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RESEARCH NOTE

Effects of *Echinostoma caproni* miracidia dose on the neutral and polar lipids of *Biomphalaria glabrata* as determined by high-performance thin-layer chromatography

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Abstract

The effects of a 5 versus 25 miracidia exposure of *Echinostoma caproni* on the lipid composition of *Biomphalaria glabrata* was studied using high performance thin layer chromatography (HPTLC)- densitometry. A 50 miracidia dose was not used because such a high level of exposure caused severe snail mortality by 3 weeks post-exposure (PE). Lipids were determined in the digestive-gland gonad complex (DGG) of the exposed snails and in the uninfected matched controls at 2 and 4 weeks PE. Extraction of lipids from DGGs was carried out by the Folch method with chloroform-methanol (2:1), and extracts were analyzed on Analtech HPTLC-HLF pre-adsorbent silica gel plates with measurement of separated bands using a CAMAG Scanner 3. For neutral lipids the mobile phase was petroleum ether-diethyl ether-glacial acetic acid (80:20:1) and the detection reagent was 5% ethanolic phosphoric acid, and for polar lipids chloroform-methanol-deionized water (65:25:4) mobile phase and 10% cupric sulfate in 8% phosphoric acid detection reagent were used. No significant differences in the concentrations of free sterols, free fatty acids, triacylglycerols, phosphatidylcholine, and phosphatidylethanolamine were seen at 2 weeks PE in any of the groups. At 4 weeks PE, the free fatty acid concentration increased significantly in the snails exposed to 25 miracidia compared to that of the 5 miracidia/snail group or the controls. Elevation of the free fatty acid fraction in the high dose snail group suggested that some changes occurred in the lipid metabolism of the snails in that group as a function of miracidia dose.

Keywords

Biomphalaria glabrata, *Echinostoma caproni*, high performance thin-layer chromatography, trematodes, miracidia-dose, neutral lipids, polar lipids.

Recent studies have been concerned with the effects of different miracidia doses of a Brazilian echinostomid, *Echinostoma parensei*, on various aspects of the biology and chemistry of *Biomphalaria glabrata* snails (Tunholi-Alves *et al.* 2011 a, b). In these studies, the low and high dose used was 5 and 50 miracidia per snail, respectively. In a biological study on *E. parensei* in *B. glabrata*, the high dose caused adverse effects on the reproductive biology of the snails not seen in snails exposed to the low dose (Tunholi-Alves *et al*., 2011a). In a biochemical study, the high dose exposure of *E. parensei* in *B. glabrata* caused a significant reduction in the concentrations of cholesterol and triacylglycerols in that group compared to the snails exposed to the low dose (Tunholi *et al.* 2011b).

Our laboratory has been doing similar work on low versus high miracidia doses of an Egyptian echinostomatid, *E. caproni*, in *B. glabrata*. However, as reported in an earlier biological study by Hunsberger *et al.* (2012), attempts to infect *B. glabrata* with 50 miracidia/snail of *E. caproni* were soon discontinued because such a high miracidia dose caused snail mortality of greater than 80% by 3 weeks post-exposure (PE). Therefore, we changed our miracidia dose protocol to that of 5 miracidia per snail for the low dose and 25 miracidia per snail for the high dose. With this new protocol, we used high performance thin-layer chromatography (HPTLC)-densitometry to determine the effects of a low and high dose exposure of *E. caproni* miracidia on the neutral and polar lipid content of the snails.

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Ninety *B. glabrata* snails (NMRI strain), 8–12 mm in shell diameter, were used in this study. The snails were obtained from Dr. Fred A. Lewis, Head, Schistosomiasis Laboratory, Biomedical Research Institute Rockville, MD, USA. The aquarium water used to maintain the cultures was artificial spring water (ASW) as described by Ulmer (1970). Each culture contained approximately 800 mL of ASW in a 1000 mL glass mason jar. During the course of this work, 3 trials were done with each trial consisting of 10 snails per group. The groups were designated as A, B, and C. The A group consisted of 10 unexposed snails (controls). The B group consisted of 10 snails each exposed individually in a single well of multiwall chamber to 5 miracidia in 2–3 mL of ASW for 6 to 8 hours. The C group consisted of 10 snails each exposed to 25 miracidia per snail as described for B. Snails were maintained on a 12 hour day/night regimen at 25±1°C under artificial fluorescent light. Snails were fed a diet of boiled romaine lettuce leaves *ad libitum*, and the ASW was changed every 2–3 days.

Snails were necropsied at both 2 and 4 weeks PE to obtain the digestive gland-gonad complexes (DGGs) used in this study. The total number of DGGs used at 2 weeks and 4 weeks PE was 43. Each sample consisted of the DGG of a single snail. The DGG was dissected free of the visceral mass and extracted and prepared for HPTLC as follows. Each sample was homogenized in a 7-mL glass homogenizer (Wheaton, Millville, NJ, USA) with 4 mL chloroform-methanol (2:1). The homogenate was filtered through cotton wool, and Folch wash (0.88% KCl) was added in an approximate volume of 25% of the chloroform-methanol (2:1) used. The lipophilic layer was separated from the hydrophilic layer using a Pasteur pipet and blown down in a warm water bath (approximately 40˚C) with N_2 gas. Samples were reconstituted in chloroform-methanol (2:1) in a ratio of 10 µL mg^{-1} sample. Reconstitution volumes ranged from 299.0 to 1208.0 µL as necessary for the densitometric scan area of at least one spotted aliquot of each sample to be bracketed within the scan areas of the standard zones during HPTLC analysis.

The neutral lipid standard, nonpolar lipid mixture B (No. 1130, Matreya, Inc., Pleasant Gap, PA, USA) contained a total of 25 mg of cholesterol, oleic acid, trolein, methyl, oleate, and cholesteryl oleate in equal amounts (20% each) as marker compounds for the free sterol, free fatty acid, triacylglycerol, methyl ester, and steryl ester fractions, respectively. The neutral lipid standard solution was prepared at 0.200 µg/µL for each lipid by diluting the 1.00 mL standard to 25.0 mL with chloroform-methanol (2:1) in a volumetric flask.

The standard for phospholipid analysis was Polar Lipid Mix No. 117 (Matreya Inc.) containing 25% each of cholesterol, phosphatidylcholine, phosphatidylethanolamine, and lysolecithin in a total of 25 mg in 1.00 mL. The polar lipid standard solution was prepared at 0.125 µg/µL of each component by dissolving the 1.00 mL standard with chloroform-methanol (2:1) in a 50 mL volumetric flask.

HPTLC analysis was performed on 10 × 20 cm HPTLC-HLF silica gel plates (Analtech Inc., Newark, DE, USA) that contained 19 scored lanes and a preadsorbent zone spotting area (catalog No. 61927). Before use, plates were precleaned by development to the top with dichloromethane-methanol (1:1), dried with a stream of air, and activated for 30 min on a CAMAG (Wilmington, NC, USA) plate heater at 120˚C. The lanes and preadsorbent zone facilitate reproducible manual application of sample and standard initial zones in order to obtain the preferred band shape upon development, and the lanes facilitate initial line up of densitometer source slit for densitometric scanning.

Standard and reconstituted samples were applied in 2.00, 4.00, 8.00, and 16.0 µL aliquots (0.200 to 3.20 µg for neutral lipids and 0.250 to 2.00 µg for polar lipids) to the preadsorbent zone of separate lanes on the HPTLC plates using a 10-µL Drummond (Broomall, PA, USA) digital microdispenser. A gentle stream of air was directed across the preadsorbent zone during application of the sample to quickly dry the standards and samples as they were applied. Plates were developed to a distance of 8 cm beyond the preadsorbent zone-silica gel interface with 50 mL of the Mangold (1969) mobile phase, petroleum ether-diethyl ether-glacial acedic acid (80:20:1), for determination of neutral lipids, or the Wagner *et al.* (1961) mobile phase, chloroform-methanol-deionized water (65:25:4), for phospholipids. Ascending, one dimensional development was carried out in a twin trough HPTLC chamber (CAMAG) containing a saturation pad (Analtech Inc.). Prior to insertion of the spotted plate for development, the chamber was equilibrated with the mobile phase for 20 min; development required approximately 10–15 min.

After development, neutral lipid plates were dried with a stream of cool air from a hair dryer for about 10 min, sprayed with 5% ethanolic phosphomolybdic acid solution, and heated on a CAMAG plate heater at 115°C for 10 min to detect neutral lipids as blue bands on a yellow background. After development, plates containing polar lipids were dried with a stream of cool air from a hair dryer for about 10 min, sprayed with 10% cupric sulfate in 8% phosphoric acid solution, and heated on a plate heater at 140°C for approximately 30 min until the bands were detected as brown-black bands on a white background.

Neutral and polar lipids were quantified by slit-scanning densitometry in the absorbance-reflectance mode using a CAMAG TLC Scanner 3 set at slit dimensions 4.00 × 0.45 mm, and scanning rate of 20 mm/s. The tungsten light source set at a wavelength of 610 nm was used for neutral lipid determination, and the deuterium light source set at a wavelength of 370 nm was used for the polar lipids. The winCATS software automatically generated polynomial regression calibration curves (standard zone masses versus peak areas) and interpolated sample zone weights based on their peak areas. The percentage by mass of lipid in each tissue sample was calculated using the equation:

$$\text{Percent lipid} = \frac{(w) \bullet (R) \bullet (100)}{\mu g \ tissue}$$

Where w = lipid mass (µg) of sample interpolated from the calibration curve and R = reconstitution volume (µL)/spotted volume (µL).

A one-way ANOVA analysis was done on each lipid class to determine the *P*-value in Microsoft Excel. Because we simultaneously compared all three cultures in the interpretation of our results, we divided the accepted value of $P < 0.05$ by 3 to yield a significance value of $P < 0.017$. Further rational for such statistical treatment was given by Beideman *et al.* (2013).

Greater than 90% of our snails survived until necropsy at 2 and 4 weeks PE. This high survival allowed for ample material for our HPTLC analysis. Lipid analysis was done at 2 and 4 weeks PE. At both 2 and 4 weeks PE, 43 of 45 snails survived for analysis. Therefore, the number (*n*) used to analyze the lipids at 2 and 4 weeks PE ranged from 13–15. The major neutral lipid fractions quantified by HPTLC were free sterols, free fatty acids, and triacylglycerols. Each sample had neutral lipid zones that matched the R_f values of the standards, cholesterol (0.19), oleic acid (0.38), and triolein (0.57). The methyl ester and steryl ester bands were faint and were were not considered further in this study. The major polar lipid fractions that were quantified by HPTLC were phosphatidylcholine ($R_f = 0.46$) and phosphatidylethanolamine ($R_f = 0.66$). The results of our HPTLC analyses are summarized in Tables I and II. At 2 weeks PE, there were no significant differences in any of the lipid classes in the experimental groups versus the controls (Table I). At 4 weeks PE, there was a significant increase in the free fatty acid fraction of the high dose group (C) compared to the low dose group (B) and the controls (A).

The results indicated relatively minor changes in lipid concentrations as a result of miracidia doses of 5 versus 25. In fact, the only significant change we noted was in the free fatty acid fraction at 4 weeks PE in the high versus low miracidia groups. Our results contrast to what was seen in the *E. paraensei-B. glabrata* model in which marked reductions in the triacylglycerol and cholesterol concentrations were seen in the high dose group (50 miracidia/snail) (Tunholi-Alves *et al.* 2011b). Because of the high snail mortality, noted previously when using a 50 miracidia dose of *E. caproni* in *B. glabrata* (Hunsberger *et al.* 2012), we could not do lipid analysis with such heavily exposed snails at 4 weeks PE. The fact that the 50 miracidia *E. caproni* dose had more adverse effects on *B. glabrata* survivial than did a similar dose of *E. paraensei* attests to biochemical and genetical differences in these two morphologically similar 37-collar spined echinostome species.

We plan to continue our studies on low versus high miracidia dose infections with *E. caproni* in *B. glabrata*. We plan to use high miracidia doses of 30 and 40 miracidia per snail and determine if such doses are less deleterious to snail survival than 50 miracidia per snail. We hope to compare our findings with similar biological and biochemical studies published on the *E. paraensei-B. glabrata* host-parasite relationship.

Table I. Percent by mass (mean ± standard error) of the lipids studied at 2 weeks post-exposure

Lipids	A	B	C	*P*-value
Free sterols	0.0759 ± 0.0436	0.162 ± 0.138	0.0857 ± 0.0440	0.0224
Free fatty acids	0.0586 ± 0.0613	0.198 ± 0.184	0.161 ± 0.137	0.0237
Triacylglycerols	0.441 ± 0.276	0.520 ± 0.543	0.348 ± 0.199	0.473
Phosphatidylcholine	0.222 ± 0.113	0.238 ± 0.113	0.226 ± 0.162	0.943
Phosphatidylethanolamine	0.0548 ± 0.0434	0.0953 ± 0.0610	0.0956 ± 0.0733	0.121

For Culture A, n = 15. For Culture B, n = 14. For Culture C, n = 14. A = control culture (unexposed), B = low dose culture (5 miracidia/snail), C = high dose culture (25 miracidia/snail)

Table II. Percent by mass (mean ± standard error) of the lipids studied at 4 weeks post-exposure

Lipids	A	B	C	*P*-value
Free sterols	0.0945 ± 0.0509	0.104 ± 0.094	0.0910 ± 0.0734	0.886
Free fatty acids	0.0536 ± 0.0268	0.0725 ± 0.0452	0.133 ± 0.061	0.000131*
Triacylglycerols	0.151 ± 0.140	0.206 ± 0.164	0.190 ± 0.115	0.568
Phosphatidylcholine	0.220 ± 0.126	0.203 ± 0.130	0.196 ± 0.143	0.886
Phosphatidylethanolamine	0.0882 ± 0.0404	0.0729 ± 0.0479	0.0864 ± 0.0495	0.615

For Culture A, n = 15. For Culture B, n = 15. For Culture C, n = 13. A = control culture (unexposed), B = low dose culture (5 miracidia/snail), C = high dose culture (25 miracidia/snail). *Significantly different at $P < 0.017$

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