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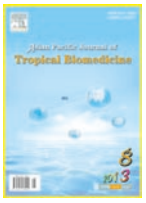

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New scope on the relationship between rotifers and *Biomphalaria alexandrina* snails

Shereen Farouk Mossallam*, Eglal Ibrahim Amer, Iman Fathy Abou-El-Naga

Medical Parasitology Department, Faculty of Medicine, Alexandria University, Egypt

PEER REVIEW

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Kálmán Imre, DVM, PhD, Department of Food Hygiene and Veterinary Public Health, Faculty of Veterinary Medicine, Banat's University of Agricultural Sciences and Veterinary Medicine Timissoara, Romania.
Tel: 40256277186
Fax: 40256277118
E-mail: kalman_imre27@yahoo.com

Comments

This is a complex study with a particular emphasis on the effect of rotifers internalization into the *Biomphalaria alexandrina* snail tissues during the development of schistosomes, which serves as an important human pathogen.
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ABSTRACT

Objective: To investigate the effect of rotifer internalization into snail tissue on the development of schistosomes.
Methods: Susceptible laboratory-bred *Biomphalaria alexandrina* (*B. alexandrina*) snails were exposed to lab-maintained rotifers; *Philodina* spp., two weeks before and after being infected with *Schistosoma mansoni (S. mansoni)* miracidia. The consequent histopathological impact on snail tissues and cercarial biology were investigated before and after emergence from snails.
Results: Contamination of *B. alexandrina* snails with philodina, two weeks before miracidial exposure, was found to hinder the preliminary development of *S. mansoni* cercariae inside the snail tissues. Furthermore, when snails were contaminated with rotifers two weeks post miracidial exposure; growth of already established cercariae was found to be retarded. The consequent influence of internalized rotifers within the snail tissue was clearly reflected on cercarial emergence, activity and infectivity along the four weeks duration of shedding. In the present study, comparison of snail histopathological findings and altered cercarial biology observed between the experimental and control groups indicated that the rotifers may have affected the levels of snail's energy reservoirs, which eventually was found to have had an adverse impact on reproduction, growth and survival of the parasite within the snail host, coupled with its performance outside the snail.
Conclusions: In future biological control strategies of schistosomiasis, ritifers should be considered as a parasitic scourge of humanity.

KEYWORDS

Biomphalaria alexandrina, Schistosoma mansoni, Rotifers, Snail histopathology, Cercarial biology

1. Introduction

Schistosomiasis is a widespread snail-borne chronic trematode infection of humans, domestic and wild animals. The disease has significant economic and public health consequences on affected populations in the disease-endemic developing countries in Africa, South America and South-East Asia. Schistosomiasis continues to be a serious health problem, where exposure to infective cercariae results in more than 200 million cases worldwide[1-3].

Control of schistosomiasis has been a challenging task for most endemic countries. A number of control strategies, including blocking parasite transmission in snail hosts; have been proved unsuccessful for long-term control of schistosomiasis[4-6].

Philodina snails are needed by *Schistosoma mansoni* (*S. mansoni*) parasites to be able to complete their life cycle[7]. Indeed, snails thrive in nature under diverse and changing environmental conditions[8]. Numerous metazoa have been described as living in close association with snails[9-11]. Occurrence of many diverse organisms within snails is not surprising, since the snail's natural habitats are shared by

*Corresponding author: Shereen Farouk Mossallam, Medical Parasitology Department, Faculty of Medicine, Alexandria University, Egypt.
Tel: +201006271788
Fax: +2034831498
E-mail: mossallamsh@hotmail.com
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a variety of flora and fauna. The effects of such unintended contaminants on the growth, survival and fecundity of snails have always been of wide interest[8]. Biological control of snail borne diseases offers an inexpensive and environmentally acceptable control approach[7]. Thus, more emphasis should be put on studying microorganisms that may directly or indirectly affect the intermediate snail host and could be potential agents for disease control. So far, a number of biological control agents have been tried; bacteria, competitor snails, fish and rotifers[12-17].

Rotifers "wheel animals" are bilaterally symmetrical metazoans belonging to the phylum Rotifera, which comprises over two thousand species varying in shape, size and hardness. They are distinguished by a ciliated anterior corona (used in locomotion and food gathering) and a pharynx equipped with a complex set of jaws. These microscopic animals can be either freewheeling or parasitic and known to live in a variety of fresh and salt water environments[10,18].Rotifers possess wide ecological importance. They represent an important part of the freshwater zooplankton by contributing to nutrient recycling[10,19]. On the other hand, some species of rotifers are associated with organisms in a predator-prey manner[20]. The effects of rotifers on schistosomiasis were found to be quite dissimilar and their relationships with snails remain imprecisely defined; most are symbionts or commensals, although few could be pathogenic[7]. Several species of rotifers, including Rotaria and *Philodina* spp., naturally exist on the shells of snails with no proof of a predator-prey association to the snail[8].

Previous work has pointed out to the deleterious effect of rotifers on *S. mansoni* cercariae following their emergence from snails[16], whereas their pathological impact was mostly scoped on egg masses and newly hatched snails than on mature snails[17,21,22].

Much remains unknown about this exquisite phylum[10]. The effect of rotifer's internalization into the snail tissues on the development of schistosomes has not been precisely studied. Hence, this study was conducted to investigate the possible effects of invasion of susceptible laboratory-bred snails by lab-maintained rotifers; *Philodina* spp., on the development of *S. mansoni* parasites before and after their emergence from the snail host.

2. Materials and methods

2.1. Snail source and maintenance

Susceptible rotifers-free unexposed adult snails (7-8 mm) were purchased from Theodor-Bilharz Research Institute. The snails were delivered to our lab in dechlorinated tap water, which was daily checked, for one week, to make sure they were rotifer-free (described in Section 2.5.1.). Snails were maintained in black-walled covered plastic aquaria and kept at 26-28 °C in an incubator; each aquarium containing well-aerated aged dechlorinated tap water (DTW) that was changed twice a week. Fresh washed lettuce leaves were supplied as food every couple of days and soft chalk was kept in all aquaria. Snails were cleaned by brushing, wiped with a dry cloth and dead snails were regularly removed[23].

2.2. Snail exposure to S. mansoni

Five laboratory-bred Swiss strain albino mice were infected with 150 cercariae per mouse using the paddling technique[24]. Seven weeks post-infection, eggs obtained from the intestines and livers of infected mice were exposed to direct sunlight for approximately 30 min to stimulate miracidial hatching. Each snail was individually exposed to 5-6 active, vigorously swarming *S. mansoni* miracidia in 5 mL of DTW for 2 h, a period sufficient for maximal miracidial penetration[25].

2.3. Rotifers source, maintenance culture and viability

Rotifers, *Philodina*, were obtained from a batch of contaminated snails that were delivered in mud and algae from Theodor Bilharz Institute to the Medical Parasitology Department-Lab, Faculty of Medicine, Alexandria University. Contaminated snails were removed and the remaining rotifer-containing water was used as a source for rotifers cultivation, after being microscopically examined to exclude the presence of other metazoans or rotifer specie *S. rotifers* were maintained in 100 mL polyethylene screw-capped jars in filtered DTW at a temperature of 20-21 °C. They were fed every two days with a nutrient mixture of algae. For rotifers selection, approximately, 10 mL of the stirred culture water was aspirated using a pipette from a heavily populated area on the bottom of the jar. Rotifers in 0.1 mL of the aspirated water were counted using a neubaur ruling haemocytometer chamber. The number of rotifers used for snail contamination was adjusted to approximately 20 organisms/0.1 mL per snail[26].

2.4. Experimental design

A hundred and tweenty live lab-adapted rotifers-free unexposed susceptible B. alexandrina snails were divided into two groups;

2.4.1. Group I: Un-exposed snails

Group I Included 60 snails not exposed to *S.mansoni* miracidia and were subsequently divided into two subgroups (SG).

Subgroup I-A (SG I-A) included 20 un-exposed uncontaminated snails. They were neither exposed to S.mansoni miracidia nor contaminated with rotifers. They served as a control group for the histopathological study.

Subgroup I-B (SG I-B) included 40 Un-exposed contaminated snails. These snails were not exposed to *S. mansoni* miracidia, yet maintained in rotifers-culture water for two weeks duration. Snails were then brushed and transferred to rotifers-free DTW for both histopathological and viability assessments at two, four and six weeks from the day of rotifer contamination.

2.4.2. Group II: Exposed snails

Group II snails Included 60 snails exposed to *S. mansoni* miracidia and were equally divided among three subgroups.

Subgroup II-A (SG II-A) consisted of 20 exposed uncontaminated snails. They were exposed only to *S. mansoni* miracidia without rotifer contamination.

Subgroup II-B (SG II-B) included 20 rotifers pre-contaminated exposed snails. They were maintained in rotifers-culture water for two weeks duration. After that, they were brushed, exposed to S. mansoni miracidia and kept in rotifers-free DTW.

Subgroup II-C (SG II-C) were exposed snails then contaminated with rotifers. These snails were exposed to *S. mansoni* miracidia, and then maintained in rotifers-free DTW. Two weeks later, they were maintained in rotifers-culture water for another couple of weeks. Snails were finally brushed and transferred to rotifers-free DTW.

2.5. Snail assessment

2.5.1. Checking for rotifers

All through the experiment, snail aquaria were checked every two days for rotifers. Snail shells were checked for rotifer colonization on their surfaces through a dissecting microscope, while water was microscopically checked using the Leitz microscope for freely swimming rotifers[16]. A drop of the water deposit was mixed with a similar drop of 50% glycerin and covered with a cover slip, aiming to obtain a clear thin preparation. For staining, several fixation protocols were assessed for fixing rotifers using 2.5% glutaraldehyde, Bouin's solution, 70% ethanol, methyl alcohol or formalin 10%[27]. Rotifers were then stained with 0.5% eosin and finally mounted in glycerin.

2.5.2. Tissue rotifers viability

On the first, fourth and sixth weeks of contamination with rotifers, six snails from unexposed contaminated I-B subgroup (SG I-B) snails were cautiously removed from shells, individually minced, centrifuged for 7 min at room temperature to remove tissue debris. Rotifer-containing supernatant was collected and centrifuged for 10 min. The pellet obtained was resuspended in 0.1 mL PBS. Viability was evaluated using a dye exclusion test with 0.4% trypan blue within 30 min[28].

2.5.3. Snail tissue histopathology

On the second, fourth and sixth weeks of snail contamination with rotifers, six snails from the SG I-B were histopathologically assessed and compared to those of the subgroup I-A (SG I-A) at the time points indicated above. Similarly, histopathological changes in tissues of six snails from each exposed subgroup; II-A, -B and -C, crushed four weeks post exposure to *S. mansoni* miracidia, were assessed and compared with those of the un-exposed snails. Crushed snail-tissues were cautiously removed from their shells, fixed in Bouin's fluid for 5 h, and then transferred to 70% alcohol. Further procedures included dehydration in 100% alcohol, clearing in xylol, then paraffin embedding were followed. Five sections were stained with hematoxylin and eosin. Stained slides were examined under a light microscope.

2.5.4. Cercarial assessment

Four weeks post exposure to *S. mansoni* miracidia, ten uncrushed exposed snails belonging to subgroups II-A, -B and -C were checked for the following:

The mean cercarial output for each subgroup was counted once per week for four consecutive weeks. This was done by placing each subgroup of snails in 100 mL beakers containing DTW and subjecting them to a source of light for 2 h[23].

Evaluation of spontaneous swimming activities of cercariae that were shed from the exposed snails was done on a weekly basis starting from the fourth week post exposure. This was repeated with another cercaria of the same drop-sample. Another group of cercariae was then examined for the next replicate. Five replicates of cercariae from each snail subgroup were recorded at each time point by three different examiners[30,31]. The infectivity powers of cercariae shed from all exposed snails subgroups (SG II-A,-B,-C) were investigated. A total fo 120 locally bred Swiss albino mice, 4-6 weeks old and weighing 20-25 g, were infected with 80 *S. mansoni* cercariae per mouse using the paddling technique[24]. Mice were equally distributed among 4 subsequent subgroups (n=40/subgroup), to be subsequently examined along the 1-4 weeks duration of shedding. Animal study was approved by the Ethics Committee of the Faculty of Medicine, Alexandria University, Egypt. Animal experiment complied with the Egyptian National Regulations for Animal Experimentation. After performing the paddling technique, the remaining DTW content of each flask was centrifuged, the sediments were examined for any remaining cercariae and the number of non penetrating cercariae were recorded. In addition, the infectivity rate was calculated for each subgroup. The percentage of non-penetrants was calculated per flask (No. of flasks with remaining cercariae/ Total No. of flasks-100).

At the time point of 7 weeks post infection; mice were injected with heparin and sacrificed by cervical dislocation. Hepatic and portomesenteric vessels were perfused, using citrated saline, to recover adult schistosomes[24]. Infectivity rate was calculated for each subgroup (No. of mice acquired infection; having adults/ No. of mice per subgroup-100).

2.6. Statistical analysis

Data were analysed using the Predictive Analytics Software (PASW Statistics 18). Qualitative data were described using frequency and percentage, while quantitative data were described using minimum and maximum as well as mean and standard deviation. Data obtained for the different experimental and control groups was compared using ANOVA test with the *Post Hoc* test (Schpffe) to clarify the significance between groups. Significance test results are quoted as two-tailed probabilities. Values of $P \leqslant 0.05$ were considered to be statistically significant.

3. Results

3.1. Checking for rotifers

While continuous checking of SG I-A & II-A snail aquaria didn't show any sign for rotifer contamination, *Philodina* was found to colonize on SG I-B, II-B and II-C snail's shells, starting from the center of the whorl and extending over entire shell, during two weeks period of snails maintenance in rotifer-culture water. Moreover, free rotifers were frequently witnessed traversing the surface of shell and entering into mantle cavity. After being removed and brushed from rotifer-cultured water, continuous samplings from these aquaria showed no more rotifers. Through a dissecting microscope, freshly collected unstained *Philodina* rotifers from snail aquaria were viewed as active freely moving extended bodies, having a well demarcated rotatory wheel-like corona. On mounting them in glycerin without being fixed or stained, they were found to retain their extended position, and the characteristic remarkably expanded coronas were distinguished as two ciliated disks surrounding the mouth (Figure 1B). Upon fixation of fresh *Philodina* in its extended form, it failed to take up the stain. Rotifers exposed to various fixing agents in this work were found to gradually fold around themselves, ended up acquiring a folded globular-like structure.

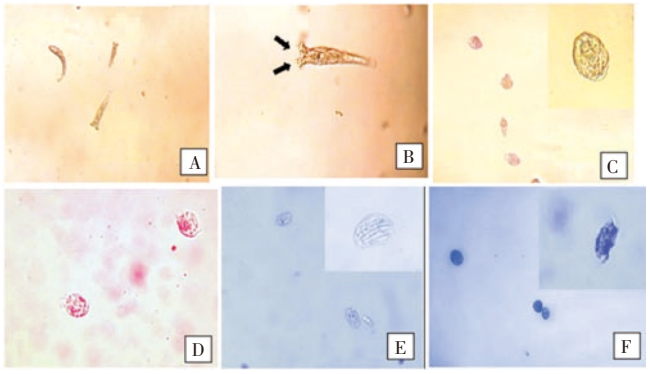

Figure 1. *Philodina* rotifers.
A: Unstained *Philodina*e in glycerin in mounted an extended form (×100); B: Higher magnification of an unstained extended *Philodina* with its remarkable expanded corona; the two ciliated disks (arrowed) surrounding the mouth (×400); C: Different folding stages of unstained fresh *Philodina* upon fixation; D: Eosine-stained folded globular fresh *Philodina* after different fixations (×400); E: Viable rotifers (inset); F: Non-viable rotifers (inset).

3.2. Tissue rotifers viability

In SG I-B, viability of rotifers was retained at the second and fourth weeks, but was almost lost at the sixth. Viable rotifers excluded the dye, while non-viable ones took it up (Figure 1E&F).

3.3. Histopathology of snail tissue

Histopathological examination of SG I-B snails, after two weeks of maintenance with rotifers, revealed the presence of cut sections through tissue-invasive organisms; whose internal details were not very clear, but their conlours could be identified. They simulated folded rotifers that were freshly stained from the contaminated aquaria. Hence, these tissue-invasive organisms appear to be folded rotifers entrapped within the loose snail connective tissue and digestive glands. On the fourth week, mild hemocytic infiltration against folded globular-like rotifers-entrapped within SG I-B snail tissues was seen. After two more weeks, rotifers looked degenerated and were surrounded by concentric layers of fibroblasts with some hemocytes encapsulated (Figure 2).

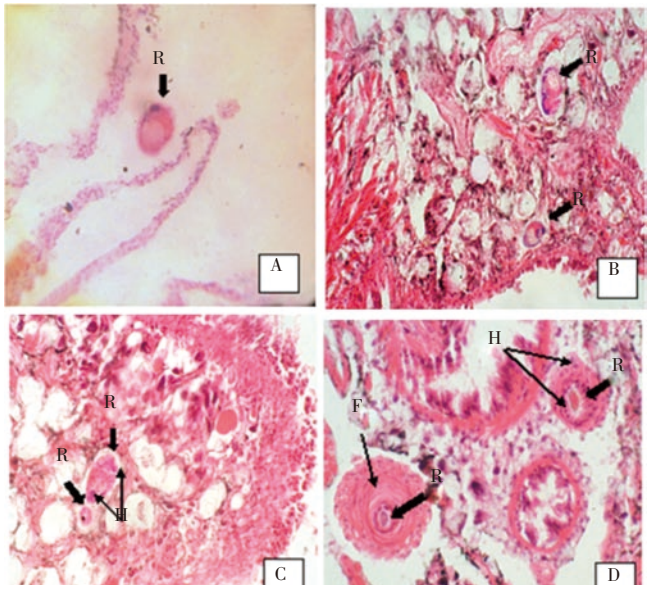

Figure 2. Histopathological changes of snails.
A: A cut section through a folded rotifer in the loose connective tissue within the mantle region of snail (×400); B: Folded rotifers entrapped within the snail digestive glands (×400); C: Mild hemocytic infiltration against folded rotifers thick arrows entrapped in snail digestive gland (×400); D: Degenerated rotifers inside granuloma-like structures of concentric layers of fibroblasts with some hemocytes (×400).

3.4. Cercarial count

The mean numbers of cercariae shedded from exposed snails in SG II-A in weeks 1, 2, 3 and 4 were (193.40 ±9.51), (212.10±19.65), (198.90±6.59) and (175.30±6.78), respectively. These values significantly declined in SG II-C to (102.50±3.89), (106.90±4.68), (91.40±3.89) and (92.70±

3.86), respectively. In SG II-B, a remarkable deterioration was noticed in the same respective order as (80.60±5.32), (77.0±4.99), (71.20±3.52) and (64.30±3.53). Upon comparing the three subgroups, statistical significant difference was found between SG II-A and II-B&-C, as well as between SG II-B and II-C (P<0.05) (Table 1).

Table 1
Cercarial counts among subgroup II at different time points.

Indicator	Shedding week			
	1st week	2nd week	3rd week	4th week
II-A				
Range	178.0-211.0	189.0-242.0	188.0-211.0	169.0-189.0
Mean±SD	193.40±9.51	212.10[a]±19.65	198.90±6.59	175.30±6.78[abc]
II-B				
Range	72.0-89.0	69.0-84.0	66.0-77.0	60.0-69.0
Mean±SD	80.60±5.32	77.00±4.99	71.20±3.52[ab]	64.30±3.53[abc]
II-C				
Range	98.0-109.0	100.0-114.0	88.0-100.0	89.0-99.0
Mean±SD	102.50±3.89	106.90±4.68	91.40±3.89[ab]	92.70±3.86[ab]

II-A: Exposed subgroup of snails; II-B: Rotifers pre-contaminated exposed subgroup; II-C: Exposed snails, then contaminated with rotifers; a: significant with 1st week at 0.05; b: significant with 2nd week at 0.05; c: significant with 3rd week at 0.05.

3.5. Cercarial activity

No definite cercarial activity was seen to be unique to all snail subgroups. Table 2 shows the various detectable cercarial attachments and free motilities of the exposed subgroups II-A, -B and -C. Free un-attached cercarial swimming actions were numerically coded, while cercariae attached-attitudes were alphabetically coded.

3.5.1. Free cercarial motility

The free-swimming performance of un-attached cercariae was thoroughly studied (Table 2). In SG II-A, two swimming motion types were the most commonly noticed throughout the four weeks; prompt linear progressive (1) and zigzag (2) swimming motions. These two swimming patterns were temporarily interrupted by cercarial pausing with random motions (4). Regarding SG II-B, straight line and zigzag swimming motions (1&2) were as detectable as pauses with intermittent spins or irregular onward movements (3&4) throughout the four weeks of shedding. Stationary cercarial pauses (5) were exclusively identified in the first two weeks of shedding in SG II-C even more than the pauses observed with intermittent spins or irregular onward movements (3&4). The latter types 3&4 were seen to even overwhelm the straight line- and zigzag-cercarial swimmings (1&2) in the last two weeks of shedding.

3.5.2. Cercarial attachment

Close observation of cercariae emerging from SG II-A and II-B snails didn't prove the presence of any attachment manners of cercariae to each other along the four studied durations (N) (Table 2). Occasionally, extremely scanty cercariae got briefly attached to each other with their heads, while showing fast tail movements. However, they immediately got dislodged once this happened (un-tabulated data).

On the other hand, several cercarial attachments, which lasted for a minute or more, were observed in SG II-C. In the first week of shedding, mutual head-tail attachment, with or without sluggish central twitches, was uniquely noted (Figure 3A). Another two types of cercarial behaviors; attachment through the heads to curly stationary curved bodies /or coiled tails (Figure 3B) and to stationary non-curled tails (Figure 3C), were further observed along the first two weeks of shedding. In the next two weeks, attachment was seen to occur through the heads and this was accompanied with fast or slow lateral tail movements perpendicular to the position of the body (Figure 3D&E).

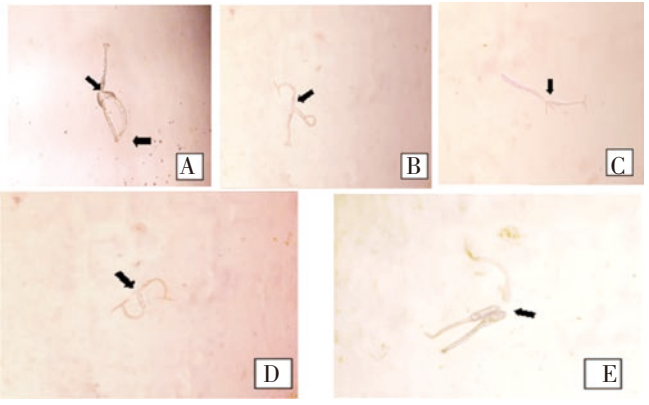

Figure 3. Various identified cercarial attachments.
(A) Mutual cercarial head-tail attachment (×100); (B) Cercariae attached through their heads, with stationary curved bodies and coiled tails (×100); (C) Cercarial head to tail attachment (×100); (D&E) Cercariae attached together through their heads. (×100) (Arrows show the points of attachments).

3.5.3. Animal infectivity

As shown in Table 2, infectivity rates of mice belonging to

Table 2
Cercarial activities and animal infectivity among subgroups II-A, -B and -C at the different studied time intervals.

Subgroups		II-A				II-B				II-C			
Shedding weeks		1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Cercarial activity	Free motility	1,2>4	1,2>4	1,2>4	1,2>4	3,4=1,2	3,4=1,2	3,4=1,2	3,4=1,2	5>3,4	5>3,4	3,4>1,2	3,4>1,2
	Attachment	U	U	U	U	U	U	U	U	ABC	BC	DE	DE
Animal infectivity	Rate%	100	100	100	100	70	60	80	70	0	0	40	50
	Non-penetrating cercariae%	0	0	0	0	10	30	20	20	100	80	50	30

II-A: Exposed subgroup of snails; II-B: Rotifers pre-contaminated exposed subgroup; II-C: Exposed snails, then contaminated with rotifers

SG II-A was 100% throughout the 4 weeks of shedding. This coupled with the absence of any non-penetrating cercariae remaining in the flasks after mice infection in this subgroup. In SG II-B, infectivity decreased to 70, 60, 80 and 70% in the four sheddings, respectively. At the same examined time points, non-penetrating cercariae were 10 and 30% (in the first two weeks, respectively) and 20% (in the later couple of weeks). As regards SG II-C, shed cercariae failed to induce animal infectivity in the first couple of weeks, yet infectivity rates relatively increased in the next two weeks to 40 and 50%, successively. The percentages of flasks showing remaining cercariae in this subgroup were 100, 80, 50, and 30% in weeks 1, 2, 3 and 4 of shedding, respectively. The observed slight mis-matching between the infectivity rates and the percentage of non-penetrating cercariae is currently under further investigation.

4. Discussion

Preceding work on the relationship between rotifers and schistosomiasis has focused on their effect on cercarial emergence from snails. As reported, cercariae; produced from snails carrying rotifers on their shells or from those deposited in water previously containing rotifers, were shown to lose their activity[16,21]. Nevertheless, a demanding inquiry for precise effect of such highly motile metazoa on internal snail tissue still needs to be investigated. The current study verified the impact of internalization of rotifers; *Philodina*, on the tissues of snails, a step which influenced the snail's ability to harbor *S. mansoni* infection.

Maintenance of snails with *Philodina* allowed colonization of the latter on the surface of snail shells. Free rotifers were seen traversing the surface of the snail's shell and occasionally entering into the mantle cavity. Likewise, a similar performance of copepods invading snails was previously noticed by investigators; copepods-aided by their vigorous movements-were able to penetrate through the surface epithelium of the snail's body wall and invade the underlying tissues[32]. In another study, it was noticed that attachment of rotifers to could induce a hole in the snail's umbilicus[21]. These two studies may explain why the above dynamically motile organisms have the ability to invade the tissues of snails.

After two weeks of maintaining SG I-B snails with rotifers, folded globular forms of tissue-entrapped rotifers were histologically detected. In contrast, snails in the SG I-A didn't show any proof of invasion into their tissues. The presence of rotifers in a globular form is mostly assumed to be because they hardly lend themselves to being fixed in extension, due to the effect of dryness caused by the fixatives used in staining procedure[27]. Rotifers are known to have a remarkable ability to survive dryness through a process known as anhydrobiosis, where they resist environmental adverse conditions by contracting into an inert form and losing almost all their body water[33]. Apparently, a state of tolerance occurred between the snail and the invader rotifer, because it didn't induce any detectable host response. Co-adaptations within the snail host have been suggested in different snail species[11].

Examination of the SG I-B snails after four weeks revealed that some hemocylic reactions started to grow around the rotifers entrapped within their tissues. After six weeks, degenerating rotifers were heavily encapsulated by granuloma-like structures of hemocytes. Degeneration of rotifers at this stage was further verified by the loss of their viability using the dye exclusion test, as compared to examinations done at the two preceding time points. This kind of delayed-developing resistance may represent a sort of host defense mechanism[34].

Apparently upon establishment of rotifers in the snail, a remarkable decline in the snail's glycogen content was noticed, as compared to the uncontaminated snails (SG I-A). Nevertheless, this didn't cause any lethal effect on the snail. This is most probably because glycogen is used to a much lesser extent than lipids to maintain the metabolism of gastropods, thus is not of such significant importance as a reserve[35]. The pathological impact of rotifers isolated from snails has been reported to affect egg masses and newly hatched snails, rather than mature snails[22]. It was reported that *Chaetogaster* metazoan could protect B. *glabrata* against infection with *S. mansoni* without interfering with the snail growth or fecundity[11].

In the present study, infection with *S. mansoni* was successfully established in SG II-A snails, as proved by the histological appearance of well developed cercariae inside the snails, along with the remarkable cercarial output throughout the four weeks shedding duration. Two types of swimming were noticed in cercariae shed from these snails without any proof of attachments; Cercariae swam in a prompt linear progressive and zigzag motion or with temporary interruption by pauses followed by random motions. This represents the normal *S. mansoni* cercarial behaviors; typically known to swim to the surface of water, rest momentarily by lying quiescent before resuming their swimming. By resting for a while, cercariae are therefore adapted to store their energy in order to maximize their chances to encounter their specific definitive host species[36]. Cercarial swimming behavior was studied in many species and the enormous diversity of movement patterns and responses to environmental and host cues was noticed[36,37]. The normal swimming attitude of cercariae is belonging to SG II-A accounts for the shown successful infectivity of all mice in the present study, with the absence of any remaining non-penetrating cercariae at the end of the infectivity experiment in this subgroup.

SG II-B snails were contaminated with rotifers two weeks prior to exposure to *S. mansoni* miracidia. Four weeks post infection, histological examination of snails belonging to the SG II-B subgroup revealed several profound changes; the

first was the presence of a host tissue reaction of hemocytes enclosing degenerated rotifers, associated with diminution of snail's glycogen. It is well known that rapidly developing cercariae depends on the snail's glycogen stores[35]. Hence, it seems that rotifers entered the snail tissues and consumed the host's glycogen and this is assumed to have hindered the proper establishment of *S. mansoni* parasites within the snail tissue, as proved by the presence of few developed cercariae together with some malnourished degenerated ones. Presumably, rotifers affected the levels of the snail's energy reservoirs, which eventually might have adversely affected reproduction, growth and survival of the parasite within the snail host. This accounts for the sharp decline in the cercarial yields along the four-weeks of shedding in SG II-B snails compared to those of SG II-A. The impact of rotifer contamination on the numbers of cercariae emerging from the snails has been formerly approved[16,21].

Cercariae released from SG II-B snails didn't exhibit any attachments along the four studied shedding durations. Nevertheless, they showed signs of slight abnormal swimming manners, as compared to SG II-A. They paused with either intermittent spins or irregular random motions. Such lazy cercariae were most probably developed weakly. Their malnourishment could have resulted from competition with entrapped rotifers for the snail's glycogen. Accordingly, the poor cercarial emergence together with the lazy cercarial swimming observed with cercariae shed from SG II-B snails, could account for the decline in the infectivity rates and the existence of non penetrating cercariae seen in this subgroup, as compared to SG II-A. Learning to distinguish normal from abnormal cercarial behavior is very important, since abnormal cercarial behavior likely pinpoints problem areas that might adversely affect infectivity[36,37]. The most influential anti-schistosome effect of the rotifer infestation is known to be the restriction of cercarial motility, which was subsequently accused of the decreased penetration of skin by rotifer contaminated-cercariae[16,21].

SG II-C snails have been exposed to *S. mansoni* miracidia, and then were contaminated with rotifers two weeks later. Examination of their tissue sections on the fourth week post infection revealed the presence of rotifers, yet no noticeable host response. This coincided with the presence of many well developed cercariae together with some degenerated ones. Apparently, the two weeks interval-lapsed before rotifers contamination-allowed the establishment of infection within the snails as nothing competed with the rapidly developing *S. mansoni* parasites on the snail host's glycogen stores[35]. We assume that upon invasion of the snail tissues with rotifers, the latter started to consume a portion of the snail's glycogen at the expense of the growing parasites. This might have led to the degeneration (of some cercariae) witnessed on histopathological examination. This assumption could explain the moderate reduction of the cercarial output from SG II-C snails compared to the more pronounced reduction observed in the SG II-B. In the latter subgroup, few parasites were allowed to develop from the start, whereas in SG II-C, a considerable number of cercariae seem to have had the chance to primarily develop, yet a portion of them later degenerated because of the rotifers invasion.

Cercariae emerging from SG II-C snails exhibited remarkable behavioral alterations, which were notably variable over the whole shedding period. In the first couple of weeks, cercariae attained the worst attachment attitudes (mutual head-tail attachments, attachment of the heads with stationary curved bodies /or coiled tails and heads attached to stationary tails). This coincided with most lethargic swimming patterns of free cercariae (stationary pauses and pauses with either intermittent spins or irregular random movement). Although the cause of such attachment behavior is uncertain, yet a relationship between rotifers and the existence of cercarial aggregations has been suggested[16]. Cercariae of schistosomes possess tails with longitudinal striated muscles extending beneath the tegument and used by cercariae to swim in their journey searching for the definitive host. Glycogen stores inside cercarial tail provide a non-renewable energy source, which is depleted during active swimming[36]. Cercarial swimming behaviors are known to be influenced by several stimuli; including intensity of light, chemical signals produced from the host and biotic pressures (for example competition with other organisms for common nutritional resources within the host)[38]. Since current data pointed out that rotifers consume a portion of the snail's glycogen, we think that rotifers would also compete with cercariae for their non-renewable glycogen reserves, hence resulting in attachment of some shed cercariae. In this work, cercarial attachment coincided with their failure to accomplish any animal infectivity, coupled with the presence of non-penetrating cercariae (cercariae failed to penetrate the mouse skin and cause the infection). Cercarial activity is known to relate to infectivity[36]. Conversely, cercarial attachment was shown to negatively correlate to infectivity to the definitive host, since altered cercarial behavior was shown to limit or completely inhibit their skin penetration capability[30]. Time spent attached is likely to represent an approach of energy conservation in the absence of host cues[38].

The above mentioned alterations of SG II-C cercariae shown in the first two weeks of shedding coincided with the entrapped non-degenerated viable rotiferS. rotifers, most probably released active byproducts that directly influenced the cercarial swimming performance and consequently their infectivity. They were accused of being one of many factors that are known to be able to reduce the infective indexes under field environment, as well as laboratory-adjusted conditions[8]. This has been attributed to the rotifer's ability to emit small molecular weight components, whose effect could extend to cause reversible paralysis of *S. mansoni* cercariae[16].

On the later two weeks, cercariae released from SG II-C performed slightly better than the preceding couples spent

weeks on their bed; attachments were seen through the cercarial head, yet tails can be moved freely. On the other hand, free cercariae paused with intermittent spins or random movements, rather than swimming in a straight line or zigzag patterns. We assume that this improved cercarial performance may be due to the snail host tissue response against the degenerated non-viable rotifers that led to reduction of release of active byproducts. This may justify the partial retention of infectivity powered by the cercariae in the later two weeks of shedding. A previous research reported that normally active cercariae showed abnormal motility when they were placed in rotifer conditioned water, from which the rotifers had been filtered out. Therefore, the observed anti-schistosome effect may not have resulted from the direct physical contact of cercariae with rotifers in snails, but rather the presence of some factors produced by the rotifers or related to their infestation of the snails[16].

Comparison of the data obtained in the present study affirmed the evident impact of *Philodina* rotifer invasion into *Biomphalaria* snails on retarding the development and growth of *S. mansoni* inside the snails. We showed that exposure of snails to rotifers before or during establishment of the parasite within the snail tissues, adversely affected the progress of cercarial development within the snails. This was found to have an un-doubtable influence on altering cercarial emergence, activities and consequently suppressing their infectivity power. We believe that it would be worthwhile to seriously consider rotifers; such priceless highly reproducible organisms, in future biological control strategies of schistosomiasis, which continues to be a parasitic scourge of humanity. Being easier, cheaper, safer and more promising than other schistosome control methods and since there is a high degree of specificity of schistosomes to their intermediate snail hosts, the concept of snail control has now gained a considerable interest,

Further work is currently being conducted to evaluate the effect of rotifers on the maturation of penetrating cercariae within the definitive host. The current results from the adverse impact of rotifers on energy stores of snails will be verified by conducting supplementary biochemical analysis of the carbohydrate levels in rotifer-exposed versus control snails.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Schistosomiasis is an important snail-borne parasitic disease of humans and other mammalian hosts, caused by trematode belonging to the genus *Shistosoma*. This disease is considered endemic with important socioeconomic and public health impact in many developing countries like Africa, Asia and South America.

Research frontiers

The study was conducted in order to verify the impact of internalization of rotifers *Philodina* in the tissues of snails, as important biological agents to prolong the life cycle of snail (one of the most important etiological agents of schistosomiasis).

Related reports

Scientific literature showed that the influence of contamination with *Philodina* in trematoda infected snail tissues have already been studied, but information's with special emphasis on internalizations of rotifers in snail tissues two weeks before and after exposure to *S. mansoni* miracidia is scanty.

Innovations and breakthroughs

The authors clearly demonstrated that the internalized rotifers (*Philodina*) within the snail tissues two weeks before and after exposure to *S. mansoni* miracidia, were reflected in emergence, activity and infectivity of trematoda.

Applications

Data presented in the current study indicate the possible future application of highly reproducible organisms like rotifers in biological control of schistosomiasis.

Peer review

This is a complex study with particular emphasis on the effect of rotifer internalization into the snail tissues during the development of schistosomes as an important human pathogen. The premise is sound and the results are interesting.

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