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Effects of ozone and ultraviolet radiation treatments on the infectivity of *Toxoplasma gondii* oocysts

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Abstract

Clinical toxoplasmosis in humans has been epidemiologically linked to the consumption of drinking water contaminated by *Toxoplasma gondii* oocysts. We evaluated killing of *T. gondii* oocysts after ultraviolet (UV) or ozone treatments by bioassay in mice and/or cell culture. A 4-log inactivation of the oocyst/sporozoite infectivity was obtained for UV fluences >20 mJ cm^{-2}. In contrast, oocysts were not inactivated by ozone with an exposure (*Ct*) up to 9.4 mg min l^{-1} in water at 20 °C. In conclusion, UV treatment can be an effective disinfection method to inactivate *T. gondii* oocysts in drinking water, but ozone did not show promise in this research.
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Keywords: *Toxoplasma gondii*; Oocyst; Water; UV; Ozone; Inactivation

1. Introduction

Toxoplasma gondii is a widely prevalent protozoan that can be responsible for severe infections in congenitally infected children and in immunocompromised people (Tenter et al., 2000). Asymptomatic and symptomatic toxoplasmosis have been associated with the consumption of chlorinated drinking water contaminated with *T. gondii* oocysts excreted in cat faeces (Bowie et al., 1997; Bahia-Oliveira et al., 2003; de Moura et al., 2006; Palanisamy et al., 2006; Sroka et al., 2006) and viable *T. gondii* was isolated from samples of drinking water (de Moura et al., 2006). Under laboratory conditions, oocysts treated for 4 h with different concentrations of chloramine, free chlorine or chlorine dioxide are still infective for mice (Villena et al., 2002). Therefore, most of chlorination conditions used in drinking water plants would not be appropriate for inactivation of *T. gondii* oocysts and other related protozoa such as *Cryptosporidium* species (Wainwright et al., 2007b; Betancourt and Rose, 2004).

Apart from chlorine treatment, effects of other water disinfection methods (ultraviolet radiation and ozonation) on the infectivity of *T. gondii* oocysts are relatively unknown (Wainwright et al., 2007a,b). UV at 254 nm induce DNA/RNA damages depending on UV fluence

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expressed in mJ cm^{-2}. Studies indicate that *C. parvum* oocysts are no longer resistant to UV above 12 mJ cm^{-2} contrary to viruses and bacterial spores (Hijnen et al., 2006). Ozone is a strong oxidant of organic and inorganic compounds and can be a more effective primary disinfectant than chlorination. Ozonation requires an evaluation of the minimum exposure (*Ct*, the product of dissolved ozone concentration and contact time) to inactivate micro-organisms and for complying with stringent regulations on disinfection by-products such as bromate (von Gunten, 2003).

In this paper, we report successful disinfection of *T. gondii* in water by UV treatment but not by ozone treatment.

2. Materials and methods

2.1. Toxoplasma gondii oocysts

Oocysts of the VEG strain were obtained from the faeces of experimentally infected cats, sporulated in 2% H_2SO_4, and stored at 4 °C for 6 months as described previously (Dubey et al., 1996). Oocysts were washed and suspended in distilled water (pH 7.1–7.2) for present experiments.

2.2. UV experiments

2.2.1. Low-pressure radiation system

UV inactivation of oocysts was conducted using a low-pressure mercury vapor lamp emitting monochromatic UV light (253.7 nm). The collimated beam apparatus consisted of a single 15-W G15T8 UV tube (Phillips) that was suspended horizontally in a box and emitting UV irradiation directed through a circular opening controlled by a shutter according to exposure time. The UV intensity was determined with a calibrated UV 254 nm detector (CX-254, Vilber Lourmat, Marne-la-Vallée, France) by placing the radiometer (VLX-3W, Vilber Lourmat) at the same location and elevation as the water surface of the irradiated sample. The UV average irradiance (E) in the suspension was determined using a form of Beer's law integrating the suspension absorbance (A) measured by a spectrophotometer at 254 nm, the depth of the irradiated sample (l), and the incident average irradiance (E_0):

$$\frac{E}{E_0} = \frac{1 - 10^{-Al}}{Al \ln 10}$$

Required exposure times were calculated by dividing the desired UV fluence by the average UV irradiance (Craik et al., 2001). For uniform lamp output, the lamp was warmed up for at least 15 min before each experiment. A stir plate was placed directly under the collimated beam for slow stirring of the oocyst suspension. Experiments were performed at 0, 5, 10, 15, 20, and 40 mJ cm^{-2}, in non-buffered distilled water (pH 7.1–7.2) in Petri dishes at room temperature (20–25 °C) with continuous stirring. Infectivity analysis was performed within minutes after treatment.

2.2.2. In vitro infectivity of irradiated oocysts

Infectivity of oocysts irradiated at 0, 5, 10, 20, and 40 mJ cm^{-2} was assessed by inoculating murine L20B fibroblasts with freshly excysted sporozoites as described (Dubey and Beattie, 1988). For this, cells were grown in eightwell chamber slides in minimum essential medium (MEM) supplemented with 5% foetal calf serum (FCS), L-glutamine, non-essential amino acids and sodium bicarbonate at 37 °C at 5% CO_2. Irradiated oocysts were centrifuged, suspended in Hanks' balanced salt solution (HBSS), and sonicated 5 min at full power (40 W) in a water bath sonicator (Branson 3200, Branson, Danbury, CT, USA) to release the sporocysts. Sporocysts were incubated in acidified HBSS (pH 2.7) for 30 min at 37 °C, washed and then incubated in HBSS containing 0.75% (w/v) of bile salts (sodium taurocholate, glycocholate, deoxycholate, and cholate, Sigma #S9875) for 2 h at 37 °C. Excysted sporozoites were washed, suspended in MEM-5% FCS and numerated in Malassez hemacytometer. 10^2, 10^3, 10^4, and 10^5 sporozoites were inoculated onto confluent cell monolayers in triplicate for each UV dose. Cells were incubated 2 days at 37 °C at 5% CO_2.

T. gondii tachyzoite foci were detected by an immunofluorescence assay (IFA). Slides were fixed with 100% methanol at −20 °C for 10 min and probed for 45 min at 37 °C with a rabbit polyclonal anti-*T. gondii* antibodies (Derouin, unpublished data) diluted at 1:150 in PBS–1% BSA–0.1% Tween 20. After washings with PBS–0.1% Tween 20, the slides were incubated with a goat anti-rabbit IgG-FITC diluted at 1:150 in PBS–1% BSA–0.1% Tween 20 for 30 min at 37 °C. Tachyzoite foci were counted at ×400 magnification under the following epifluorescence filter set: exciter filter, 450–480 nm; dichroic beam splitting, 500 nm; barrier filter, 515 nm.

The reduction of the sporozoite infectivity was interpreted as the inactivation ratio (N/N_0), where N is the number of foci-forming sporozoites after UV exposure, and N_0 is the number prior UV exposure. Two independent experiments were performed.

2.2.3. In vivo infectivity and pathogenicity of UV-treated oocysts

In addition to *in vitro* evaluation, infectivity of oocysts irradiated at 0 and 40 mJ cm^{-2} was assessed by oral inoculation of 5, 50, 500, 5000, and 50,000 sporulated oocysts into five Swiss–Webster female mice for each dose. Five mice fed distilled water were used as negative controls. Lung and mesenteric lymph node imprints of mice that died were examined for *T. gondii* tachyzoites. Survivors were bled 6 weeks post-inoculation (p.i.) and their serum was tested for *T. gondii* antibodies using twofold dilutions from 1:20 to 1:320 with the direct agglutination test (Desmonts and Remington, 1980). Seropositive mice were killed, their brains removed and homogenized in 1 ml of saline (0.9% NaCl). Tissue cyst number was estimated by counting 5 × 20 μl of the brain suspension.

2.3. Ozone experiments

Ozone gas was generated from extra dry air using a water-cooled discharge generator (type 5LO, Trailigaz, Garges-les-Gonesse, France). The concentration of the product gas was continuously measured by UV absorption at 254 nm (UVOZON TLG200, Trailigaz), and ozone was bubbled into a thermostatic flask containing 2 l of sand-filtered water maintained at 20.0 ± 0.1 °C (pH 7.7–7.8). The concentration of dissolved ozone (i.e., residual ozone) in water was measured by an ozone sensor (sensor 3660, Orbisphere, Trappes, France) and was maintained at 0.5 mg/l. Water was continuously homogenized with a pump set at 250 ml/min. About 10 millions of sporulated oocysts were injected in the 2-l flask, homogenized and a 50-ml aliquot was extracted every 4 min during 20 min. Residual ozone was immediately quenched by adding 25 μl of ethylenediamine (EDA) at 100 mg/l. Oocyst infectivity was evaluated *in vitro* as described above, within 24 h of sample collection.

The reduction of the sporozoite infectivity was interpreted as the inactivation ratio (N/N_0), where N is the number of foci-forming sporozoites after ozonation, and N_0 is the number prior ozonation. Three independent experiments were performed.

2.4. Statistical analysis

Data were analyzed using GraphPad Prism software Version 4.03 to generate dose–response curves and their respective equation, calculate infective dose 50 (ID50) and lethal dose 50 (LD50) for mice, and ozone exposure (Ct).

3. Results

3.1. UV experiments

3.1.1. In vitro evaluation of the sporozoite infectivity

Infective sporozoites differentiate into tachyzoites in 48 h in L20B cells to form parasite foci detected by IFA. In preliminary experiments, the inoculum was adapted since only 10–15% of non-irradiated sporozoites differentiated into tachyzoites in our cell culture assay. For example, 100–150 foci developed after cell inoculation of 1000 sporozoites. UV treatment did not influence the excystation rate as determined microscopically (data not shown). Tachyzoites developed from sporozoites excysted from oocysts were exposed at 5–15 mJ cm^{-2} but not at 20 and 40 mJ cm^{-2}. Minimal UV fluences of 7.9, 11.8, 15.3, and 17.5 mJ cm^{-2} were required to obtain a 1-, 2-, 3-, and 4-log reduction of the sporozoite infectivity, respectively (Fig. 1).

3.1.2. Mouse bioassay

In titration experiments with non-irradiated oocysts, at least five oocysts were infective for mice but mortality was observed for a dose ≥50 oocysts (Table 1). The ID50 was estimated at 6.2 oocysts (CI 95%, 3.3–11.7) and the LD50 at 335.4 oocysts (CI 95%, 118.0–952.9). Tissue cysts ranged from 645 to 3735 in brains of infected mice and were dose dependent (Table 1). In contrast, mice fed with 5000 and 50,000

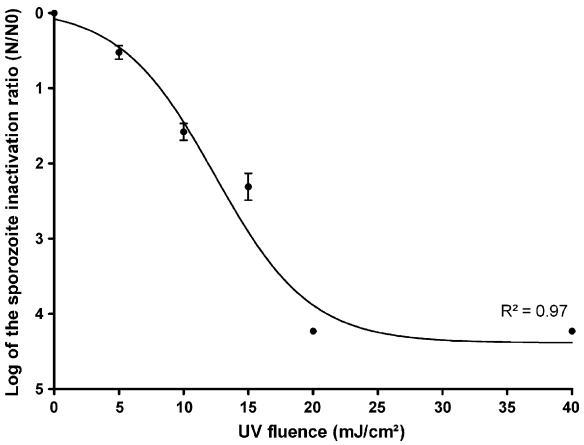

Fig. 1. *In vitro* inactivation of the infectivity of excysted sporozoites following UV exposure of oocyst suspensions at 5–40 mJ cm^{-2}. The reduction of the sporozoite infectivity was interpreted as the inactivation ratio (N/N_0), where N is the number of foci-forming sporozoites in L20B cells after UV exposure, and N_0 is the number prior exposure. The curve represents best fit of data using sigmoidal dose–response with variable slope.

Table 1
Infectivity and pathogenicity of UV-treated oocysts to mice[a]

No. of oocysts fed/mouse	UV fluence (mJ cm^{-2})	No. infected	No. died	Day of death (average)	Tissue cyst number at 45 dpi[b]
0	0	0	0	NA	NA
5	0	2	0	NA	645-930
50	0	5	1	14.0	1442-2678
500	0	5	3	11.3	2856-3042
5000	0	5	4	8.5	3735
	40	0	0	NA	NA
50,000	40	1	0	NA	210

[a] Five mice were fed with each dose of oocysts.
[b] Day post-infection.

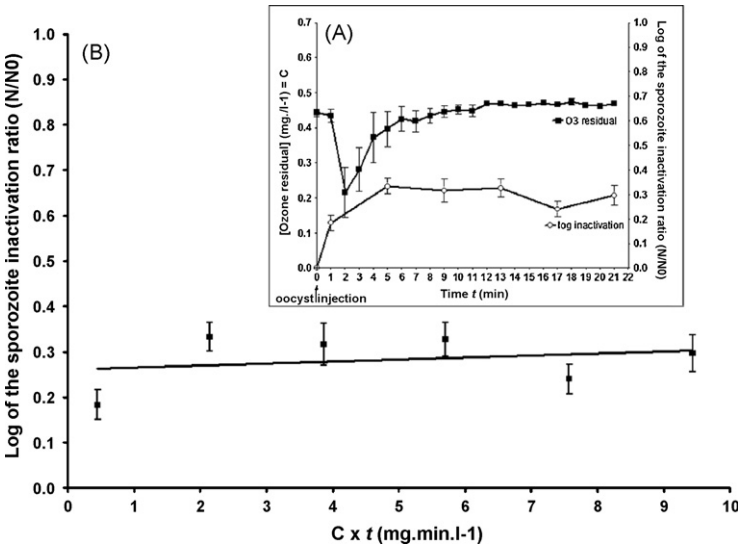

Fig. 2. Effects of ozone on the infectivity of excysted sporozoites. The reduction of the sporozoite infectivity was interpreted as the inactivation ratio (N/N_0), where N is the number of foci-forming sporozoites in L20B cells after ozone exposure, and N_0 is the number prior exposure: (A) variation of the dissolved ozone residual (C) and the sporozoite inactivation with time (t); (B) effects of integrated ozone exposure (Ct) on the infectivity of excysted sporozoites. Black line represents best fit of data using linear regression.

oocysts irradiated at 40 mJ cm^{-2} were not infected except one mouse fed with 50,000 oocysts (Table 1). The infective dose for this mouse was estimated from the dose-infectivity curve at 1.6 oocyst and tissue cyst number at 210. Thus, oocyst infectivity was 31,250-fold diminished after UV radiation which corresponds to a 4.5-log reduction.

3.2. Ozone treatments

Fig. 2 shows the variation of the dissolved ozone residual (C) with time (t). Oocysts were injected in water at 0 min, and residual was rapidly re-stabilized between 0.4 and 0.5 mg l^{-1} after 5 min of ozonation. 50-ml aliquots were analyzed by cell culture every 4 min during 20 min (Fig. 2A). At this concentration, ozone treatment did not influence the excystation rate of the sporozoites as determined microscopically (data not shown). After ozone Ct calculation by integration, negligible variation within a 1.0-log unit was observed in the infectivity of sporozoites excysted from ozone treated oocysts up to a Ct of 9.4 mg min l^{-1} compared to non-treated sporozoites (Fig. 2B).

4. Discussion

Results of the present study showed that continuous UV exposure is very effective to inactivate >99.9% of *T. gondii* oocysts with relatively low fluences (>15 mJ cm^{-2}) compared to minimal fluence (40 mJ cm^{-2}) recommended for drinking water treatment in France, as previously reported for *C. parvum*

(Hijnen et al., 2006). We obtained a 4-log reduction of the infectivity both by mouse bioassay and cell culture assay at 40 mJ cm^{-2}. This efficiency was also recently obtained with pulsed UV radiations, but not after continuous UV treatment since infective *T. gondii* oocysts were detected after 731 mJ cm^{-2} exposure (Wainwright et al., 2007a). These disparities can be explained by the different methods (UV radiation system, cell culture and/or bioassay procedures). In a cell culture model, Grimwood (1980) observed that *T. gondii* tachyzoites were not able to divide above 70 mJ cm^{-2}.

Compared to UV radiation, *T. gondii* oocysts were highly resistant to an ozone exposure (*Ct*) of 9.4 mg min l^{-1}. Recently, Wainwright et al. (2007b) failed to inactivate *T. gondii* oocysts following exposure to *Ct* of ozone up to 69 mg min l^{-1}. Studies with *C. parvum* oocysts indicated a 2-log inactivation at 5.6 mg min l^{-1} at 20 °C (Baudin et al., 2001). This ozone treatment is at least three times higher than the *Ct* commonly used in drinking water plants and can enhance the formation of undesirable bromates in drinking water (von Gunten, 2003). The higher resistance of *T. gondii* oocysts could be enhanced by the particular structure of the oocyst and the sporocyst walls which protect the sporozoites from chemical damages such as acids, solvents, and other oxidant compounds (e.g., sodium hypochlorite) (Dubey, 2004; Wainwright et al., 2007b). Other investigations are needed to evaluate their resistance at higher ozone exposures. Our experimental conditions of ozone treatment allowed a 5-log inactivation of MS2 phages with $Ct > 1$ mg min l^{-1} as determined by cell culture and RT-PCR (Moulin and Duguet, unpublished data).

In conclusion, UV treatment can be an effective technique to inactivate *T. gondii* oocysts under routine treatment conditions (i.e., low-turbid waters). UV produce less by-products compared to chlorination and ozonation, and allow the reduction of post-ozonation conditions in order to comply with regulations for bromate (von Gunten, 2003).

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