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Original article

Photodynamic therapy of red and blue lights on *Malassezia pachydermatis*: an *in vitro* study

W.R. Kim*, S.G. Bae*, T.H. Oh

Department of Veterinary Internal Medicine, College of Veterinary Medicine, Kyungpook National University, Daegu 41566, Republic of Korea

Abstract

In veterinary medicine, infection caused by *Malassezia pachydermatis* is spreading and necessity of alternative treatment is emphasized. Photodynamic therapy (PDT) is therapeutic method using specific spectrum of light with photosensitizer. In this study, applying PDT not only using red light which is used in human medicine commonly, but also using blue light into skin infection causative microorganism with photosensitizer, confirm the effect of PDT and possibility of being an alternative treatment. Four isolates of *M. pachyderematis* were collected from canine skin and used into this study. Light emitting diode with 495 nm, 625 nm spectrum was applied, and final concentration of δ-aminolevulinic acid (ALA), which is used as a photosensitizer, was adjusted into 20%. To confirm effectiveness of PDT, the number of colony forming unit was checked and variation of optical density values was measured. Antifungal effect of PDT on both spectrums was presented in all condition, and it makes best result when using blue light applied with ALA. Through outcome of this study, PDT using light in 465 nm, 625 nm wavelength combinations with ALA can interrupt proliferation of *M. pachydermatis* considerably. In consequence, PDT can be alterative treatment of canine *Malassezia* infection.

Key words: dog, LEDs, PDT, *Malassezia* infection

Introduction

During the past decades, *Malassezia* species have emerged as increasingly important pathogens in dogs. The lipid-independent species *Malassezia pachydermatis* is commonly recovered from the ears, skin and anal sacs of dogs not only diseased region but also normal region (Scott 1992, Guillot and Guého 1995).

Under normal circumstances, *Malassezia* infection is treated topically, systemically, or both of these methods. Among these ways, systemic antifungal agents are used commonly (Ayers et al. 2005) because it has good medicinal effect all over the body and can affect various strains of *Malassezia* species. However, the standard therapeutic regimen for *Malassezia* infection is expensive, prolonged and moreover, some clinicians prescribe antifungal agent without fungal susceptibility test which is most valuable and useful tool in skin infection. Consequentially, the prevalence of drug-resistance strains of *Malassezia* has increased (Johnson et al. 1995) so the skin infection is not cured completely and relapsed in many cases. Besides, some

Correspondence to: T.H. Oh, e-mail: thoh@knu.ac.kr, tel.: +82 53 950 5959

* These authors contributed equally to this work

patients can not intake antifungal agent because of poor compliance such as gastric discomfort, hepatotoxicity concerns (Gupta and Cooper 2008, Havlickova et al. 2008). Because of these therapeutic weaknesses, alternative therapeutic options are needed.

Using light as a therapeutic method for the treatment of localized fungal infection means a proposing new area. Photodynamic therapy (PDT) is one of alternative remedy that uses a photosensitizer, which is degraded by light illumination in a particular wavelength. The decomposition of the photosensitizer induces several chemical reactions, such as formation of singlet oxygen which is highly reactive to peripheral tissues. The existence of these toxic molecules in the region to be cured result in the destruction of target cells (Moreira et al. 2008).

Among various wavelengths, considering their abilities of absorption and penetration, the blue and red light is regarded good for fungicidal effect and useful for the remedy of skin infection (Ito 1981, Prates et al. 2009).

In human medicine, many cases of skin infections are treated with photodynamic therapy and have an excellent result (Maisch et al. 2004, Kharkwal et al. 2011) for a long time. In these days, to maximize effect of PDT, most clinicians are adding δ-aminolevulinic acid (ALA) as the photosensitizer (Gaullier et al. 1997, Peng et al. 1997) but there are just a few studies about photodynamic therapy and *Malassezia* infection (Kim and Kim 2007, Lee et al. 2010). Besides, these studies use just red light alone. What was worse, in veterinary medicine, the study on the phototherapy is still in the early stage and there are no studies about photodynamic therapy and *Malassezia* infection.

As many studies showed that growth of various fungal species is restricted by visible wavelength photodynamic therapy (Dovigo et al. 2011, Lyon et al. 2011), hypothesis that visible light also restricts growth of *Malassezia* species which is major pathogen of the canine otitis externa and skin infection can be formulated.

In this study, to prove anti-*Malassezia* effect of visible light, change of colony forming units (CFU) and the value of optical density (OD) at 530 nm was measured. Moreover, antifungal effect of red and blue light was compared so we can find suitable wavelength to apply on infected skin.

Materials and Methods

Isolation of *Malassezia pachydermatis*

Four samples were collected from the dogs which *Malassezia* infection was suspected based on the history, microscopic examination of tape imprint specimen, and physical examination. After *Malassezia* infection had been confirmed, samples were collected aseptically from external ear canal, axillary skin and inter-digital skin region by using sterile cotton wool swabs, and inoculated in Sabouraud Dextrose Agar (SDA; Difco, USA). Samples were cultured aerobically at 35° for 72 h. *M. pachydermatis* was identified by gross colony, microscopic morphology and its ability to grow on SDA.

Illumination method

Illumination was carried out using LED system consist of 4*1W for blue light, 5*0.8W for red light power LED (Seoul Semiconductor Co., Korea). The blue lamps had a symmetrical peak wavelength of 465 ± 10 nm and the red lamps had an asymmetrical peak wavelength of 625 +5/-7 nm. At a distance of 5 cm from the light source, total irradiance of both lamps was 62.9 mW/cm.

– Photosensitizer

ALA (Sigma, USA) is used as a photosensitizer. Stock solution is made by diluting ALA in sterile distilled water and stored in refrigerator to prevent chemical change by ambient light and heat. New ALA stock solution (40% in sterile distilled water) was prepared every 5 days and a new working solution (20% in sterile distilled water) was prepared daily.

– Illumination to Agar

Single colony was diluted 10000 times with normal saline to make suitable concentration for manual counting (150–300 CFU/petri dish), and 10 µl of yeast diluted solution was inoculated onto 90 mm petri dishes of SDA.

The SDA petri dishes were divided into 6 groups as follows:

* Group A: The control group (Non-treatment)
* Group B: The group treated red light alone
* Group C: The group treated red light with ALA
* Group D: The group treated blue light alone
* Group E: The group treated blue light with ALA
* Group F: The group treated ALA alone

Group B and C were illuminated to red light for 30, 60, 90 and 120 min just once, followed by incubation at 35° for 48 h. Group D and E were treated same way as Group B and C, except red light switched to blue light. After incubation, colonies of each petri dish were counted.

– Illumination to Broth

To confirm efficacy of growth inhibition effect of PDT, measuring value of OD at 530 nm was taken. Single colony of each isolates was diluted and then

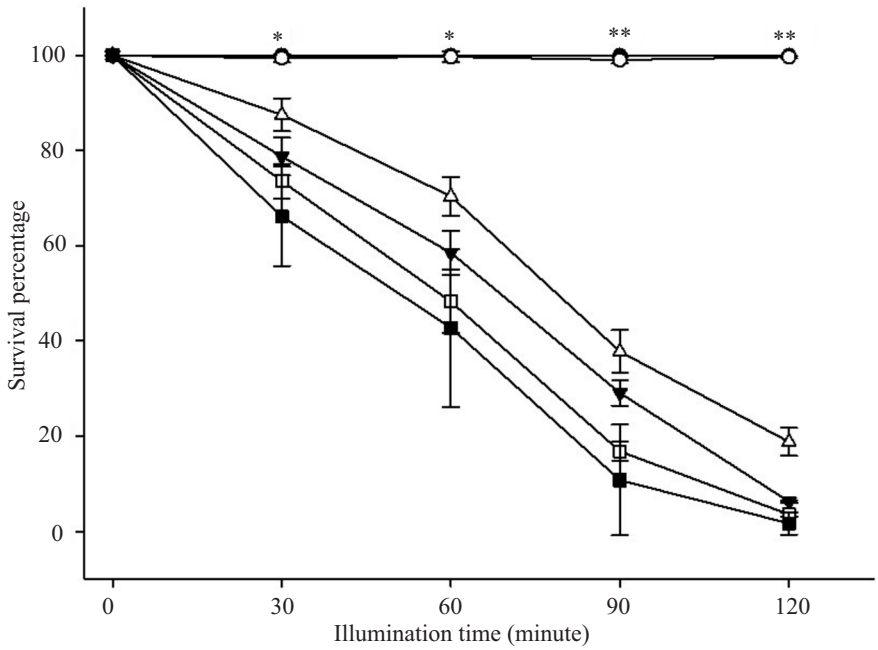

Fig. 1. Survival percent of *M. pachydermatis* after 30, 60, 90, and 120 min of illumination compared to control. ○: Group A (non-treatment), ●: Group F (ALA alone), Δ:Group B (red light illumination), ▼: Group D (blue light illumination), ❑: Group C (red light illumination with ALA), ■: Group E (blue light illumination with ALA), *: $p<0.05$ in Groups B, C, D, and E compared to the non-treatment group, **: $p<0.05$ in Groups C, D, and E compared to the non-treatment group.

100 µl of each yeast diluted solution was distributed into 96 well titer plates. 96 well titer plates were divided into 6 groups same as SDA petri dish test (Group A, B, C, D and E) except Group F. Group B and C were illuminated to red light, Group D and E were illuminated to blue light also same as petri dish test and then incubated at 35° for 48 h. After incubation, efficacy of growth inhibition effect of PDT was confirmed by measuring OD_{530nm} at 8 h intervals over a period of 48 h.

– Statistical method

For statistical analysis, commercial statistical analysis software (SigmaPlot 12.0, Systat Software Inc., USA) were used in all tests. Repeated measure ANOVA was used to analyze survival percent between test groups. Dunnett's test was performed to compare treatment groups and control group and when p value was smaller than 0.05, the result was considered significant.

Results

– Plate colony counting

Fungal survival was measured after 48h of incubation by standard plate counting. Each count was conducted in duplicate and the survival rate was calculated for each treatment group compared to the control group (Fig. 1). The survival rate of Group F, treated with ALA alone, was similar to that of Group A, the control group, regardless of exposure time. Groups B and D, which were treated with light alone, revealed a correlation between light dose and antifungal effect. This effect was improved when applied with ALA together, as presented in the result for Groups C and E.

– Optical density

To estimate effect of PDT on *M. pachydermatis*, turbidity of the broth was measured by determining the OD_{530nm} values for samples under different treatment conditions. Experimentally measured values of OD_{530nm} are shown in Fig. 2-5. Red and blue light inhibited the growth of *M. pachydermatis* under all conditions; when ALA was added, growth inhibition was increased.

Discussion

The results of this study show that illumination with visible spectrum light, particularly red and blue light, had a light dose-dependent fungicidal effect on *M. pachydermatis* when applied with ALA.

All eukaryotic cells naturally produce ALA, which converted to porphyrins by intracellular metabolism. These porphyrins absorb mainly blue and red light and promote the formation of singlet oxygen, causing photoactivation in microorganisms

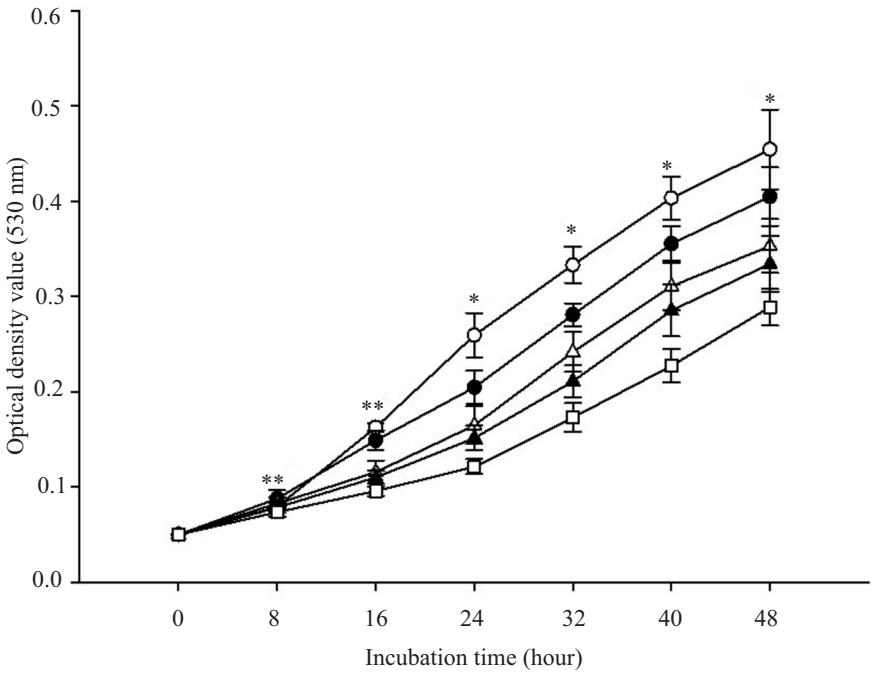

Fig. 2. After 30 min of illumination, changes in optical density values at 530 nm were measured at 8-h intervals for 48 h. ❍: Group A (non-treatment), ●: Group B (red light illumination), ∆: Group D (blue light illumination), ▲: Group C (red light illumination with ALA), ❑: Group E (blue light illumination with ALA), *: $P<0.05$ in Groups B, C, D, and E compared to Group A, **: $p<0.05$ in Groups C, D, and E compared to Group A.

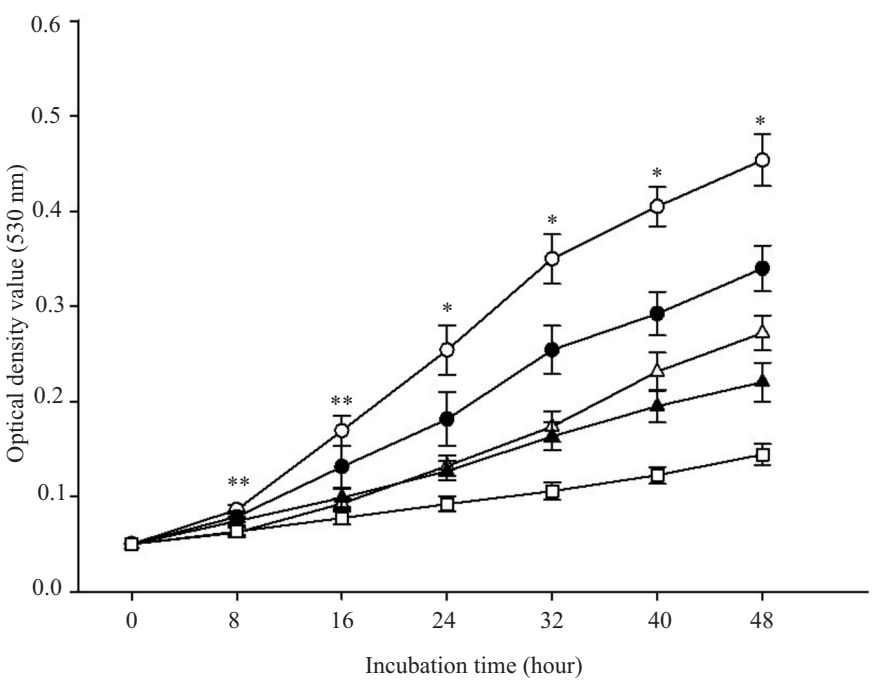

Fig. 3. After 60 min of illumination, changes in optical density values at 530 nm were measured at 8-h intervals for 48 h. ❍: Group A (non-treatment), ●: Group B (red light illumination), ∆: Group D (blue light illumination), ▲: Group C (red light illumination with ALA), ❑: Group E (blue light illumination with ALA), *: $p<0.05$ in Groups B, C, D, and E compared to Group A, **: $p<0.05$ in Groups C, D and E compared to Group A.

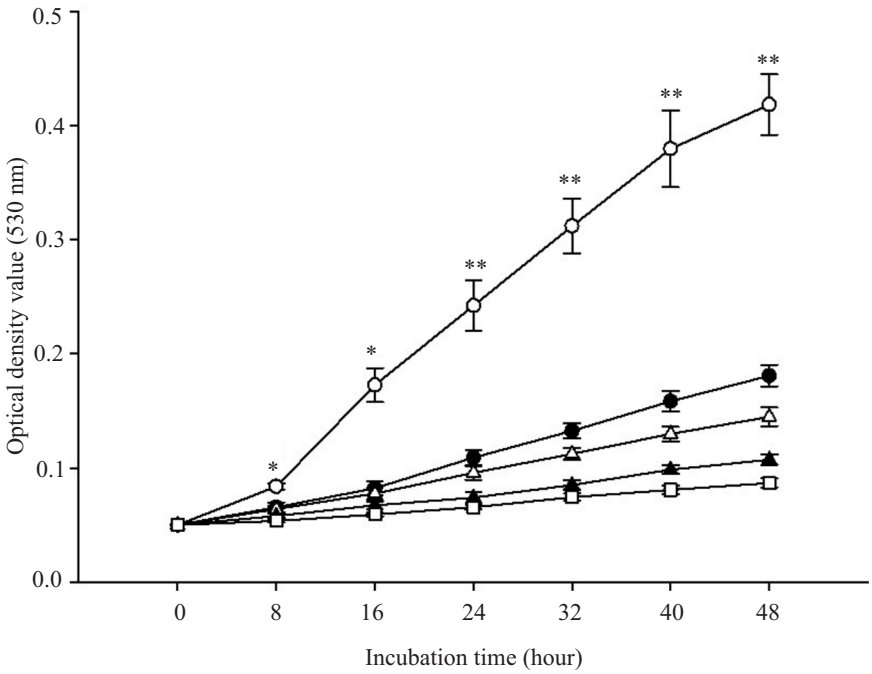

Fig. 4. After 90 min of illumination, changes in optical density values at 530 nm were measured at 8-h intervals for 48 h. ❍: Group A (non-treatment), ●: Group B (red light illumination), ∆: Group D (blue light illumination), ▲: Group C (red light illumination with ALA), ❑: Group E (blue light illumination with ALA), *: $p<0.05$ in Groups B, C, D, and E compared to Group A, **: $p<0.05$ in Groups C, D, and E compared to Group A.

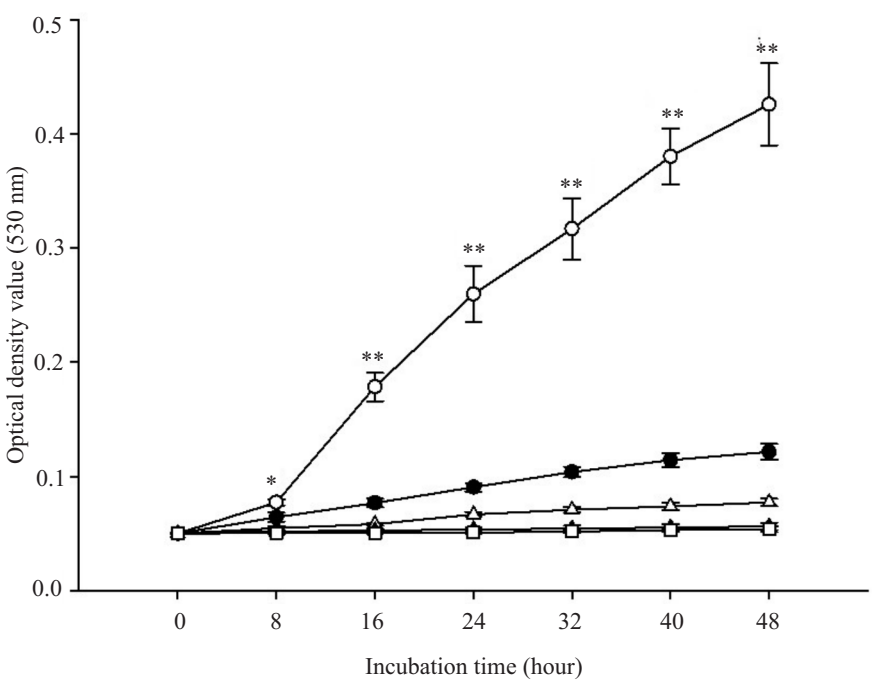

Fig. 5. After 120 min of illumination, changes in optical density values at 530 nm were measured at 8-h intervals for 48 h. ❍: Group A (non-treatment), ●: Group B (red light illumination), ∆: Group D (blue light illumination), ▲: Group C (red light illumination with ALA), ❑: Group E (blue light illumination with ALA), *: $p<0.05$ in Groups B, C, D, and E compared to Group A, **: $p<0.05$ in Groups C, D, and E compared to Group A.

(Kennedy et al. 1990, Kalka et al. 2000, Fukuda et al. 2005, Steinbauer et al. 2010). However, the quantity of naturally produced ALA is not sufficient to have a fungicidal effect. Therefore, adding exogenous ALA can lead to increased intracellular ALA in fungi and improve the photodynamic effect of red and blue light. As in other eukaryotic cells, *M. pachydermatis* also metabolizes ALA into photosensitizing porphyrin (Brouillet et al. 1975). Thus, PDT with adding ALA is expected to have a good fungicidal effect to *M. pachydermatis*.

In this study, the output of both lamps was 4 W; after illumination of samples for 30, 60, 90, and 120 min, the amount of energy was calculated to be 63.7, 127.4, 191.1, and 254.8 J/cm, respectively. Plate colony count examination revealed that the maximum fungicidal effect was observed in Group E, with *Malassezia* colonies reduced by as much as 99.75%. In the comparison of blue and red light, the fungicidal effect of blue light was superior to that of red light under all conditions and more suitable for treating *M. pachydermatis* infection. We also examined whether ALA has fungicidal effects when used alone. The results for Group F, which was treated with ALA alone, were not significantly different from those of Group A, the no treatment control group. Thus, ALA as a photosensitizer has nearly no fungicidal effect when used alone.

To assay yeast growth, optical density was measured at 530 nm. The OD value increased as the number of yeast in the broth increased. The OD values showed that as light dose increased, the gradient of the yeast growth curve decreased compared to in the control group. The degree of decrease was enhanced by adding ALA as a photosensitizer. Under all conditions, blue light showed more significant photodynamic effects than red light. These results demonstrate that PDT can suppress the proliferation of *M. pachydermatis*.

In previous human medicine studies, PDT showed an excellent ability to kill pathogenic microorganisms (Kharkwal et al. 2011, Harris and Pierpoint 2012). However, in veterinary medicine, few studies have examined using PDT to treat microbial infection. In humans, there are two *in vivo* studies of *Malassezia* infection treated with PDT, which used red light rather than blue light. In the first study (Lee et al. 2010), six patients with recalcitrant *Malassezia* folliculitis were treated using red light (average wavelength 630 nm) with ALA three times. In the second study (Kim and Kim 2007), one man suffering from pityriasis versicolor was treated with PDT using the same procedure and wavelength. In that studies, red light with ALA showed significant photodynamic effects on organisms. However, they also suggested that the environmental conditions differed according to the patients or lesion locations. Thus, the outcome of PDT may vary in different patients. Thus, additional *in vitro* or pilot studies needed before PDT can be used in veterinary clinics.

The results of this and former studies reveal the following. First, blue light has better antifungal effects than red light and considerably inhibits the proliferation of *M. pachydermatis*. Second, ALA itself does not have antifungal effects, but when used in combination with PDT significantly increases the effect of PDT. Third, the effect of PDT is light dose-dependent under all conditions.

The antifungal effect of PDT varies *in vivo*; however, in this study, we confirmed the effectiveness of PDT using two wavelengths of light with ALA, demonstrating that this method is an alternative treatment modality for canine *Malassezia* infection.

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