Acanthamoeba keratitis is a medical emergency. The main risk factors are contact lens wearing and corneal trauma. The pathogenesis is characterized by two events: (1) trophozoites adhere to the epithelial surface and invade then degrade the stromal extracellular matrix and (2) encystment of the protozoan, which promotes resistance and recurrence of infection. Currently, the treatment involves topical antimicrobials, such as chlorhexidine, polyhexamethylene biguanide, diamidines, and antifungal agents belonging to membrane-acting agents. Contrary to bacterial or fungal keratitis, failure of topical therapy is common. Based on the severity of Acanthamoeba keratitis and as a last resort, the only viable surgical option is penetrating keratoplasty. Among the novel therapeutic approaches, corneal cross-linking seems promising in the management of Acanthamoeba keratitis.

Titanium dioxide (TiO\textsubscript{2}) is a fine crystal powder. There are two forms of TiO\textsubscript{2}: micro particles, widely used in surface coatings and cosmetic materials, and nanoparticles. TiO\textsubscript{2} nanoparticles have photocatalytic activity: they produce oxygenated free radicals under UV-A radiation. Oxygenated free radicals cause cell lysis by lipid peroxidation, release of intracellular components, and nuclear acid and protein damage.

Acanthamoeba antimicrobial activity is known and commonly used as a self-disinfecting surface option. TiO\textsubscript{2} is used in number of materials, including health care items.

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.

This study presents results of a series of in vitro experiments evaluating the amoebicidal effects of TiO\textsubscript{2} 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 amoeba pathogens isolated by corneal scraping from patients with severe amoeba 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 trophozoites in a monolayer. Cell supernatants were discarded, excystment process was observed followed by growth of viable cysts, determined with trypan blue staining. The 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, chemiluminescence 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 30 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...
Acanthamoeba and Titanium Dioxide

Table 1. Percentage of Trophozoite Viability and Percentage of Encystment at 24 hours*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acanthamoeba T4</th>
<th>Acanthamoeba hatchetti</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trophozoite Viability</td>
<td>Encystment</td>
</tr>
<tr>
<td>Control</td>
<td>48 ± 20 %</td>
<td>47 ± 18 %</td>
</tr>
<tr>
<td>UV-A</td>
<td>52 ± 13 %</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TiO₂</td>
<td>45 ± 16 %</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TiO₂ and UV-A</td>
<td>34 ± 13 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C</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>
</tr>
<tr>
<td>C+UV-A</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>
</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.

† 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.

The 2-way ANOVA test was used for the analysis, assuming a standard deviation (SD) of the triplicate of the three repeats. 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 2. Percentage of Cyst Viability of Acanthamoeba T4 and Acanthamoeba hatchetti at 48 hours*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acanthamoeba T4</th>
<th>Acanthamoeba hatchetti</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyst Viability</td>
<td>P value</td>
</tr>
<tr>
<td>Control</td>
<td>94 ± 6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>UV-A</td>
<td>95 ± 5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TiO₂</td>
<td>85 ± 5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TiO₂ and UV-A</td>
<td>51 ± 15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C</td>
<td>27 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C+TiO₂</td>
<td>22 ± 16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C+UV-A</td>
<td>21 ± 19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C+TiO₂+UV-A</td>
<td>11 ± 16</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* 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.
‡ P values are comparisons between control and each condition. Significant P values are in bold. Means and SD from triplicate experiments are shown.
amoebicidal effects: on *Acanthamoeba* T4, there were 49% of dead cysts with TiO$_2$ + UV-A against 6% with control (P < 0.001); and on *Acanthamoeba castellanii* batchetti, there were 31% of dead cysts with TiO$_2$ + UV-A against 3% with control (P < 0.001). TiO$_2$+UV-A was better than TiO$_2$ alone (P < 0.001). Each group with chlorhexidine was better than the control (P < 0.001). There was a synergistic effect of chlorhexidine with TiO$_2$+UV-A: after 48 hours, the percentage of dead cysts was higher with the combination of C+TiO$_2$+UV-A than with chlorhexidine alone and on *Acanthamoeba* T4 cysts (P < 0.05) as on *Acanthamoeba castellanii* cysts (P < 0.01).

**DISCUSSION**

The work outlined here is directed to the development of TiO$_2$ 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. *Acanthamoeba* keratitis continues to be difficult to treat despite the use of topical agents and adjunctive surgery, such as corneal transplantation. Many studies worked on the photocatalytic utility of TiO$_2$/UV in a disinfection system, with efficacy on bacteria, fungi, and viruses. Sökmén et al. used TiO$_2$ for photocatalytic disinfection of *Giardia intestinalis* and *Acanthamoeba castellanii* cysts in water with UV-C exposure. Imran et al. synthesized TiO$_2$ nanoparticles and demonstrated their inhibitor effects on *Acanthamoeba castellanii* trophozoite growth and viability. The antimicrobial effect of photocatalysis is a reason why we tested TiO$_2$ with UV-A on cysts and trophozoites of *Acanthamoeba castellanii* and *Acanthamoeba* T4 in vivo.

*Acanthamoeba* keratitis has been characterized as a painful and vision-threating disease. The infection cascade starts with the adhesion of protozoa to the corneal surface, and the infection involves the invasion and destruction of the corneal stroma. In our study, we demonstrated an antitrophozoite effect of the combination of TiO$_2$ and UV-A. Nevertheless, the usual treatment by chlorhexidine is better than TiO$_2$ 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.

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 cellular layer, and an endocyst, an internal fibrillar layer, which together provide amoebic resistance to physical and chemical compounds. Cyst persistence in tissue is common and a recurrence of infection can follow prolonged topical therapy or surgical therapy. We suspect that lipid peroxidation, release of intracellular components, and nucleic acid and protein damage are the photocatalytic effects on cysts of the TiO$_2$/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 TiO$_2$ with UVA observed in vitro on cysts. Another theory is that there was a conjugation between chlorhexidine and TiO$_2$ and, thus, nanoparticles of TiO$_2$ 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.

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.

In conclusion, the combination TiO$_2$+ UV-A presents antitrophozoite and an adjunctive anticyst activity in vitro when applied with the parameters used in the present study.

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**References**


