別記様式第 6 号（第 16 条第 3 項，第 25 条第 3 項関係）

論 文 審 査 の 結 果 の 要 旨

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学 位 授 与 の 条 件	学位規則第４条第①・２項該当		

論　文　題　目

Antimicrobial Photodynamic Therapy with the photosensitizer TONS504 eradicates *Acanthamoeba*
(アカントアメーバに対する光線力学的抗微生物化学療法（PACT）の効果)

論文審査担当者

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〔論文審査の結果の要旨〕

Acanthamoeba species are the causative agents of a sight-threatening infection of the cornea known as Acanthamoeba keratitis (AK). Acanthamoeba has two forms, an active trophozoites 25-40 μm in diameter and dormant cyst stage with diameter 13-23 μm. Trophozoites do active functions in favorable environments (food supply, neutral pH and temperature about 30°C) and change into a double-walled cyst form in harsh conditions. The risk factor of AK is ocular trauma that exposed with contaminated water, it is also associated with poor hygiene of contact lens (CL) wearers. The incidence of Acanthamoeba keratitis on the previous reports is widely variable around the countries. In the United States, the Acanthamoeba keratitis incidence was 1.5–2.01 per million and 1.4–42 cases per million inhabitants in the United Kingdom. Patients with AK may experience pain with photophobia, ring-like stromal infiltrate and epithelial defect. Diagnosis of AK is challenging, and the available treatments are lengthy and not fully effective against this microorganism. This is of particular concern in the absence of available alternative treatment.

Photodynamic therapy that they proposed has also historically been applied to the inactivation of microorganisms, for which it is termed photodynamic antimicrobial chemotherapy (PACT). PACT is a promising technology that can target pathogens and thus has the potential for use in therapeutic interventions. The basis of this technology is that light of an appropriate wavelength activates the photosensitizing compound, resulting in the production of singlet oxygen and other reactive oxygen species (ROS) that induce cell death in the target pathogen/tissue.

Recently, they have been developing PACT with the combination of a newly developed photosensitizer (PS), called TONS504, and light source using a light-emitting diode system that provides single beam with wavelength of 660nm, as a potential approach to treat corneal

infection. In the previous reports showed that this approach was effective for the inactivation of several pathogens, such as methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus,* acyclovir-sensitive or acyclovir-resistant herpes simplex virus type 1, *Pseudomonas aeruginosa*, filamentous fungi *Fusarium solani* and *Aspergillus fumigatus* in vitro.

This study was aimed to evaluate the effectiveness of TONS504-PACT in *Acanthamoeba* in vitro. *Acanthamoeba castellani* (ATCC) 50370) trophozoite form was grown in xenic culture at 26°C with a lawn of heat-inactivated *Escherichia coli* (ATCC 8739) in 10-cm tissue culture petri dishes containing CHROMagar Candida Medium. Encystment was induced by transfer of trophozoites from CHROMagar to *Acanthamoeba* saline and incubation for at least 2 weeks at 26°C. *Acanthamoeba* trophozoites or cysts (1×10^6/mL) in *Acanthamoeba* saline were transferred to 35-mm petri dishes. TONS 504 (1000 µL) at various concentrations (0, 1, or 10 mg/L for trophozoites, or 0, 1, 10, or 20 mg/L for cysts) in *Acanthamoeba* saline was then added to each dish, and the dishes were placed uncovered below the LED system for irradiation at 10 J/cm^2 (single 3-min exposure), 30 J/cm^2 (three 3-min exposures separated by two 1-min rest periods), or 60 J/cm^2 (six 3-min exposures separated by five 1-min rest periods). The cultures were then maintained at 26°C for 3 h before measurement of viability and determine the *Acanthamoeba* cell death.

The viability of *Acanthamoeba* trophozoites or cysts was determined on the basis of trypan blue exclusion. The *Acanthamoeba* viable and nonviable cells were counted separately, and the viable cell ratio was calculated. The death of *Acanthamoeba* after subjected with TONS504-PACT was the evaluated by Apoptosis and Necrosis staining. The apoptotic cells were stained green with fluorescein isothiocyanate–conjugated annexin V as well as late apoptotic and necrotic cells red with ethidium homodimer III. The stained cells were examined with a laser-scanning fluorescence microscope.

Acanthamoeba trophozoite and cysts treated with either TONS504 or LED irradiation alone manifested no inhibition of their growth. In contrast, *Acanthamoeba* trophozoite and cyst subjected to TONS504-PACT showed reduction in viability. At a TONS 504 concentration of 10 mg/L, LED exposure at 30 J/cm^2 successful to inhibit about 77% of trophozoite. Cysts are a dormant stage of *Acanthamoeba* that are more difficult to eradicate than trophozoites. At a TONS 504 concentration of 20 mg/L, LED exposure at 60 J/cm^2 successful to inhibit about 42% of cyst viability. Fluorescence microscopic analysis of stained cells was performed to determine whether the cell death induced by TONS 504–PACT was mediated by apoptosis or necrosis. Apoptosis was apparent in trophozoites at a TONS504 concentration of 1 mg/L and light energy of 10 J/cm^2, whereas necrosis of trophozoites was observed at 10 mg/L and 30 J/cm^2. Apoptosis induction in cysts required a TONS504 concentration of 10 mg/L and light energy of 30 J/cm^2, while necrosis of cysts was not achieved at any dose of TONS and light energy.

The results suggest that the amoebicidal effect of TONS504-PACT is based on irradiation of the photosensitizer at the appropriate wavelength. The efficacy of this approach may be due in part to the positive charge of the photosensitizer, TONS504, which facilitates

electrostatic interaction with negatively charged cellular components including the cell wall, mitochondria, nucleus, and lysosomes. Such activation of the photosensitizer generates singlet oxygen and other reactive oxygen species that can induce damage to the cell wall or cell membrane, with this damage eventually leading to the death of the target pathogen.

This study has several limitations, first the TONS504-PACT is not adequate to achieve 90-100% elimination. Further study is required to identify the optimal dose of photosensitizer to effectively reduce the viability of *Acanthamoeb*a in both stages. They might also consider about whether current antiamoebic drugs have a synergistic effect with TONS504-PACT. Secondly, the corneal and retinal safety is a very important concern in developing this current treatment for clinical application. In the previous report, they found that TONS504-PACT had no detrimental effect in the Human Corneal Fibroblast in vitro.

Based on these results, this dissertation greatly contributes to suggested the usefulness of TONS 504–PACT on treating *Acanthamoeba* infection. Therefore, all the committee members admitted that this dissertation is of sufficient value to confer the Doctor of Philosophy in Medical Science to Yunialthy Dwia Pertiwi.