REDUCTION OF INFECTIVITY OF SCHISTOSOME CERCARIAE BY APPLICATION OF CERCARICIDAL OIL TO WATER

JEAN MARIE NAPLES,* CLIVE SHIFF, AND ROLF U. HALDEN
Department of Molecular Microbiology and Immunology, and Department of Environmental Health Sciences, Center for Water and Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland

Abstract. Schistosomiasis continues to plague populations living in disease-endemic areas, and exposure to infective cercariae results in more than 200 million cases worldwide. Laboratory experiments were conducted to test whether a cercaricidal film applied directly to the water surface can reduce viability of cercariae. A distillate from inexpensive cedarwood oil enriched for cedrol in a mixed oil fraction was formulated (1:5) with the surfactant Tween 80. When applied to the surface of clean and turbid water in test vessels, the formulation spread across and just below the air-water interface, causing inactivation of Schistosoma mansoni cercariae within minutes. The active ingredient was heat stable and reduced schistosome survival and infectivity by 90% and 99.2%, respectively in a mouse model. The effective dose (13 µg/cm²) was dependent on surface area rather than volume of water treated. We conclude that application of the biodegradable formulation to the surface of schistosome-infested waters may be an effective, economical, and safe means of reducing human infections.

INTRODUCTION

Schistosomiasis is a water-borne disease that continues to affect the lives, health, and productivity of millions of people living mainly in rural areas where the parasite is endemic. People get infected by contact with freshwater infested with free-swimming larvae (cercariae) released into the water by infected aquatic snails. Cercariae are short-lived, active organisms that localize at or near the water surface where they contact and penetrate the intact skin of human or animal hosts. Skin lipids facilitate cercarial penetration across the skin, and in a process of metamorphosis that includes the shedding of their protective glycocalyx, the invading cercariae become schistosomula that grow into adult worms in the infected host.1-3

Several topical agents have been evaluated to prevent infection via the blocking of cercarial penetration into skin.4-12 However, topical anti-schistosome products require product application to the entire body surface, and repeated application that can exceed chronic use guidelines.13,14

Here we report a technique to avoid infection by cercariae that exploits normal host-seeking behavior of cercariae at or near the water surface and the stimulatory activity of lipid components of red cedarwood oil. To attack cercariae as they orientate to the water surface, this technique involves the targeted delivery of a distillate of red cedarwood oil to the water surface. Red cedarwood oil is cercaricidal.8,15 The specific gravity of this oil and its components is 0.940,16 such that oil droplets can be expected to float when applied to water. We sought to develop a cercaricidal formulation with enhanced dispersal and effectiveness when compared with cedarwood oil.15 Our approach was to apply a formulation of red cedarwood oil components plus surfactant to the surface of water containing actively swimming cercariae to inactivate the larvae.

Cercariae exposed to this oil show early phases of their normal penetration response, followed rapidly by death. Disruption of the cercarial glycocalyx, a consequence of the penetration process, alters the osmoregulatory processes of the intact organisms. This increases the absorption of the toxic substances in red cedarwood oil and results in rapid cercarial death.8

Components of red cedarwood oil include terpenes, terpene alcohols, sesquiterpenes, and unsaturated fatty acids.16 The most active components are believed to be cedrol and widdrol, which can be concentrated in a mixed oil fraction (MOF) by distillation. Red cedarwood oil has been approved as a flavor additive and fragrance agent by the U.S. Food and Drug Administration and by the U.S. Environmental Protection Agency for use in topically-applied agents (soap, detergent, cosmetics) for humans and animals (flea collars).17 The surface-active agent used in this study, Tween 80, is a biodegradable food-grade surfactant.18

METHODS

Preparation of MOF from red cedarwood oil. Red cedarwood oil, also known as cedar oil, was obtained from Giles and Kendall, Inc., (Huntsville, AL). The MOF derived from cedar oil was used as the biologically active oil in all studies. It was obtained by heated vacuum distillation of crude cedar oil (130 mL) using a dry ice vacuum (25 liters/minute) over a two-hour period. The biologically inactive terpenes (α-cedrene, thujoisene) (Naples JM, unpublished data) were removed via distillation between 60°C and 135°C (approximately 100 mL). The remaining 30-mL fraction was the MOF.

Identification of cedar oil, terpene, and MOF was verified by gas chromatography/mass spectrometry (GC/MS) analysis (GCMS-QP5050; Shimadzu, Tokyo, Japan) of each sample and comparison of α-cedrene and cedrol fragment particles with spectra in the NIST98 spectral library.18

Red cedarwood oil is biodegradable. Prolonged exposure (greater than one year) to atmospheric oxygen, direct intense sunlight, and temperatures > 30°C over time will result in oxidation of secondary alcohols (cedrol, widdrol) to aldehydes and ketones19 that do not appear to be biologically active toward schistosome cercarie.

Preparation of MOF emulsion and dosing for cercariae. The MOF was formulated with Tween 80 (Aldrich, Milwaukee, WI), a common lipophilic agent.20 The MOF-containing formulations were developed using a range of MOF and Tween

* Address correspondence to Jean Marie Naples, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, 615 North Wolfe Street, Baltimore, MD 21205-2179. E-mail: jnaples@jhsph.edu

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80 concentrations in 10 mL of spring water. These mixtures were sonicated for five seconds to form the working concentrate, which was generally stable over the experimental time frame of several hours.

Evaluation of the effect of the test formulation on cercariae was conducted in vessels containing commercial spring water. Freshly shed Schistosoma mansoni cercariae were derived from infected Biomphalaria glabrata snails (Biomedical Research Institute, Bethesda, MD). For each treatment evaluation, cercariae were introduced into test vessels prior to addition of test formulation. Numbers of cercariae were estimated by counting aliquots withdrawn from the bulk of cercariae shed from a batch of infected snails. When the MOF formulation was added dropwise to the water surface, a suspension formed at and just below the meniscus. Cercariae were subsequently observed over time with a low-power dissecting microscope.

In addition to various MOF formulations, treatment controls were assessed for cercaricidal effects and included Tween 80 in spring water and spring water alone. Preliminary formulation and dose-ranging studies found the optimum ratio of MOF and Tween 80 to be 1:5 (10 mg of MOF and 40 mg of Tween 80). This was formulated with 10 mL of water to make the MOF test concentration 1 mg of MOF/mL. Dose-ranging studies showed that reproducible effects on cercariae were achieved using 13 μg of MOF/cm². This was the standard dose/surface area ratio used in all subsequent experiments unless otherwise noted.

Experimental cercarial behavior patterns. The effect of MOF formulation on cercarial motility/viability was monitored. Three types of motility/viability patterns of cercariae were observed. A type 1 pattern (normal motility) was random swimming movements from the water meniscus throughout the water with no attempt at penetration of any nearby surface. A type 2 pattern was initiation of the penetration response with cercariae observed to swim from the meniscus, and when reaching a surface, to probe and adhere to the side or bottom of the vessel by the oral sucker, with little or no inclination to swim away. A type 3 pattern was cercarial immobilization with no movement or an occasional tail twitch.

Experimental conditions. Ordinary laboratory glassware and plastic materials were used in all experiments. The concept of surface versus volume treatment was tested in beakers and glass dishes of various surface areas (15, 87, 235, and 255 cm²) and plastic dishes (1,780 cm²). The smaller diameter vessels were ideal for observation of cercarial response to the surface treatment. In the large vessels, 20-mL samples were drawn from 1 cm deep (near the surface) and 4 cm deep (near the bottom). Bottom samples were augmented by scraping the area to be sampled with a small spatula to release cercariae that might have adhered to the surface and thus would be missed in the normal pipette drawn samples. Sampling was done in parallel rows at depths of 1 cm, 4 cm, and at the bottom for each sampling point. Cercariae were then observed, stained, and counted under a microscope.

To examine the long-term stability of the distilled oil, two sample vials of MOF were sent to Ghana and exposed to environmental conditions for six weeks. One vial was exposed to direct heat and sunlight while the other was covered in aluminum foil and exposed only to direct heat. The efficacy was compared with similar MOF maintained in an amber bottle at 25°C in Baltimore. The oil was also tested in a 0.033% (w/v) loamy soil water mixture to see if the active ingredient was adsorbed and removed from the treated surface in turbid waters. Testing under these conditions was carried out at room temperature (23–25°C). Controls using spring water with and without Tween 80 were tested simultaneously in each experiment.

Exposure conditions were replicated at least five times as follows. Vessels were filled to the appropriate depth and allowed to stand. Cercariae up to three hours post-shedding were used. In the small vessels, approximately 100 cercariae were added to each container and left for 30 minutes to aclimatize. The MOF was added dropwise from the stock solution described earlier and timing commenced. Vessels were observed microscopically after 3 minutes, 10 minutes, and serially until 60 minutes, and the numbers and activity patterns were observed. Final numbers were counted after 60 minutes after staining with Lugol’s iodine. In larger vessels (36 × 50 × 10 cm deep), cercariae were added to approximate a density of 10/mL. The MOF formulation was applied in droplets to the surface in rows and allowed to disperse naturally. Since direct observation of cercariae in the large vessels was impossible, samples were taken 60 minutes prior to MOF application, and 60 and 120 minutes post-application. Experiments were repeated in similar vessels in the presence of several shedding snails that allowed them to aclimate and produce cercariae for 60 minutes prior to the application of the test formulation. Counts were made prior to MOF application and 60 and 120 minutes post-application.

Cercarial infectivity was measured by mouse exposure to 100 cercariae in 1.5-cm diameter vials. Test vials were treated with MOF and allowed to stand for 60 minutes prior to tail immersion in the test vessel. Controls were carried out with 100 S. mansoni cercariae in clean spring water.21,22

RESULTS

Gas chromatography/mass spectrometry results demonstrated the successful enrichment of the active ingredient, cedrol, from red cedarwood oil during the vacuum distillation process. Prior to distillation, the cedarwood oil contained significant levels of various terpenes, most of which eluted from the column in the retention time window of 13–14 minutes (Figure 1A). After vacuum distillation, the remaining viscous residue, the MOF, was depleted in terpenes and enriched in cedrol (Figure 1B), a nontoxic, natural compound with known cercaricidal activity.8

When the MOF was applied directly to spring water either by itself or contained in an alcoholic solution, the oil tended to form droplets that were visible with the naked eye. In contrast, when the MOF was applied as a mixture with Tween 80 (1:5), the compound rapidly spread across the water surface to form a thin surface film that also extended just below the meniscus. The cercaricidal activity of this was investigated using S. mansoni cercariae.

Cercaricidal effects of the surface-active formulation were observed very rapidly within a time frame of minutes (Figure 2). The dose response was constant with surface area and independent of water depth. This relationship was demonstrated in experiments where the dose per surface area was held constant and the volume of water was increased successively by five-fold increments. In these experiments, the pro-
cess of inactivation that was detected after exposure for 60 minutes varied slightly from 90% to 100%. The theoretical bulk concentration of active ingredient calculated assuming homogeneous compound distribution in the water column varied from 1.6 to 7.6 mg/L (Figure 3). Interestingly, the overall effect of the formulation was maintained, as judged by microscopic examination, even in the presence of infected snails that continuously released cercariae into the water column (Figure 3 and Table 1).

A cercaricidal effect was also determined in exposure experiments with BALB/c mice (Figure 4). Whereas tail exposure of mice to untreated, schistosome-infested water resulted in infection with an average of 12 worms per mouse, only one worm was detected in the single infected mouse exposed to cercariae previously treated with MOF and Tween 80 (Figure 4B). Tween 80 alone did not decrease the overall rate of infection (100% of animals infected; Figure 4A). A reduction in worm burden of 99.2% was observed after the MOF:Tween 80 treatment (Figure 4C).

The active ingredients in MOF showed excellent stability to both heat and light exposure. Aliquots of MOF exposed continuously to high environmental temperatures and direct sunlight during a six-week field trip to Ghana lost less than 10% of their cercaricidal activity. Analysis of the heat- and sunlight-exposed samples of MOF by GC/MS for cedrol content showed a cedrol loss of 4% compared with the cedrol concentration in MOF sample maintained at 25°C in an amber bottle. Similarly, the presence of soil suspension and particles in treated spring water caused only moderate reductions in the effectiveness of the test formulation (P = 0.168; Table 2).

DISCUSSION

The unique aspect of this work is that it exploits and interrupts a mechanism for osmoregulation that is essential for the freshwater existence of schistosome cercariae and essentially reduces their ability to infect the definitive host. The cercarial glycocalyx enables the cercariae to survive in freshwater. During the process of host invasion, the integrity of the glycocalyx is interrupted and the surface coat has been shed by the time the cercaria is within the skin of the human host. Observation of cercariae that have been stimulated by unsaturated fatty acids clearly shows that this process is sequential

![](image1.png)

**Figure 2.** Behavioral response in exposed cercariae after application of the surface-active formulation (1:5 mixture of mixed oil fraction and Tween 80) to simulated surface water containing Schistosoma mansoni. The observed type 2 behavior (penetration response) irreversibly leads into inactivation and death of exposed larvae. The surface-active formulation took effect after as little as three minutes (min) of exposure and after approximately 20 minutes reached a sustained maximum. Error bars show the standard error of the mean.

![](image2.png)

**Figure 3.** Inactivation activity of a mixture of mixed oil fraction and Tween 80 against cercariae of Schistosoma mansoni in experiments conducted at a fixed ratio of cercaricide mass per surface area (13 μg/cm²). Activity was independent of the theoretical or apparent concentration of the active ingredient in bulk water.

### Table 1

Effect of the surface-active formulation on Schistosoma mansoni cercariae that were continuously shed from patent snails over a one-hour time period

<table>
<thead>
<tr>
<th>Effect</th>
<th>Prior to exposure (%)</th>
<th>Variable exposure (60 minute maximum) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Penetration response</td>
<td>0%</td>
<td>16 ± 0.03%</td>
</tr>
<tr>
<td>Inactivation</td>
<td>0%</td>
<td>84 ± 0.02%</td>
</tr>
<tr>
<td>Overall effectiveness</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>824</td>
<td>422</td>
</tr>
</tbody>
</table>

* Counts were made 60 minutes postexposure and after the snails were removed from the test vessel.  
† Values are percentages ± SEM.
and irreversible, and if the cercariae remain in an aqueous environment after stimulation they soon absorb water, swell, and die. The process takes place in a matter of minutes.\(^3\) We demonstrate here that the same process takes place in the presence of the MOF of red cedarwood oil.

Oily substances generally have specific gravities less than 1 and thus remain at or near the meniscus. This is close to the water compartment that cercariae normally occupy because they tend to orientate to the water surface to be appropriately located for the best chance to encounter a likely host. The toxicology of MOF is known\(^8\) and provides a means to attack the schistosome cycle at the cercarial stage. This means that only the water surface, irrespective of volume, needs to be treated for an effective attack on cercariae. The work reported here provides compelling evidence for the feasibility of this strategy and encourages further research to be conducted under field conditions.

Crude red cedarwood oil and the constituents cedrol and widdrol are used extensively as fragrance agents in soaps, detergents, and perfumes. Annual U.S. production is greater than 1 million kg and the annual world production is 2.7 million kg valued at $10 million.\(^24\) Other use includes the addition of the compounds to alcoholic and non-alcoholic beverages and foods as flavor ingredients.\(^25,26\) The acute 50% lethal dose for the oil is \(> 5\) grams/kg of body weight and the maximum tolerated dose (representing a measure of chronic toxic exposure) is 0.005 mg/kg/day.\(^19\) The main constituents of red cedarwood oil (\(\alpha\)-cedrene and thujopsene) are considered non-toxic.\(^17,27\) The dose limits specified by the industrial classification of these compounds are based on the metabolism and pharmacology of the reactive functional groups present.\(^13,14,28\) Vacuum distillation of red cedarwood oil serves to remove the terpenes having no cercaricidal activity and concentrates the active ingredients in the MOF.

It was important to observe the actual behavior of cercariae after contact with the treated surface water. Therefore, a significant part of the initial work was done in small 50-mL beakers. Normal cercarial behavior consists of swimming up and drifting down in a column of water, thus most organisms will move into the treated area during these activities. After exposure to MOF for a minimum of three minutes, cercariae alter their behavior and appear to attempt penetration. This behavior was observed as vigorous swimming to the side of the vessel or to the bottom where they attached to the glass surface and attempted to penetrate. Other investigators have observed this activity with collection and analysis of cercarial secretory enzymes deposited on glass surfaces.\(^29\) This aggrivated activity continued usually \textit{in situ} for 30–40 minutes, after which the cercariae become immobilized and apart from an occasional twitch, will rapidly die. In considering the rationale for this study, it is important to focus on cercarial behavior as an indicator of impending and rapid death. When experiments were carried out in larger vessels, cercariae were not observed directly, but samples taken from the vessel were first observed microscopically to ascertain the type of activity prior to the addition of Lugol’s iodine to enable counting of the organisms.

The concept of surface water treatment was validated experimentally by using a constant dose per surface area. We used a standard treatment of 1.3 mg of MOF per 100 cm\(^2\) in all experiments, which would extrapolate to the use of approximately 130 mg of active ingredient per square meter of surface water treated. The efficacy of this dose was independent of the theoretical bulk water concentration that was calculated to have ranged from 1.6 to 7.6 mg/L (Figure 3).

We acknowledge that this work needs to be expanded as a field study that will be designed to test application techniques in still and moving bodies of water, as well as the affects of the formulation on cercariae and other freshwater biota. However, attacking schistosome cercariae in the water rather than by topical application of a barrier cream is a new aspect for schistosomiasis control. Barrier creams have been tested extensively,\(^7,9–12\) but there have been no attempts to introduce them for parasite transmission control. The idea of covering all surface skin each time water immersion is contemplated is

| Table 2 |

Comparison of the effectiveness of the surface-active formulation on cercariae of \textit{Schistosoma mansoni} in clean spring water (\(n = 153\)) and in simulated turbid spring water (\(n = 599\))^*^ (Figure 4).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Spring water</th>
<th>Spring water plus 0.003% (w/w) of soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0 ± 0%</td>
<td>0 ± 0%</td>
</tr>
<tr>
<td>Penetration response</td>
<td>7 ± 2%</td>
<td>11 ± 2.5%</td>
</tr>
<tr>
<td>Inactivation</td>
<td>93 ± 2%</td>
<td>89 ± 2.5%</td>
</tr>
<tr>
<td>Overall effectiveness</td>
<td>100 (100%)</td>
<td>100 (100%)</td>
</tr>
</tbody>
</table>

*Values are percentages ± SEM.
challenging and may not be properly accomplished. This might be helpful for a technician who needs to work under water for a specific project in a potentially infested area, but for the public at large, it is often not a viable option.

The approach described here does open several new doors that may help in the control of this insidious parasitic disease. Field trials are planned and it will be important to determine optimum methods for application to water as well as the effect of the formulation on aquatic biota in general. Stability of the oil is good as we have demonstrated, but the durability of the formulation in situ needs to be evaluated. Since the oil is effective against cercariae, it is likely that application will be focal and directed to likely transmission sites. It is necessary to evaluate formulation efficacy as a function of time under natural conditions to see in at what intervals the formulation needs to be reapplied or whether it can be delivered in a controlled-release matrix. As a tool to reduce cercarial populations in specific foci, it could be a means to augment other modes of schistosomiasis control. Transmission control through mass-targeted chemotherapy is under way in several countries, but as yet we do not have good data on the long-term effect. In well-sustained control operations, snail control did reduce transmission, but this activity has only been sustained in Egypt, which does have an effective infrastructure and research base. We do know, however, that transmission is focal, seasonal, and diurnal such that a targeted approach against cercariae is not unreasonable.

We have experimented with cedarwood oil, which is a natural product. There are numerous similar natural oils extractable from plants in all parts of the world. Some of these oils are consumed, many are used for cosmetics and anointments, and some even stimulate cercarial penetration processes. There is much to be done in developing this concept. However, in areas that are endemic for schistosomiasis, local customs exist that could become the starting point for community-based control of cercariae.

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Authors’ addresses: Jean Marie Naples and Clive Shiff, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205-2179. Rolf U. Halden, Department of Environmental Health Sciences, Center for Water and Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205-2179.

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