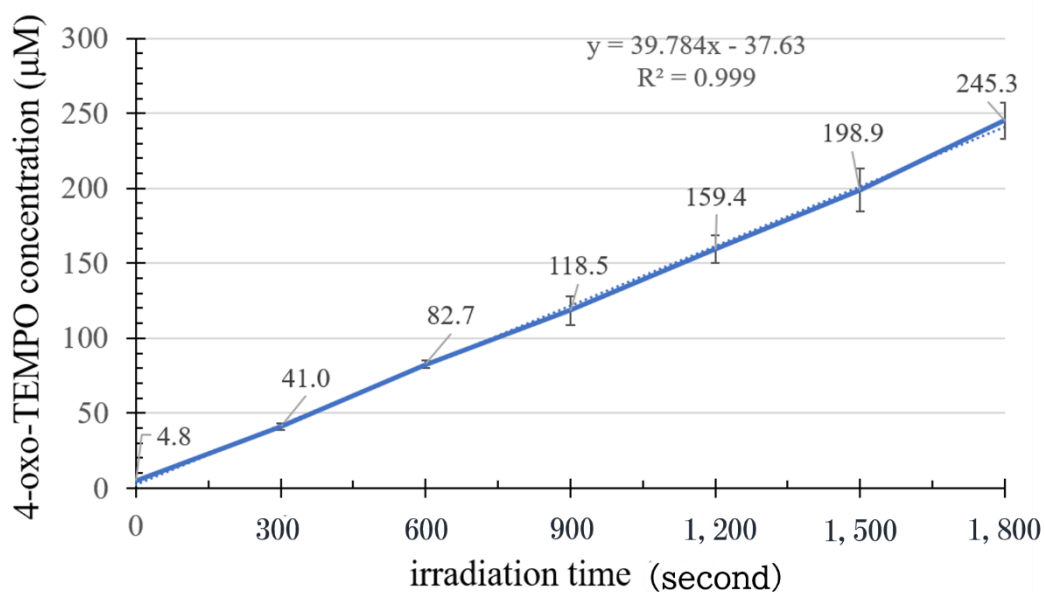
nitroxide radical, respectively.

Figure 4    The relationship between laser irradiation time and 4-oxo-TEMPO

The amount of generated $^1O_2$ increased with laser irradiation. A positive correlation was

observed between the amount of generated $^1O_2$ and 0.01% MB ($R^2$=0.999).

The equation of the line is $y = 39.784x - 37.63$. The data points indicate the mean values

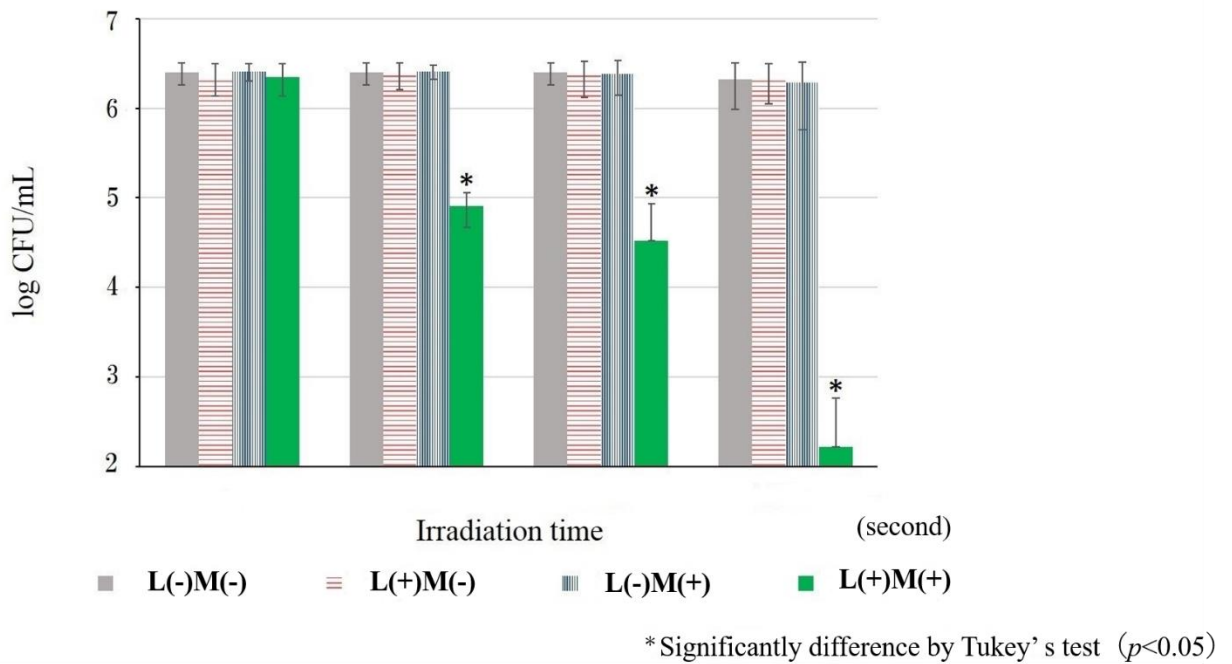(n=6) with standard deviation bars.

Figure 5　　　Numbers of CFU/mL in the suspension after a-PDT

In the L(+)M(+) group, the number of *C. albicans* cells decreased with a >4-log

reduction within 1,800 seconds. The data points indicate the mean values (n=6) with

standard deviation bars. The numbers of CFU/mL decreased significantly in the

L(+)M(+) group at 600, 1,200 and 1,800 seconds, compared to all other groups
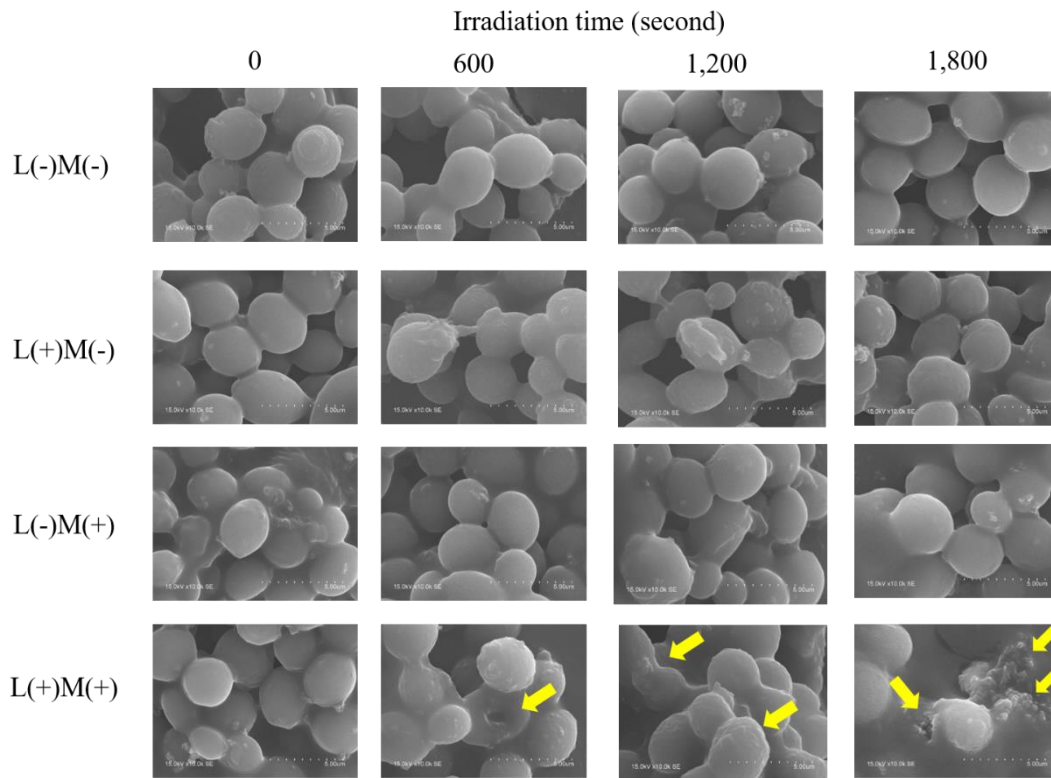
($p<0.05$).

Figure 6     Typical SEM images of *C. albicans* surface on each conditions

The yellow arrows indicated fused cells and bumpy shapes.

The experimental groups were defined as follows: with laser-irradiation, L(+); without

Laser-irradiation, L(-); containing MB, M(+); and not containing MB, M(-).