In Vitro Amoebicidal Activity of Titanium Dioxide/UV-A Combination Against Acanthamoeba

Gabrielle Gomart,1,2 Julie Denis,2 Tristan Bourcier,1,3 Anne Dory,4 Ahmed Abou-Bacar,2 Ermanno Candolfi,2 and Arnaud Sauer1,3

Purpose. To assess the amoebicidal effect of titanium dioxide (TiO2)/UV-A combination against Acanthamoeba sp trophozoites and cysts.

Methods. The amoebicidal effect of the TiO2/UV-A combination was tested on trophozoites and cysts of clinical isolates of Acanthamoeba baileyi and Acanthamoeba sp genotype T4, obtained from two severe cases of ulcerative keratitis. Samples of cultured Acanthamoeba were transferred to a 96-well plate. We tested the effect of sterile water (blank control), TiO2 alone, UV-A alone, TiO2 and additional UV-A exposure, chlorhexidine 0.02% alone, chlorhexidine 0.02% and TiO2, chlorhexidine 0.02% and UV-A, chlorhexidine 0.02% and TiO2, and additional UV-A exposure. Cell viability assessment was done using the trypan blue dye exclusion method.

Results. The combination of TiO2 with UV-A demonstrated antitrophozoite and anticyst activity ($P < 0.05$). This in vitro study showed a synergistic effect of the association of chlorhexidine with TiO2 and UV-A on cysts ($P < 0.001$).

Conclusions. Given the in vitro synergistic effectiveness of the association of chlorhexidine with TiO2 and UV-A against cysts, the treatment of Acanthamoeba keratitis could be improved by this new therapeutic approach.

Keywords: Acanthamoeba, cornea, infection, titanium dioxide, ultraviolet rays

Acanthamoeba of the Acanthamoeba genus are free-living, aerobic, eukaryotic organisms and are widely dispersed in nature and can inhabit terrestrial and aquatic environments.1 Acanthamoeba species present two morphologic stages in their life cycle: a vegetative trophozoite stage, in which there is reproduction by binary fission, and a cyst stage that is resistant to environmentally adverse conditions.2 Acanthamoeba protozoan are involved in keratitis and encephalitis.3 Acanthamoeba keratitis is a medical emergency.4 The main risk factors are contact lens wearing and corneal trauma.5 The pathogenesis is characterized by two events: (1) trophozoites adhere to the epithelial surface and invade then degrade the stromal extracellular matrix6 and (2) encystment of the protozoan, which promotes resistance and recurrence of infection.7 Currently, the treatment involves topical antimicrobial agents, such as chlorhexidine, polyhexamethylene biguanide,8 diamidines,9 and antifungal agents10 belonging to membrane-acting agents.11 Contrary to bacterial or fungal keratitis, failure of topical therapy is common.9 Based on the severity of Acanthamoeba keratitis and as a last resort, the only viable surgical option is penetrating keratoplasty.12 Among the novel therapeutic approaches, corneal cross-linking seems promising in the management of Acanthamoeba keratitis.13,14 Miltefosine (hexadecylphosphocholine), an alkylphosphocholine, showed interesting results too, in topical15 and systemic therapy.16 The development of antiacantheamoebic agents remains a challenge in order to improve the visual prognosis of the disease.

Titanium dioxide (TiO2) is a fine crystal powder. There are two forms of TiO2: micro particles, widely used in surface coatings and cosmetic materials, and nanoparticles. TiO2 nanoparticles have photocatalytic activity17: they produce oxygenated free radicals under UV-A radiation. Oxygenated free radicals cause cell lysis by lipid peroxidation,18 release of intracellular components,19 and nucleic acid20,21 and protein damage.22-23 TiO2 antimicrobial activity is known and commonly used as a self-disinfecting surface option.17 TiO2 is used in number of materials, including health care items.24

The antimicrobial properties of some types of nanoparticles, mainly metallic ones, have been recently demonstrated in vitro, notably against Gram-negative and Gram-positive bacteria.25

This study presents results of a series of in vitro experiments evaluating the amoebicidal effects of TiO2 with UV-A on Acanthamoeba cysts and trophozoites in an effort to ultimately expand the armamentarium of antimicrobial agents for the treatment of amoebic keratitis.

Methods

Acanthamoeba Strains

Two Acanthamoeba strains were selected from a panel of human clinical ocular amoebic pathogens isolated by corneal scraping from patients with severe amoebic keratitis and...
identified using the routine procedure (PCR) of the parasitology laboratory. The pathogens were one isolate of Acanthamoeba sp belonging to T4 genotype (Acanthamoeba T4) and one of Acanthamoeba hatchetti.

Acanthamoeba Axenization Assay

Briefly, the amoeba was isolated from corneal tissue by plate-culture procedure onto an nonnutrient agar medium covered with an avirulent, plasmid-less, heat-inactivated Escherichia coli strain without antibiotic resistance at 25°C. A piece of agar culture was picked up aseptically from nonnutrient agar medium and transferred to a tissue culture flask (Dutscher, Brumath, France) containing 5 ml of peptone-yeast extract glucose (PGY)26 broth medium with penicillin-streptomycin to proceed with the axenization process of primary isolation.

For trophozoites, after 48 hours of incubation at 35°C, the excystment process was observed followed by the growth of trophozoites in monolayer. Trophozoites were harvested by draining the supernatant medium and placing the tissue culture flask on ice for five minutes. Trophozoites were pelleted and washed in diluted saline solution twice by centrifugation at 405g for 10 minutes. The washing process was followed by cell counting and quantitative standardization of both the total and viable trophozoites, determined with trypan blue staining. The experimental viable trophozoites concentration was adjusted to $1 \times 10^5$ trophozoites/mL.

For cysts, after 48 hours of incubation at 35°C, the excystment process was observed followed by growth of trophozoites in monolayer. Cell supernatants were discarded, and the encystment of adherent trophozoites was performed in fresh PGY medium culture. Viable cysts were harvested and washed in diluted saline solution twice by centrifugation at 405g for 10 minutes. The washing process was followed by cell counting and quantitative standardization of both the total and viable cysts, determined with trypan blue staining. The experimental viable cyst concentration was adjusted to $1 \times 10^5$ cysts/mL. For each trial, the count of the chambers was performed in triplicate.

Titanium Dioxide

The TiO2 eye drop solution was prepared by the pharmacy department (Hôpitaux Universitaires de Strasbourg, France) by dissolving titanium powder (Inresa, Bartenheim, France; conformed to European pharmacopeia standard 9.2) in saline water and caromer gel (Gel Larmes; Théa, Clermont-Ferrand, France) at a 0.8-mg/mL final concentration. This final concentration was chosen because of imperatives of fabrication (it was the highest final concentration that can be produced)27 and the potential efficacy of TiO2 eye drops with UV-A. This potential efficacy was evaluated by oxygenated free radical production measured by chemiluminescence. Briefly, luminescence is based on photon emission when an excited molecule returns to its lowest energy state.28 Luminol (97%; Sigma-Aldrich Chimie, Saint-Quentin-Fallavier, France) produces photons in attendance of oxygenated free radicals.29 The photon production is measured by a luminometer (Glomax 96 Microplate Luminometer; Promega, Madison, WI, USA), and chemiluminescence is expressed in related light unit (RLU). We tested the production of oxygenated free radicals by TiO2 eye drop at final concentrations of 0.2, 0.4, and 0.8 mg/mL with and without irradiance of UV-A for 50 minutes. The experiment was repeated three times. There was minimal chemiluminescence without UV-A. The result of the three concentrations with UV-A is represented in the Figure.

In Vitro Testing

To begin with, we tested the in vitro effect of TiO2 with UV-A exposure on trophozoites (Acanthamoeba T4, then Acanthamoeba hatchetti). The second step was the in vitro test on cysts (Acanthamoeba T4 then Acanthamoeba hatchetti).

Eight groups were tested as follows: sterile water (blank control), TiO2 alone (T), UV-A alone (UV-A), TiO2 and additional UV-A exposure (T+UV-A), chlorhexidine 0.02% alone (C; Gilbert, Herouville-Saint-Clair, France), chlorhexidine 0.02% and TiO2 (C+T), chlorhexidine 0.02% and UV-A (C+UV-A), and chlorhexidine 0.02% and TiO2 with additional UV-A exposure (C+T+UV-A). Each group was assayed in triplicate.

An aliquot of 30 μL of an Acanthamoeba-containing solution was placed into each well of a sterile 96-well microplate (Dutscher). We used three wells for each group. For a blank control, 30 μL of saline solution was added. For the TiO2 groups (T and T+UV-A) 30 μL of the TiO2 eye drop was added (with a final concentration of 0.8 mg/mL). For the chlorhexidine groups (C and C+UV-A), 30 μL of chlorhexidine was added (with a final concentration of 0.2 mg/mL). For the chlorhexidine and TiO2 groups (C+T and C+T+UV-A), 15 μL of the TiO2 eye drop (with a final concentration of 0.8 mg/mL) and 15 μL of chlorhexidine (with a final concentration of 0.2 mg/mL) was added. Irradiance of UV-A light source with a 365-nm wavelength at a power...
density of 2.2 mW/cm² was dispensed on the four groups with UV-A exposure for 30 minutes. For cysts, based on the classical clinical treatment regimen, we retreated after 24 hours, following the same steps.

### Analysis of Tests

After the 24-hour incubation period for trophozoites and the 48-hour incubation period for cysts, the cell viability of each assay was measured with the trypan blue dye exclusion method in a counting chamber (Dutscher) under microscope. Trypan blue is a vital stain that colors only dead cells. Living amoebae have a refringent appearance under light microscopy, whereas dead amoebae exhibit a blue color (see Supplementary Fig. 1 for photographs of live and dead trophozoites and cysts).

### Statistical Analysis

The analysis focuses on the following *Acanthamoeba* outcomes: trophozoites persist, die, or encyst; and cysts persist or die. Descriptive statistics were expressed as the mean and standard deviation (SD) of the triplicate of the three repeats. The 2-way ANOVA test was used for the analysis, assuming a theoretical normal population. Multiple comparisons were made post hoc between the different groups in order to find significant differences. A $P < 0.05$ was considered statistically significant.

### RESULTS

Regarding *Acanthamoeba* T4 trophozoites (Table 1), after 24 hours of incubation, the combination of TiO₂ and UV-A demonstrated antitrophozoite activity: the difference between the control group and the TiO₂+UV-A group was statistically significant ($P < 0.001$). Each group with chlorhexidine (versus control) was an amebicide ($P > 0.001$). All the trophozoites died in the presence of chlorhexidine.

Regarding *Acanthamoeba* hatchetti trophozoites (Table 1), after 24 hours of incubation, the in vitro experiment showed no difference among the groups UV-A, TiO₂ alone, and TiO₂+UV-A: UV-A versus control ($P > 0.05$), TiO₂ versus control ($P > 0.05$), and TiO₂+UV-A versus control ($P > 0.05$). The encystment was statistically lower in the TiO₂+UV-A group than in the control group ($P < 0.05$). Each group with chlorhexidine (versus control) was an amebicide ($P > 0.001$).

Regarding *Acanthamoeba* T4 (Table 2) and *Acanthamoeba* hatchetti (Table 2) cysts, after 48 hours of incubation, the in vitro experiment showed no difference among the groups UV-A and TiO₂ alone: UV-A versus control ($P > 0.05$) and TiO₂ versus control ($P > 0.05$). TiO₂+UV-A had the following an

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>Mean ± SD, %</th>
<th>$P$ value</th>
<th>Mean ± SD, %</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48 ± 20</td>
<td>&gt;0.05</td>
<td>47 ± 18</td>
<td>&gt;0.05</td>
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<tr>
<td>UV-A</td>
<td>52 ± 13</td>
<td>&gt;0.05</td>
<td>44 ± 14</td>
<td>&gt;0.05</td>
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<tr>
<td>TiO₂</td>
<td>45 ± 16</td>
<td>&gt;0.05</td>
<td>19 ± 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TiO₂ and UV-A</td>
<td>34 ± 13</td>
<td>&lt;0.05</td>
<td>17 ± 17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C</td>
<td>0 ± 0</td>
<td>&lt;0.001</td>
<td>0 ± 0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C+TiO₂</td>
<td>0 ± 0</td>
<td>&lt;0.001</td>
<td>0 ± 0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C+UV-A</td>
<td>0 ± 0</td>
<td>&lt;0.001</td>
<td>0 ± 0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C+TiO₂+UV-A</td>
<td>0 ± 0</td>
<td>&lt;0.001</td>
<td>0 ± 0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*P values are comparisons between control and each condition. Significant $P$ values are in bold. Means and SD from triplicate experiments are shown.

### Table 2. Percentage of Cyst Viability of *Acanthamoeba* T4 and *Acanthamoeba* hatchetti at 48 hours

<table>
<thead>
<tr>
<th>Treatment‡</th>
<th>Mean ± SD, %</th>
<th>$P$ value</th>
<th>Mean ± SD, %</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>94 ± 6</td>
<td>&gt;0.05</td>
<td>97 ± 5</td>
<td>&gt;0.05</td>
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<tr>
<td>UV-A</td>
<td>95 ± 5</td>
<td>&gt;0.05</td>
<td>100 ± 0</td>
<td>&gt;0.05</td>
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<tr>
<td>TiO₂</td>
<td>85 ± 5</td>
<td>&gt;0.05</td>
<td>88 ± 8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TiO₂ and UV-A</td>
<td>51 ± 15</td>
<td>&lt;0.001</td>
<td>69 ± 15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C</td>
<td>27 ± 12</td>
<td>&lt;0.001</td>
<td>39 ± 9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C+TiO₂</td>
<td>22 ± 16</td>
<td>&lt;0.001</td>
<td>36 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C+UV-A</td>
<td>21 ± 19</td>
<td>&lt;0.001</td>
<td>38 ± 9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C+TiO₂+UV-A</td>
<td>11 ± 16</td>
<td>&lt;0.001</td>
<td>25 ± 6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*$P$ values are comparisons between control and each condition. Significant $P$ values are in bold. Means and SD from triplicate experiments are shown.

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* † Retreatment at 24 hours.
‡ † UV-A, 30 minutes of initial exposure, 365-nm wavelength, at a power density of 2.2 mW/cm²; TiO₂ eye drop at 0.8 mg/ml final concentration; C, Chlorhexidine eye drop at 0.2 mg/ml final concentration.
§ † Retreatment at 24 hours.

amoebicidal effects: on *Acanthamoeba* T4, there were 49% of dead cysts with TiO2 +UV-A against 6% with control (*P* < 0.001); and on *Acanthamoeba batetti*, there were 31% of dead cysts with TiO2 +UV-A against 3% with control (*P* < 0.001). TiO2+UV-A was better than TiO2 alone (*P* < 0.001). Each group with chlorhexidine was better than the control (*P* < 0.001). There was a synergistic effect of chlorhexidine with TiO2+UV-A: after 48 hours, the percentage of dead cysts was higher with the combination of C+TiO2+UV-A than with chlorhexidine alone and on *Acanthamoeba T4* cysts (*P* < 0.05) as on *Acanthamoeba batetti* cysts (*P* < 0.01).

**Discussion**

The work outlined here is directed to the development of TiO2 and UV-A as a new adjunctive method for the treatment of *Acanthamoeba* keratitis, which is a cause of significant morbidity worldwide and can cause rapid and devastating vision loss. 6 *Acanthamoeba* keratitis continues to be difficult to treat despite the use of topical agents and adjunctive surgery, such as corneal transplantation.50 Many studies worked on the photocatalytic utility of TiO2/UV in a disinfection system, with efficacy on bacteria, fungi, and viruses. Sökmen et al.35 used TiO2 for photocatalytic disinfection of *Giardia intestinalis* and *Acanthamoeba castellanii* cysts in water with UV-C exposure.31 Imran et al.52 synthesized TiO2 nanoparticles and demonstrated their inhibitor effects on *Acanthamoeba castellanii* trophozoite growth and viability. The antimicrobial effect of photocatalyse is a reason why we tested TiO2 with UV-A on cysts and trophozoites of *Acanthamoeba batetti* and T4 in vivo.

*Acanthamoeba* keratitis has been characterized as a painful and vision-threatening disease. The infection cascade starts with the adherence of protozoa to the corneal surface, and the infection involves the invasion and destruction of the corneal stroma.1 In our study, we demonstrated an antitrophozoite effect of the combination of TiO2 and UV-A. Nevertheless, the usual treatment by chlorhexidine is better than TiO2 with UV-A exposure. In the in vitro experiment, most of the trophozoites died after 24 hours in the presence of chlorhexidine. The antimicrobial activity of chlorhexidine is known. Chlorhexidine is a polyhexamethylene biguanide compound that is positively charged and ionic with the negatively charged plasma membrane of the parasite, resulting in structural and permeability changes, ionic leakage, cytoplasmic disruptions causing cellular damage, and cell death.11

The encysted stage is the second step of *Acanthamoeba* keratitis after the adhesion and multiplication of the trophozoite. *Acanthamoeba* cysts are composed of an ectocyst, an external cellularic layer, and an endocyst, an internal fibrillar layer, which together provide amoebic resistance to physical and chemical compounds.35 Cyst persistence in tissue is common and a recurrence of infection can follow prolonged topical therapy or surgical therapy.7 We suspect that lipid peroxidation,18 release of intracellular components,79 and nucleic acid20,21 and protein damage22,23 are the photocatalytic effects on cysts of the TiO2/UV-A combination, as described by bacterial (*E. coli*) studies. The cytoplasmic membrane damage due to chlorhexidine may facilitate entry into the cysts of oxygenated free radicals and could explain the synergistic effect of chlorhexidine and TiO2 with UVA observed in vitro on cysts. Another theory is that there was a conjugation between chlorhexidine and TiO2 and, thus, nanoparticles of TiO2 facilitated chlorhexidine driving to the site of action. The conjugation of chlorhexidine with gold nanoparticles has demonstrated a significant increase in its amoebicidal and cystidal potency, with minimal associated host-cell cytotoxicity.10,34

Results obtained in vitro do not always correlate with in vivo efficacy; therefore, further tissue culture models and animal studies are under way to test the efficacy of this treatment for infectious keratitis. Furthermore, it was important to determine the cytotoxicity of TiO2. Eom et al.53 evaluated the effect of TiO2 nanoparticle exposure on the ocular surface in vivo on 40 rabbits. Of the five toxicity criteria, two increased after TiO2 exposure. Given that we were able to demonstrate in vitro activity of the TiO2/UV-A against *Acanthamoeba*, it is necessary to establish safety with other in vivo tests on corneal epithelia cells and animal studies.

In conclusion, the combination TiO2+ UV-A presents antitrophozoite and an adjunctive anticyst activity in vitro when applied with the parameters used in the present study.

**Acknowledgments**

The authors thank Nicolas Meyer (Laboratoire de Biostatistique, Faculté de Médecine de Strasbourg, Université de Strasbourg), Philippe André (Laboratoire de Biophotonique et Pharmacologie, Faculté de Pharmacie de Strasbourg, Université de Strasbourg), and Nicolas Keller (Institut de Chimie et Procédés pour L’Energie, L’Environnement et la Santé, Université de Strasbourg) for technical assistance.

Disclosure: G. Gomart, None; J. Denis, None; T. Bourcier, None; A. Dory, None; A. Abou-Bacar, None; E. Candolfi, None; A. Sauer, None

**References**


