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Effects of albendazole, artesunate, praziquantel and miltefosine, on *Opisthorchis viverrini* cercariae and mature metacercariae

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ABSTRACT

Objective: To explore larvicidal effects of anthelmintic drugs on *Opisthorchis viverrini* (*O. viverrini*) for alternative approach to interrupting its cycle for developing a field-based control program.

Methods: The larvicidal activities of albendazole (Al), artesunate (Ar), praziquantel (Pzq) and miltefosine (Mf) on *O. viverrini* cercariae and mature metacercariae were investigated. Lethal concentrations (LC_{50} and LC_{95}) of these drugs were determined. Mature metacercariae previously exposed to various concentrations of the drugs were administered to hamsters. Worms were harvested 30 d post infection and worm recovery rates calculated. Al, Ar, Pzq and Mf produced morphological degeneration and induced shedding tails of cercariae after 24 h exposure.

Results: The LC_{50} and LC_{95} of Al, Ar, Pzq and Mf on cercariae were 0.720 and 1.139, 0.350 and 0.861, 0.017 and 0.693, and 0.530 and 1.134 ppm, respectively. LC_{50} and LC_{95} of Ar on mature metacercariae were 303.643 and 446.237 ppm and of Mf were 289.711 and 631.781 ppm, respectively but no lethal effect in Pzq- and Al-treated groups (up to 1 ppt). No worms were found in hamsters administered Pzq-treated metacercariae. The adult worms from Al-treated metacercariae were significantly bigger in size compared to the control group ($P < 0.05$). Fecundity and body width were greater in adults from Mf-treated metacercariae compared to the control group ($P < 0.05$).

Conclusions: The larvicidal effects of these drugs were high efficacy to *O. viverrini* cercariae but lesser efficacy to metacercariae. It should be further studied with the eventual aim of developing a field-based control program.

1. Introduction

Opisthorchiasis, caused by infection with *Opisthorchis viverrini* (*O. viverrini*), is a major risk factor for cholangiocarcinoma (CCA), an important public health problem in northeastern Thailand [1]. Humans acquire infection by eating raw or undercooked cyprinid fish containing the infective stage, mature metacercariae [2]. The parasite life cycle requires the snail *Bithynia siamensis goniomphalos* (*B. siamensis goniomphalos*) as first intermediate host in the northeast region of Thailand and many species of cyprinid fishes as second intermediate host [3].

In the snail host, germinal cells in sporocysts and rediae proliferate asexually, ultimately producing many free-swimming cercariae. Numerous cercariae of *O. viverrini* are shed daily from naturally infected snails, with an average of 1728 cercariae/snail, at the daily peak shedding time of 8.00–10.00 AM [4]. After finding a cyprinid fish host, cercariae penetrate and encyst to become metacercariae, mainly in the head portion and muscles [5]. This can lead to a high prevalence of *O. viverrini* metacercariae [3]. For treatment of opisthorchiasis

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cases, praziquantel has high efficacy [6]. Other drugs widely used for many species of trematodes, including *O. viverrini*, are albendazole and artesunate [7,8]. Efficacy of miltefosine against trypanosomes, schistosomes and *Entamoeba histolytica* has been studied [9,10]. Miltefosine has larvicidal effects on eggs, miracidia and cercariae of *Schistosoma mansoni* (*S. mansoni*) and *Schistosoma haematobium*, and lethal effect to *Biomphalaria alexandrina*, the snail hosts [11]. To date, there has been no study on the effects of any of these drugs against larval stages of *O. viverrini*.

This study investigated *in vitro* larvicidal effects of albendazole, artesunate, praziquantel and miltefosine on field-collected *O. viverrini* cercariae and metacercariae. If these drugs are effective against swimming cercariae and mature metacercariae, their use in this way could be incorporated into effective strategic control of opisthorchiasis in humans.

2. Materials and methods

2.1. Sample preparation

2.1.1. Collection of *O. viverrini*-infected *B. siamensis goniomphalos*

Snails were collected by hand from water bodies in endemic areas of Khon Kaen Province and brought back to the Malacology Laboratory, Khon Kaen University in plastic bags labeled for each locality. Snails were identified as *B. siamensis goniomphalos* based on morphology of their shells [3,12]. They were rinsed with tap-water and maintained in dechlorinated tap-water for a few days before examination for *O. viverrini* infection.

2.1.2. Collection of *O. viverrini* cercariae

Snails were placed individually in plastic cups (3 cm in diameter and 2.5 cm in height) contained dechlorinated tap-water. Cercarial shedding was induced by exposure to electric light (40 W) for 2–3 h, after which the water in the cup was examined for the presence of cercariae. Cercariae were identified as *O. viverrini* by morphology [3] and confirmed by polymerase chain reaction (PCR) using species-specific primers [13].

2.2. Life span of *O. viverrini* cercariae at various temperatures

A suitable temperature for use in the cercaricidal study was investigated. Freshly shed *O. viverrini* cercariae were maintained at various water temperatures from 4 to 52 °C (8 °C intervals, 100 cercariae in five replicates at each temperature). Their activity was observed and changes in morphology, such as tail shedding or body degeneration, were noted.

2.3. Effects of albendazole, artesunate, miltefosine, and praziquantel on *O. viverrini* cercariae

Albendazole, artesunate, praziquantel, (Sigma–Aldrich Shanghai Trading Co Ltd Shanghai, China) and miltefosine (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) were used for this study. Cercariae (n = 1380) were divided into four groups, one for each drug. Groups 1, 2, and 3 (360 cercariae/group), cercariae in those groups were divided to 6 sub-groups (60 cercariae/sub-group, 3 replicates, 20 cercariae/replicate) Group 1 and 2: cercariae were exposed to concentration of albendazole and artesunate (dissolved in 10% DMSO) of 0.0 (2 control groups, water and solvent of 10% DMSO), 0.2, 0.4, 0.6 and 0.8 ppm. Group 3: cercariae were exposed to praziquantel at 0.0000 (2 control groups, water and solvent of 10% DMSO), 0.0125, 0.0250, 0.0500, and 0.1000 ppm. Group 4 (300 cercariae), cercariae were divided to 5 sub-groups (60 cercariae/sub-group, 3 replicates, 20 cercariae/replicate) to expose to miltefosine at 0.0000 (control in water), 0.1375, 0.2750, 0.5500, and 1.1000 ppm. To study the effects of drugs, cercariae were exposed for 24 h then rinsed with dechlorinated tap water and observed for 24 h. The criteria for death of a cercaria were lack of movement and physical degeneration.

2.4. Effects of albendazole, artesunate, miltefosine, and praziquantel on *O. viverrini* metacercariae

2.4.1. Effect of drugs on mature metacercariae

This study consisted of two experiments. Experiment Ⅰ determined survival of mature metacercariae *in vitro* and Experiment Ⅱ determined infectivity and development of worms in infected hamsters. Mature *O. viverrini* metacercariae were obtained from natural infected cyprinid fish by the pepsin digestion method [14].

Experiment Ⅰ: The metacercariae (n = 3600) were divided into 4 groups (900 metacercariae/group), one for treatment with each drug. Within each group there were six sub-groups (150 metacercariae/sub-group, 3 replicates, 50 metacercariae/replicate), each exposed to praziquantel, miltefosine, artesunate and albendazole at 0 (2 control groups, water and 10% DMSO), 50, 100, 150 and 200 ppm. Criteria for death of metacercariae were no movement of larvae and degeneration.

Experiment Ⅱ: Infectivity and development of drug-treated mature metacercariae in hamsters.

Metacercariae were exposed to drugs for 24 h as in Experiment I, then washed with water and administered to hamsters (4 hamsters/sub-group, 50 metacercariae/hamster). Hamsters were housed in the Animal House, Faculty of Medicine, Khon Kaen University. Food and water were provided *ad libitum* for 30 d, after which hamsters were euthanized with diethyl ether in a fume hood and *O. viverrini* adults collected from the livers. The protocol for *O. viverrini* infection and sacrifice of hamsters was approved by the Animal Ethics Committee of Khon Kaen University, Thailand (record No. ACUC-KKU-34/2558).

2.5. Morphology and fecundity of adult worms

Morphology of adult worms from hamster livers was studied for ten adult worms from each concentration of each drug (miltefosine, artesunate and albendazole). Worms were fixed in 10% neutral buffered formalin, stained with Semichon's acetic carmine, dehydrated in an alcohol series and permanent slide mounts made. The length and width, body area, area of testes (anterior and posterior testes), and area of ovaries were determined for each worm under a microscope (Olympus Co., Tokyo, Japan) using the program DP2-BSW (Olympus Co., Tokyo, Japan). Eggs-per-worm was calculated from ten worms from each hamster of each concentration of each drug and control groups. The uterus of each worm was minced individually in a plastic tube containing a known number of drops of normal saline solution (NSS). One drop of this mixture was randomly

sampled and placed on a glass slide, covered with a cover glass and the number of *O. viverrini* eggs was counted (two replicates for each worm). The number of eggs per worm (EPW) was calculated as follows:

EPW = average number of *O. viverrini* eggs in 1 drop × total number of drops of NSS.

2.6. Calculations and statistical analyses

The effects of albendazole, artesunate, miltefosine, and praziquantel on cercariae and metacercariae were evaluated by examination under a stereomicroscope. The percentages of dead cercariae and metacercariae at each drug concentration were counted to determine 50% and 95% lethal concentrations (LCs) for each drug. Probit analysis in SPSS version 16 was used. The effects of these drugs on the ability of mature metacercariae to establish in hamsters were determined by counting the number of worms in hamster livers at 30 d post-infection. Fecundity of the worms was determined by counting uterine eggs in each. Two-way ANOVA in SPSS Version 16 was used to calculate the significance of effects of each drug concentration at $P < 0.05$.

3. Results

Genomic DNA of *O. viverrini* cercariae was amplified using species-specific primers. In each case, PCR products of 330 bp were obtained, confirming their identity [14].

3.1. Life span of O. viverrini cercariae at various temperatures

Cercariae survived longest at 12 °C (75 h), 73 h at 20 °C and for about 49 h at room temperature (28 °C). Longevity gradually decreased below 12 °C and above 20 °C. Nearly all cercariae died immediately at 52 °C (Figure 1). For the larvicidal study, room temperature of (28 ± 2) °C was used.

3.2. Cercaricidal effects of albendazole, artesunate, miltefosine, and praziquantel on O. viverrini

Cercariae exhibited abnormal behaviors after 24 h contact with a drug: abnormal movement, shedding of the tail and remaining at the bottom of their container. All cercariae in the experimental groups were dead compared to active movement in control group (Figure 2). The highest cercaricidal effect in this study was praziquantel with LC_{50} and LC_{95} of 0.017 and 0.693 ppm, respectively. The order from high cercaricidal effects were artesunate, miltefosine, and albendazole with LC_{50} and LC_{95} of 0.350 and 0.861, 0.530 and 1.134, 0.720 and 1.139 ppm, respectively. The number of dead cercariae was increased to the increasing concentrations of drugs (Figure 3) but slowly decreasing in higher concentrations of artesunate and praziquantel.

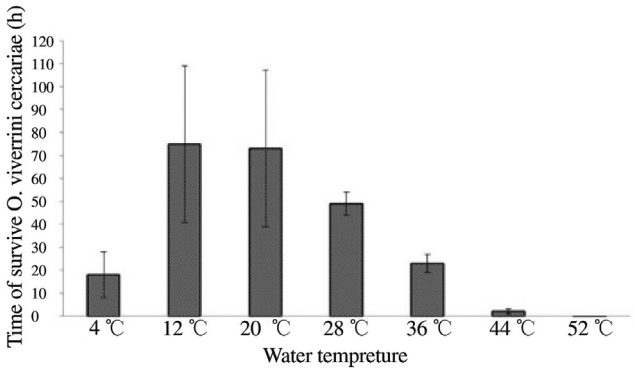

Figure 1. Effects of various temperatures on life span of *O. viverrini* cercariae in various temperatures water.

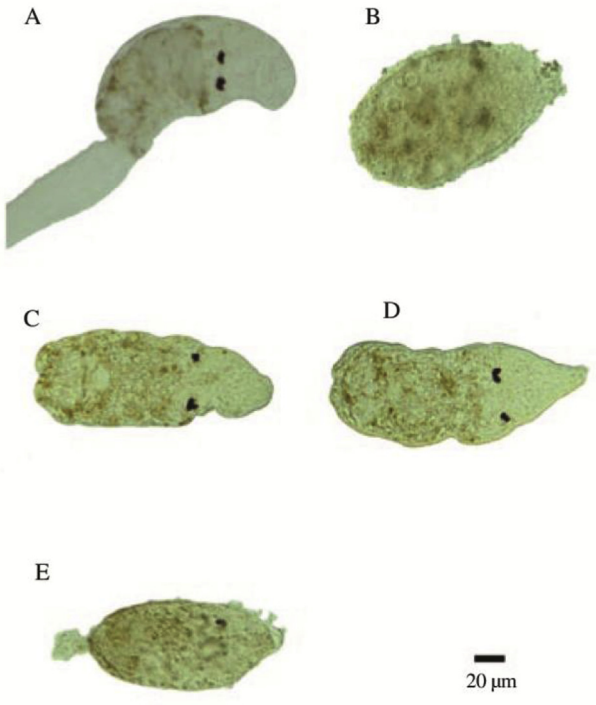

Figure 2. Photographs of *O. viverrini* cercariae in control group (A) and after 24 h exposed to (B) albendazole, (C) artesunate, (D) miltefosine, and (E) praziquantel, showing the death of cercariae and tail shedding.
B and E: cercariae were degenerated; C and D: tails of cercariae were shed and no movement (death). Scale bar = 20 μm.

3.3. Effects of albendazole, artesunate, miltefosine, and praziquantel on O. viverrini metacercariae

3.3.1. Observed effects on mature O. viverrini metacercariae

In Experiment I, after 24 h exposure mature *O. viverrini* metacercariae to albendazole, artesunate, praziquantel, and miltefosine, the effective metacercaricidal was found in artesunate and miltefosine. Lethal percentages of *O. viverrini* metacercariae in artesunate treated groups of 0, 0 (10% DMSO), 50, 100, 150 and 200 ppm were 0, 0, 1.32, 0, 1.32, and 14%, respectively. And metacercaricidal effects of 0, 0 (10% DMSO), 50, 100, 150, and 200 ppm miltefosine were 0, 0, 18, 28, 24.66, and 26.66%, respectively. Miltefosine has a considerable metacercaricidal effect than artesunate in same conditions. Dead metacercariae were determined by no movement of larvae and degeneration, clear or semi-translucent inside cyst wall (Figure 4).

Artesunate had LC_{50} and LC_{95} on mature metacercariae at 303.64 and 446.24 ppm, and miltefosine, at 289.711 and 631.781 ppm, respectively. But albendazole and praziquantel treated groups did not present metacercaricidal effect on *O. viverrini* mature metacercariae.

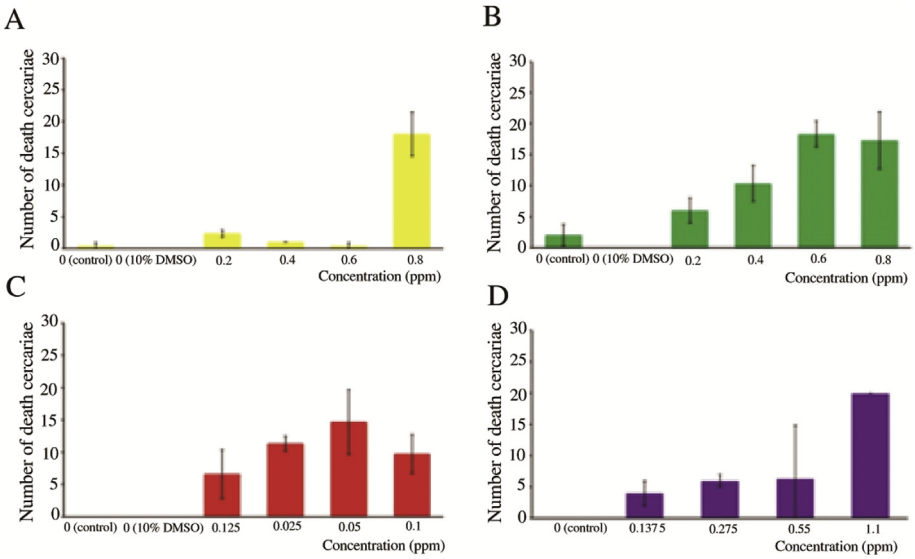

Figure 3. Number of death *O. viverrini* cercariae at various concentrations of drugs (after 24 h exposure).
A and D: cercaricidal effects of albendazole and miltefosine showing number of dead cercariae increasing with increased drug concentration; B and C: cercaricidal effects of artesunate and praziquantel showing dead cercariae increasing with increased drug concentration but the highest concentration the death were slightly decreased.

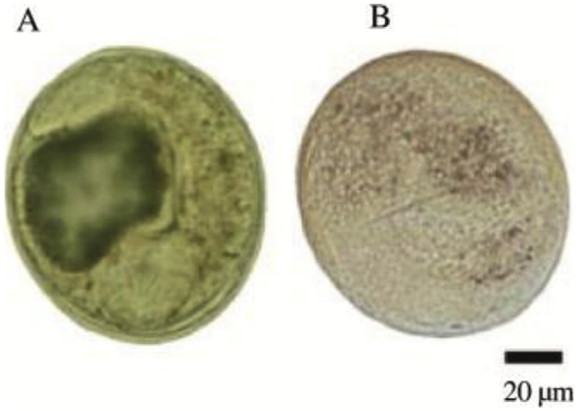

Figure 4. Photographs of *O. viverrini* mature metacercariae.
(A) normal mature metacercaria in control group; (B) degeneration of mature metacercaria after 24 h exposures with miltefosine. Scale bar = 20 μm.

In Experiment II, mature metacercariae were exposed to albendazole, artesunate, praziquantel, and miltefosine for 24 h. At that time, counts were made of the number of metacercariae exhibiting movement, number of excysted, abnormal and dead metacercariae. The percentages of metacercariae exhibiting these features at each concentration of albendazole, artesunate, praziquantel, and miltefosine are shown in Table 1.

3.3.2. Worm burdens

Albendazole: *O. viverrini* adults recovered from infected hamsters at 30 d P.I. were lancet-shaped, thin and transparent. Numbers of adult worms recovered and derived from metacercariae treated with water, 10% DMSO, 50, 100, 150, and 200 ppm albendazole were 27, 9, 48, 36, 21, and 47, respectively, representing 37%, 12%, 24%, 15.5%, 13%, and 23.5% of the inoculum, respectively. Dimensions of worms were (4–6 × 1–2) mm; the uterus was usually not full of eggs; testes and ovaries were visible in most worms.

Table 1
Characteristics of *O. viverrini* mature metacercariae after 24 h drug exposure (50 metacercariae/concentration, 4 replicates).

Concentration (ppm)	Drugs	Characteristics of *O. viverrini* mature metacercariae after 24 h drugs exposure (%)			
		Movement	Excystation	Abnormal	Death
0 (control)	–	98.5	1.5	0.0	0.0
0 (10% DMSO)	–	99.3	0.0	0.7	0.0
50	Al	97.0	3.0	0.0	0.0
	Ar	98.5	1.0	0.5	0.0
	Pzq	92.0	3.0	4.5	0.0
	Mf	93.5	0.0	0.0	6.5
100	Al	99.5	0.5	0.0	0.0
	Ar	100.0	0.0	0.0	0.0
	Pzq	90.5	9.5	0.0	0.0
	Mf	91.0	0.5	0.0	8.5
150	Al	98.0	2.0	0.0	0.0
	Ar	99.5	0.5	0.0	0.0
	Pzq	94.0	4.0	2.0	0.0
	Mf	88.0	0.0	0.0	12.0
200	Al	99.0	1.0	0.0	0.0
	Ar	96.0	0.0	0.0	4.0
	Pzq	96.5	3.5	0.0	0.0
	Mf	88.0	0.5	0.0	11.5

Al: albendazole; Ar: artesunate; Pzq: praziquantel; Mf: miltefosine.

Artesunate: Numbers of adult worms recovered and derived from metacercariae treated with water, 10% DMSO, 50, 100, 150, and 200 ppm artesunate were 23, 3, 33, 67, 77, and 91 respectively, representing 37%, 18%, 33%, 33.5%, 38.5% and 45.5% of the inoculum, respectively. Dimensions of worms were (3–4 × 1) mm; the uterus was full of eggs in most cases and testes and ovaries were always visible.

Miltefosine: Numbers of adult worms recovered and derived from metacercariae treated with water, 10% DMSO, 50, 100, 150, and 200 ppm miltefosine were 24, 6, 59, 53, 27, and 55 worms, respectively, representing 37%, 6%, 29.5%, 26.5%, 13.5%, and 27.5% of the inoculum, respectively. Dimensions of

worms were (3 × 1) mm; the uterus was full of eggs in most cases, but the testes and ovaries were indistinct.

Praziquantel: no worm was recovered from any hamster.

In the control groups (0 ppm and 10% DMSO) 74 and 18 adult worms were recovered respectively, representing 49.3% and 12% of the inoculum, respectively. Dimensions of worms were (4–5 × 1) mm; the uterus was full in most worms and testes and ovaries were visible in most (Figure 5).

3.3.3. Effects of drugs on morphology and fecundity of adult worms

Dimensions and development (area) of reproductive organs were evaluated for *O. viverrini* adults derived from metacercariae treated with albendazole, artesunate, miltefosine, and praziquantel. Numbers of uterine eggs were counted for each worm and the rest of the body was carmine-stained for morphological study. Worms from the two control groups (treated with water and with 10% DMSO) differed significantly in dimensions ($P < 0.005$), with those in the DMSO group being larger.

Adult worms from groups treated with artesunate and miltefosine had significantly more uterine eggs ($P < 0.05$) than did the control group (water only) (Figure 6A). Worms of the albendazole-treated groups exhibited significantly greater body area than did the control group ($P < 0.05$) (Figure 6B). Areas of the anterior testis of adult worms in the groups treated with albendazole, artesunate and of the posterior testis in groups treated with albendazole artesunate and miltefosine were significantly greater than in the control group ($P < 0.05$) (Figure 6C and D). The area of the ovary in adult worms in the groups treated with albendazole, artesunate and miltefosine were significantly greater than in the control group ($P < 0.05$) (Figure 6E). Adult lengths in the groups treated with albendazole and artesunate were significantly greater than in the control group ($P < 0.05$). Body widths of adults in the groups treated with albendazole and miltefosine were significantly greater than in the control group ($P < 0.05$) (Figure 6F and G).

Numbers of uterine eggs from adult worms derived from metacercariae treated with albendazole, artesunate and miltefosine did not differ significantly from numbers in the control group (10% DMSO) ($P > 0.05$). Body areas of worms treated with artesunate and miltefosine were significantly smaller than in the control group ($P < 0.05$). Areas of anterior testes of adult worms in the groups treated with miltefosine were significantly smaller than in the control group, but this difference was not seen in the posterior testes ($P > 0.05$). Area of ovary of adult worms in the groups treated with artesunate and miltefosine were smaller significantly than the control group ($P < 0.05$). Body lengths of adults raised from metacercariae treated with artesunate and miltefosine were significantly shorter than in the control group ($P < 0.05$). Width of adults raised from metacercariae treated with albendazole and artesunate were significantly narrower than in the control group ($P < 0.05$).

Numbers of uterine eggs from adult worms derived from metacercariae treated with albendazole and with artesunate at 50 and 150 ppm, and miltefosine at 150 and 200 ppm were significantly greater than in the control group (10% DMSO) ($P < 0.05$). Adult worm body area was significantly greater than controls in all albendazole-treated groups ($P < 0.05$). The areas of anterior testes were significantly greater than controls in all albendazole-treated groups, the 50, 150 and 200 ppm artesunate-treated groups, and the 200 ppm miltefosine-treated group ($P < 0.05$). The areas of posterior testes were significantly greater than controls in all treatment groups except the 200 ppm albendazole group and the 150 ppm miltefosine group. The areas of ovaries were significantly greater than controls in the 50, 100 and 150 ppm albendazole-treated groups, the 50, 150 and 200 ppm artesunate-treated groups and the 50 and 200 ppm miltefosine-treated groups ($P < 0.05$). Lengths of adult worms were significantly greater than controls in all albendazole- and artesunate-treated groups ($P < 0.05$), and widths of adult worms were significantly greater than controls in the 200 ppm albendazole-treated group, and the 50 and 200 ppm miltefosine-treated group ($P < 0.05$).

Numbers of uterine eggs in adult worms from hamsters infected with mature metacercariae exposed to 150 ppm of albendazole and to 200 ppm of artesunate were significantly lower than in the control group (10% DMSO) ($P < 0.05$). The areas of worm bodies were significantly greater than controls in the 200 ppm artesunate-treated group but were significantly less in all albendazole- and miltefosine-treated groups ($P < 0.05$). The areas of anterior testes were significantly lower than controls in the 100 ppm artesunate-treated group and the 50 and 100 ppm miltefosine-treated groups ($P < 0.05$). The areas of posterior testes were significantly greater than controls in the 100 ppm albendazole-treated group, but significantly lower than controls in the 50 ppm miltefosine-treated group ($P < 0.05$). The areas of ovaries were significantly lower than controls in the 100 and 200 ppm artesunate-treated group and the 100 ppm miltefosine-treated group ($P < 0.05$). Adult body lengths were significantly higher than controls in the 200 ppm albendazole-treated group, but significantly lower in the 50 and 100 ppm artesunate-treated groups and in all miltefosine-treated groups ($P < 0.05$), Adult body widths were significantly lower than controls in the 50 ppm albendazole-treated group, all artesunate-treated groups and the 100 ppm miltefosine-treated group ($P < 0.05$).

Figure 5. Adult worms of *O. viverrini* 30 d post-infection from hamster livers.

C1 and C2: 0 ppm and 10% DMSO (control); Al: treated mature metacercariae with albendazole; Ar: artesunate; Mf: miltefosine. Scale bar = 200 μm.

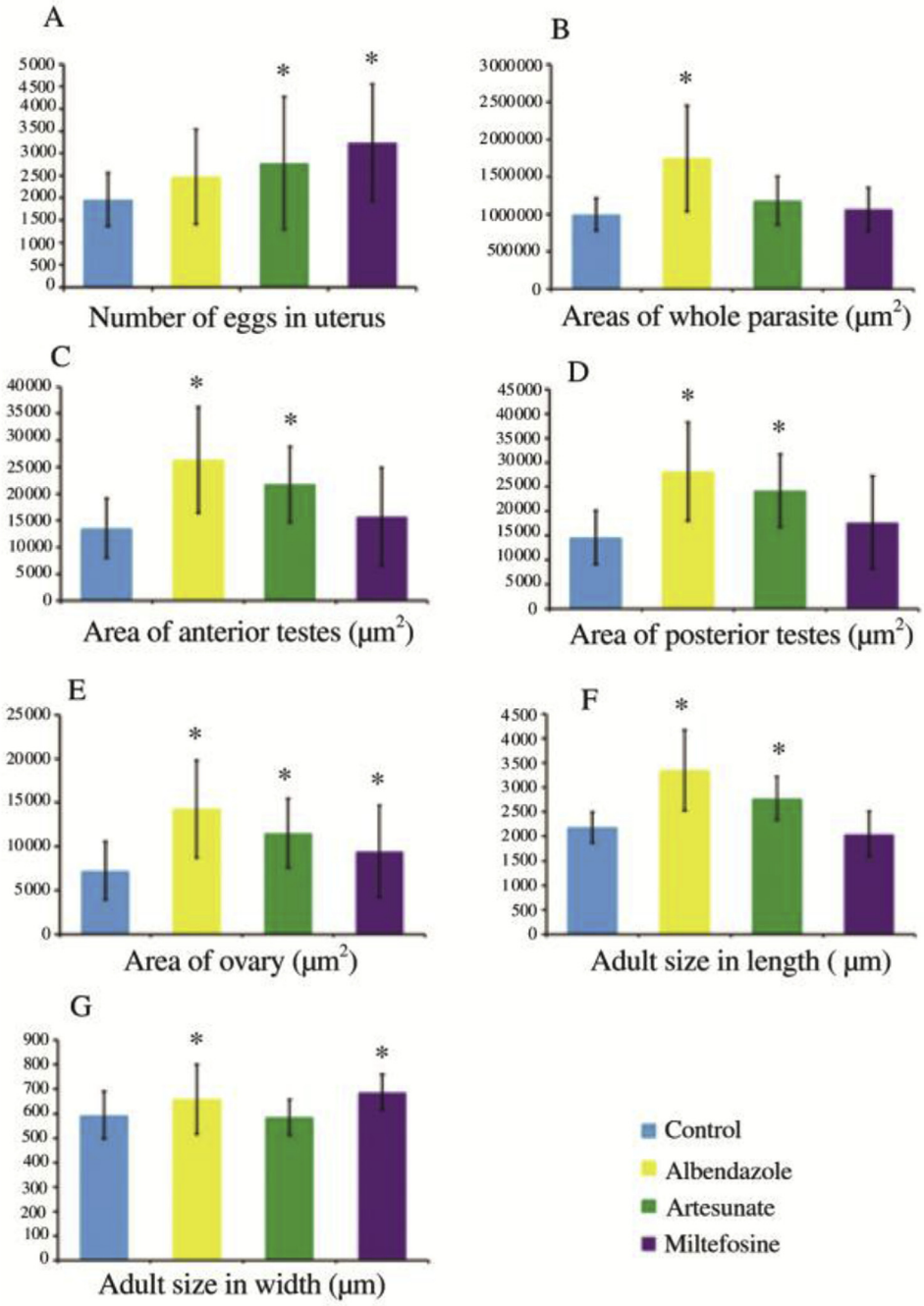

Figure 6. Morphology study of *O. viverrini* adults from hamsters infected with mature metacercariae exposed to albendazole, artesunate and miltefosine. $^{*}P < 0.05$, compared to control group (in water). A: bar charts of the number of eggs in uterus; B: the areas of parasite bodies; C and D: areas of testes (anterior and posterior testes); E: areas of ovaries; F and G: adult sizes (length and width).

4. Discussion

The cercariae in this study were proved to be those of *O. viverrini* by using species-specific primers yielding amplification products of 330 bp [13].

Cercariae of *O. viverrini* were longest survival in the group of low temperature of 12 °C in which the cercariae were low activity and used low preserve energy resulting of the longest life span. At room temperature cercariae had a life span of 49 h; in this study when cercariae were treated with drugs for 24 h and observed for their survival for another 24 h, those cercariae still survived in this period of studying time.

The drugs used were effective against *O. viverrini* cercariae. Praziquantel was most effective, with LC_{50} and LC_{95} values of 0.017 and 0.693 ppm, respectively. High cercaricidal effect was also shown by other drugs, in descending order, artesunate, miltefosine, and albendazole. Few studies have investigated effects of drugs on larval stages of trematodes. Praziquantel has been reported to have larvicidal effects on *S. mansoni* sporocysts, miracidia, and cercariae [15].

Artesunate had cercaricidal effects on *O. viverrini* cercariae with LC_{50} and LC_{95} values of 0.350 and 0.861 ppm, respectively. Artesunate is a clinically versatile artemisinin derivative utilized for the treatment of mild to severe malaria infection [16].

It was effective against *Clonorchis sinensis* and *O. viverrini* in rodent models, with reductions in worm burden of 98.6–100.0% and 77.6%, respectively [15]. Artesunate was also effective against adult *Fasciola hepatica* in the rat model and *in vitro* [8,17]. There has been no previous study of larvicidal effects of artesunate on trematodes.

Miltefosine had cercaricidal effects on *O. viverrini* cercariae with LC_{50} and LC_{95} values of 0.530 and 1.134 ppm. The drug has antiprotozoal and antineoplastic properties and has been used to treat *S. mansoni* infection, in which it damaged the outer tegument and lipid bilayers [9]. There has been no previous study of larvicidal effects of miltefosine on trematodes.

Albendazole had relatively low cercaricidal effects on *O. viverrini* cercariae with LC_{50} and LC_{95} values of 0.720 and 1.139 ppm. Albendazole is effective against *O. viverrini* in hamsters, *C. sinensis* in rats, *F. hepatica* in cattle [18–20] and schistosomiasis [21]. Li and colleagues in 1990, showed that albendazole caused the tegument of parasites to swell and adhere to gut microvilli [22]. However there has been no previous report of effects of this drug on larval stages of trematodes. This study has therefore been the first such report. In our study, the drug had direct contact with the tegument of the cercariae: the concentration of drug required was very low compared to that required for treatment of the adult stage.

With respect to metacercariae, albendazole and praziquantel had no effect: only artesunate and miltefosine were effective at all concentrations used in this study. LC_{50} and LC_{95} values of 303.643 and 446.237 ppm were determined for artesunate, 289.711 and 631.781 ppm for miltefosine, respectively. Cercaricidal and metacercaricidal effects did not coincide. Metacercariae have a protective cyst wall that might help exclude drugs, but cercariae had their tegument in direct contact with drug solutions. There has been no previous report of artesunate and miltefosine as metacercaricide of trematodes.

Mature metacercariae, treated with various drugs, were administered to hamsters, and the resulting adult worms collected 30 d later. Worms derived from metacercariae treated with albendazole, artesunate, and miltefosine were recovered from hamster livers. However, metacercariae treated with praziquantel did not yield worms in hamsters. It is possible that praziquantel adheres to the cyst wall of metacercariae and can act on larvae following excystment. If so, this would be despite the fact that metacercariae were washed with NSS 3–4 times before administration to hamsters. Alternatively, perhaps praziquantel can penetrate the cyst wall and act against the larva. However, this drug was found not be effective against *Nanophyetus salmincola* metacercariae in Chinook salmon (*Oncorhynchus tshawytscha*) at dosages of 10, 20 or 100 mg/kg body weight [23].

The highest percentage recovery of worms was from hamsters infected with artesunate-treated metacercariae. However, worms from hamsters infected with albendazole-treated metacercariae were slightly larger than those in the control group or in the artesunate- and miltefosine-treated groups. The cause of this effect is not known. In the control group, the uteri of worms were filled with eggs, but not in the drug-treated groups. Presumably, anthelmintic effects of these drugs suppress fecundity.

The sizes of reproductive organs in the treated groups were also affected by drug treatment, being smaller than controls in some cases, but larger in others. These effects need further studies.

In our *in vitro* study, albendazole, artesunate, praziquantel and miltefosine were cercaricidal; artesunate and miltefosine had observable metacercaricidal effects. But *in vivo* study is required to evaluate their efficacy in infected wild or cultured fish. The effects of these drugs on cercarial and metacercarial stages should be further studied. If we can prevent the infection of fish by treatment of cercariae with drugs, or kill metacercariae in cyprinid fishes, this could be of value for fish farms. Infection in wild cyprinid fishes is difficult and costly to control. Provision of parasitologically sterile fishes by fish farms would relieve people of concerns about opisthorchiasis, which is a risk factor of CCA, and may lead to a sustainable control strategy for opisthorchiasis.

Conflict of interest statement

We declare that we have no conflict of interest.

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