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Original Article

Viability Analysis of *Biomphalaria glabrata* Hemocytes During *Schistosoma mansoni* Infection And Glyphosate-Based Herbicide Exposure

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Abstract

Pesticides are chemical agents that have a range of harmful effects on human health and the environment. They can cause drastic changes in natural communities, such as macroinvertebrates, plankton and fish. Snails, including *Biomphalaria glabrata*, are often present in aquatic communities and have multiple roles in limnic ecosystems. *B. glabrata* is an intermediate host of several species of helminths of medical and veterinary importance, such as *Schistosoma mansoni*, the etiological agent of schistosomiasis. The original Roundup® herbicide can affect snails and directly affects *S. mansoni* hemocytes, cells that act in the snails' immune defense. Here we analyzed the effect of herbicide exposure on *B. glabrata* hemocytes divided into four groups: control group, infected-only group, treated-only group and infected+treated group. For this, flow cytometry and Neubauer-chamber counting were used to determine the morphology, viability and lectin expression profiles of hemocytes. We observed that the group infected by *S. mansoni* and treated with herbicide had a higher concentration of dead hemocytes in relation to the other groups. The treated group showed similar results to the control group, suggesting that the herbicide (Roundup™) alone does not interfere with the snails' immune system. Regarding cellular-morphological-characterization analysis, hyalinocytes were the cells most commonly found in all groups studied. These findings suggest that *S. mansoni* infection and exposure to pesticides directly the immune system of the snails, stimulating the production of hemocytes, especially hyalinocytes, which have a high phagocytic power to quell the infection, but with toxic effects on the snail.

Key-words: Mollusks, flow cytometry, pesticide, hemolymph, morphology, helminths

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INTRODUCTION

Schistosomiasis is an acute and chronic parasitic disease caused by blood flukes (trematode worms) of the species *Schistosoma mansoni*. Estimates show that at least 236.6 million people in the world required preventive treatment in 2019. Considered a neglected tropical disease by the WHO (2022), it is present in 78 countries in tropical and subtropical regions, and mainly affects people without access to basic sanitation and piped drinking water. The distribution of this disease and others caused by helminths of veterinary importance is directly related to the presence of snails of the genus *Biomphalaria spp*., especially *Biomphalaria glabrata*, the species most susceptible to infection (Lu et al., 2022). These members of aquatic communities have multiple roles in aquatic ecosystems. They are primary consumers and decomposers and serve as the main source of food for a variety of vertebrate and invertebrate predators (Gustafson et al., 2015). Given their importance in the nutrient cycle, food webs and transmission of parasitic diseases, it is important to understand the impacts that herbicides have on them.

The internal defense of snails is composed of soluble components of the hemolymph (fibrinogen-related proteins) and hemocytes, which have phagocytic properties (Borges, 2005; Lockyer et al., 2007 Cavalcanti et al., 2012). Non-self-recognition and subsequent immune activation is mediated by lectins, initially found as humoral factors or present on the surface of hemocytes (Schultz & Adema, 2017). The circulating hemocytes in the genus *Biomphalaria* consist of at least two cell subpopulations: hyalinocytes and granulocytes. However, the presence of blast-like cells has also been reported in *B. glabrata* and *B. straminea* (Barrocco et al., 1993; Cavalcanti et al., 2012; LoVerde et al., 1982). These subpopulations have variable morphology and enzyme contents, exhibiting different adhesion and phagocytosis characteristics (Van der Knaap&LoKer,1990; Matricon-Gondran & Letocart, 1999). The density, function and variety of hemocytes are stimulated by several factors, such as parasites, pathogens and xenobiotic components, including pesticides (Russo & Lagadic, 2000 Mohamed, 2011). In this context, the freshwater snail *B. glabrata* has been used as a good bioindicator, due to its well-known biology, its ease of handling in the laboratory; and the good sensitivity to physical and chemical agents, including pesticides.

In Brazil, the glyphosate-based herbicide N-(phosphonomethyl)-glycine acid (Roundup® Original (Monsanto do Brasil Ltda., Brazil; registered with the Ministry of Agriculture under no. 00898793), is widely used in agriculture for weed control. This herbicide acts on the enzyme system of plants, inhibiting the essential pathway of shikimic acid (Schönbrunn et al., 2001; Amarante Jr et al., 2002). Due to its high solubility in water, this herbicide can affect non-target organisms, such as fish (Lushchak et al. 2009; Lopes et al., 2022) and amphibians (Lanctôt et al., 2014; Navarro-Martín et al., 2014).

Helminth free -life stages (Monte et al., 2016; Monte & Maldonado Júnior, 2017) and snails (Gattás et al., 2020; Zieritz et al., 2022) are affected by glyphosate, causing biochemical changes related to detoxification mechanisms, leading to a decrease in the activity of specific enzymes that play important roles in agrochemical detoxification, making mollusks more sensitive to environmental contaminants (Iummato et al., 2018). Roundup is used to control weeds in more than 160 countries (Grossman, 2015). In addition to glyphosate, the active ingredient, Roundup® contains other substances, including surfactants, some of them extremely toxic to the environment(Diamond et al., 1997).

In the literature, there are few studies on the implications of the use of agrochemicals, especially when evaluating the transmission cycle of *S. mansoni* with the intermediate host snails. These studies have mainly reported the effect of pesticides on snails in continental and marine waters. When evaluating the limbic mollusk *Corbicula fluminea* exposed to Roundup, Santos and Martinez (2014) observed that this herbicide interfered in the anti-oxidant defenses, leading to lipid peroxidation. Genetic changes have also been observed in marine mollusks of the species *Crassostrea gigas* when exposed to pesticides (Tanguy et al., 2005).When evaluating *B. glabrata* exposure to glyphosate, it was possible to observe that the chemical agent caused cellular toxicity and endocrine disorders (Omran & Salama, 2016), while when evaluating the pesticide known as atrazine in this same snail, a significant effect on growth, reproduction and survival was observed (Gustafson et al., 2015). In addition, changes resulting from exposure to pesticides for a short or prolonged period directly and/or indirectly affect the biological cycle, in the evolutionary forms of other parasites and also in the defense cells of the intermediate host, *B. glabrata* (Monte et al., 2016, 2019). We therefore investigated the effect of exposure to the glyphosate-based herbicide Roundup® Original on *B. glabrata* snails, infected or not by *S. mansoni*, to evaluate changes in the snails' immune defense system and consequent parasitism.

MATERIALS AND METHODS

Maintenance of *Biomphalaria glabrata* and *Schistosoma mansoni* under laboratory conditions.

The *B. glabrata* snails used in this study was collected in the municipality of Sumidouro, Rio de Janeiro State, Brazil (22°02`46''S, 42° 41'42''W), and were subsequently kept in the laboratory according to the biosafety and breeding standards previously established by the Laboratory for Biology and Parasitology of Wild Mammal Reservoirs (LABPMR), Oswaldo Cruz Institute (FIOCRUZ), in the city of Rio de Janeiro, as described by (Monte et al., 2019). In brief, adult snails with approximately 8–12mm shell diameter were maintained in 2 L plastic aquariums with a layer of clay mixed with calcium carbonate (10:1) and dechlorinated water,

which was changed every 15 days. They were fed with small pieces of fresh lettuce. Water temperature was maintained at 25±1 °C (Faro et al., 2021) The biological cycle of *S. mansoni* was maintained in Swiss mice according to bioethics and biosafety standards of the Experimental Laboratory in Lauro Travassos Building of FIOCRUZ, with the authorization of the Animal Use Ethics Committee (CEUA L – 023/2015).

Experimental infection

The snails were infected with *Schistosoma mansoni* by recovering the miracidia from the eggs present in the feces of mice infected with cercariae, using Lutz's method (Neves, 2016). Afterward, the snails each were infected with 10 miracidia individually and exposed to artificial light for 2 hours to induce cercarial emergence.

Herbicide exposure experiment

The solutions of herbicide used in the experiment were prepared from the Roundup® Original (480 g/L isopropylamine salt N- (phosphonomethyl) glycine; 360 g/L equivalent acid N-(phosphonomethyl) glycine; 684g/L inert ingredients, including the surfactant polyoxyethylene tallow amine – POEA (Monsanto do Brasil Ltda, Brazil). Serial herbicide solutions were formulated in type II water (360 mg/L (0.1%) - 36 mg/L (0.01%) to determine the median lethal concentration (LC_{50}), as described (Monte et al., 2019).

Herbicide-treated snails were exposed to 43.2 mg/L (0.012%) of the herbicide solution for 24 hours individually, while the specimens of the untreated group were kept in dechlorinated water for the same period. After that, the snails were dried on paper towels and used in the experiments. The pH varied between 8.2 and 8.5 throughout the experiment, with a photoperiod of 12/12 hours.

Experimental planning

Four experimental groups were employed in the current study: control group (C), composed of uninfected-untreated-snails; (T) uninfected snails treated with herbicide; (I) *S. mansoni*-infected snails without treatment; and (I+T) *S. mansoni*-infected snails treated with the herbicide. Flow cytometry experiments were performed using 10 snails from each experimental group (40 snails total) to assess the hemocyte viability, morphology and PNA-lectin profiles.

The hemolymph was collected directly from the pericardial cavity with the help of a 1.0 mL syringe and a 0.70x30 mm needle. The snails used in the tests had shell diameter between 8 and 10 mm (Faro et al., 2013).

Hemocyte viability

Live and dead hemocytes were determined as described by Martins-Souza et al. (2009). In brief, aliquots of recovered fresh hemolymph were diluted (1:1) in trypan blue dye (0.5%) and homogenized. Then 10 µL of hemolymph was directly deposited in a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) to determine the concentration of dead cells (trypan blue-stained cells) using light microscopy (Zeiss Observer Z1 microscope; 40X magnification).

Morphological differentiation of hemocytes

First, the hemolymph freshly harvested from each snail was fixed in Carson's Millonig formalin (1:1) to maintain the integrity of the hemocytes. After this fixing, 50 µL of hemolymph was placed directly onto a glass slide and allowed to dry for 24 h at room temperature, and then post-fixed in absolute methanol for 5 min. After the fixative had dried at room temperature, the hemocytes were stained with Giemsa (diluted 2:8 in distilled water) for 5 min and the slides were promptly washed with buffered distilled water (Cavalcanti et al., 2012). Following this, the slides were mounted in Dammar gum with xylene and observed by optical microscopy with a 100X-objective, with immersion oil (Zeiss Observer Z1 microscope). Images were captured with a Zeiss Axio Cam MRc camera and analyzed using the Axio Zen 2 lite software.

Flow cytometry

The hemocytes' morphology, viability and lectin expression profile were determined by flow cytometry, as described by (Monte et al., 2019). Briefly, hemolymph samples were collected from 10 snails, diluted 1:1 with Chernin Balanced Salt Solution (CBSS), and pooled in 1.5 mL Eppendorf tubes, which were kept on ice during all processes. Three tubes with 200 µL of total hemolymph were incubated respectively with 100 µL of propidium iodate (100 µg/mL PI, Sigma-Aldrich, USA); or 20 µL of 7AAD (Life Technologies); or 5 µL of lectin PNA-Alexa Fluor 488 (Life Technologies, USA), for 20 min at 4 °C for 20 min in the dark. Then the samples were resuspended with 200 µL de CBSS and immediately submitted to flow cytometry. Twenty thousand event acquisitions were performed with a Cytoflex flow cytometer (Beckman Coulter Life Sciences, IN, USA), set to read the fluorescence signals from FITC (PNA), PI and 7-AAD through 525/40 BP, 585/42 BP and 690/50 BP filters, respectively. The acquisition and analyses were done using theh CytoExpert and Kaluza programs, respectively (both from Beckman Coulter, Inc., Brea, CA, USA), using the following gate strategy. First, a FSC-A *vs*. FSC-H dot-plot was created to provide the doublet exclusion (Fig. 1A). After gating for singlet events, three hemocyte subpopulations were defined based on their granularity through an SSC *vs.* FSC dot plot (Fig. 1B). Three regions were created based on

low SSC (channels between 20 and 140; hyalocytes); medium SSC (channels between 150 and 290; granulocytes); and high SSC (channels >300; unknown cells) (Fig. 1B). The viability of each hemocyte subpopulation was evaluated through PI (585/40 BP filter) and 7-AAD (690/50 BP filter) histograms (Figures 1C, D and E; and Figures 1F, G and H, respectively). Simultaneously, frequencies of PNA-expressing hemocyte subpopulations were evaluated through PNA histograms (525/40 BP filter) (Figure 1 I, J and K). Four replicates of flow cytometry experiments were performed.

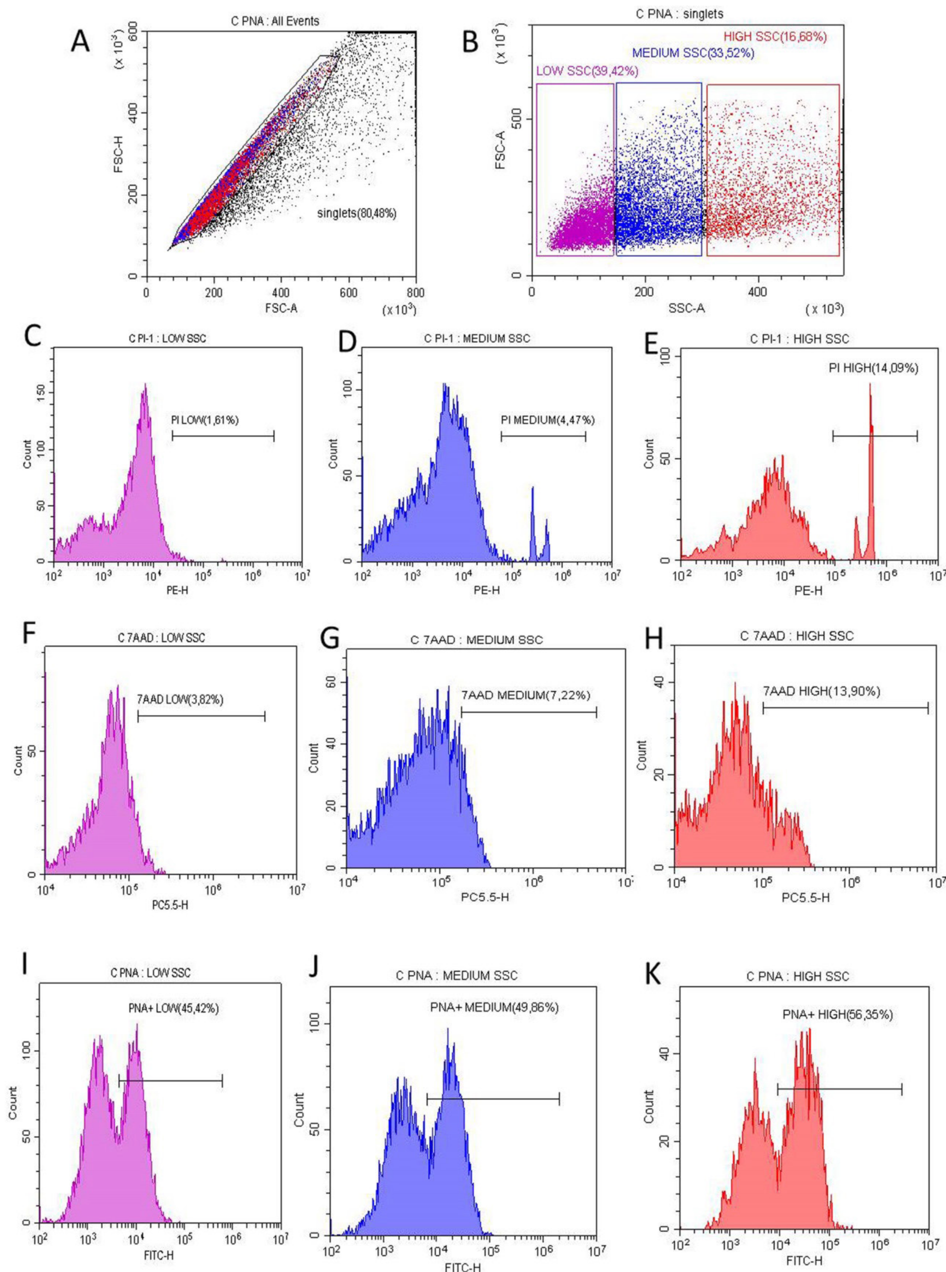

Figure 1. Flow cytometry analysis gate strategy protocol for determination of frequencies of dead (PI+ or 7AAD+) or PNA+ hemocytes from control group of *Biomphalaria glabrata* snails. (A) Dot plot FSC-A vs. FSC-H, for doublet exclusion (singlet gate); (B) Morphology dot plot (SSC-A vs. FSC-A) where three surrounding regions were created: low SSC (channels between 20 and 140); medium SSC (channels between 150 and 290); and high SSC (channels >300) for subpopulations; (C, D and E) fluorescence histograms representing PI incorporation (585/42 BP filter) staining gated on low, medium and high SSC subpopulations, respectively; (F, G and H) fluorescence histograms representing 7AAD staining (690/50 BP filter) gated on low, medium and high SSC subpopulations, respectively; and (I, J and K) histograms representing PNA staining (525/40 BP filter). Data are representative of four independent experiments.

Statistical analysis

The results obtained from the cell viability analysis (Neubauer chamber), the morphological differentiation of the types of hemocytes and the analysis of flow cytometry were expressed as mean ± standard error. All data were tested for normal distribution using the Shapiro-Wilk test, and one-way ANOVA parametric tests and the Kruskal-Wallis nonparametric test were performed followed by their respective post-tests of multiple comparisons. Values with $p < 0.05$ were considered statistically significant. The statistical analysis was performed with GraphPad Prism v. 7.0 (San Diego, CA, USA).

RESULTS

Differentiation of hemocyte types in the hemolymph of *B. glabrata*

The hemocytes have been classified by scanning electron microscopy in three different types according to their sizes and morphological characteristics (Cavalcanti et al., 2012).

When analyzing the morphological parameters through flow cytometry, we observed three different granularity-defined hemocyte subpopulations in the side scatter (SSC) versus forward scatter (FSC) dot-plot, denominated low, medium or high. All flow cytometry analyses were respectively gated on these defined regions.

According to the flow cytometry analysis, the round blast-like cells (with nucleus occupying almost the entire cell), presented the lowest SSC; the hyalinocytes, the most abundant hemocyte type, with centralized or peripheral nucleus, showed intermediate SSC; and the granulocytes, characterized by having several electron-dense granules distributed in the cytoplasm, had low SSC. These granules differed in number and were distributed either on the periphery or at the center of the cell, presenting larger SSC (Figure 2).

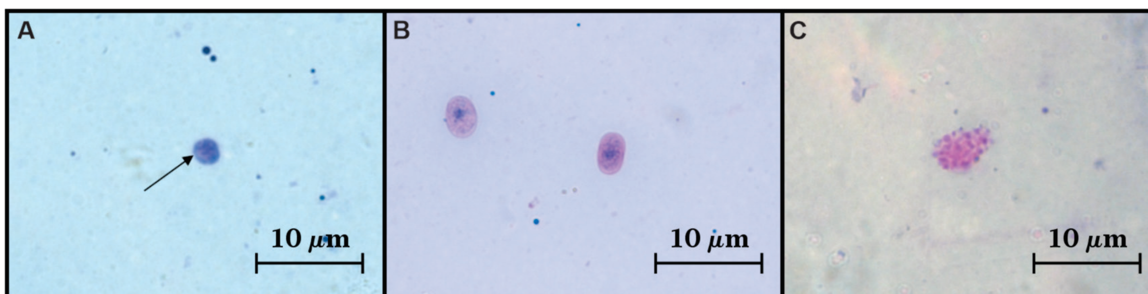

Figure 2: Morphological differentiation of hemocytes from ten-*Biomphalaria glabrata* specimens from each experimental group (n = 40): (A) Blast-like cells; (B) Hyalinocytes and (C) Granulocytes, microphotography using a Zeiss Stemi SV6 microscope (100× magnification).

Hemocyte viability by trypan blue exclusion

When analyzing the viability of hemocytes by trypan blue exclusion, we observed two-fold frequency of dead cells in groups (T) and (I) in relation to the control (Figure 3).

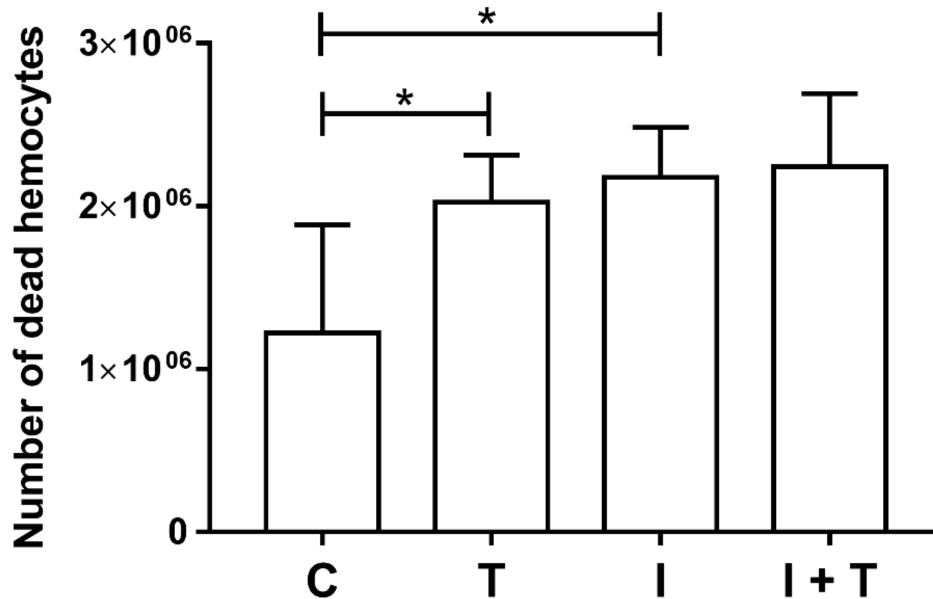

Figure 3: Analysis of the absolute number of dead hemocytes from *Biomphalaria glabrata* specimens (n=10) from each experimental group (total n=40): Control (C); Treated (T); Infected (I); Infected and Treated (I+T), using the Trypan blue exclusion method, by Neubauer chamber observation. Data are represented as mean ± standard error. Statistical analyses were performed by the nonparametric Kruskal-Wallis test, and the results were considered significant with p< 0.05 compared to the control. *(P<0.05).

Cell viability by flow cytometry

With regard to the viability of hemocytes within each studied group, we observed in the flow cytometry experiments using PI a higher frequency of dead cells in the I+T group than in the T group, in the low SSC subpopulation (p=0.008) (Figure 4A) and high SSC subpopulation (p=0.048) (Figure 4C). Similarly, using 7-AAD, we found a higher percentage of dead hemocytes in the I+T group compared to the control group in the low SSC subpopulation (p=0.020), and in the I+T group compared to group T (p=0.040) (Figure 4D). These increases in hemocytes using 7-AAD in group I+T were also seen in the mean SSC subpopulation compared to group I (p=0.028) (Figure 4E) and between group I+T with group T in the high SSC subpopulation (p=0.031) (Figure 4F).

Regarding the frequencies of PNA^+ cells, we observed high frequencies of all hemocyte subpopulations in all groups, with no difference between them (Figures 4G, 4H, 4I).

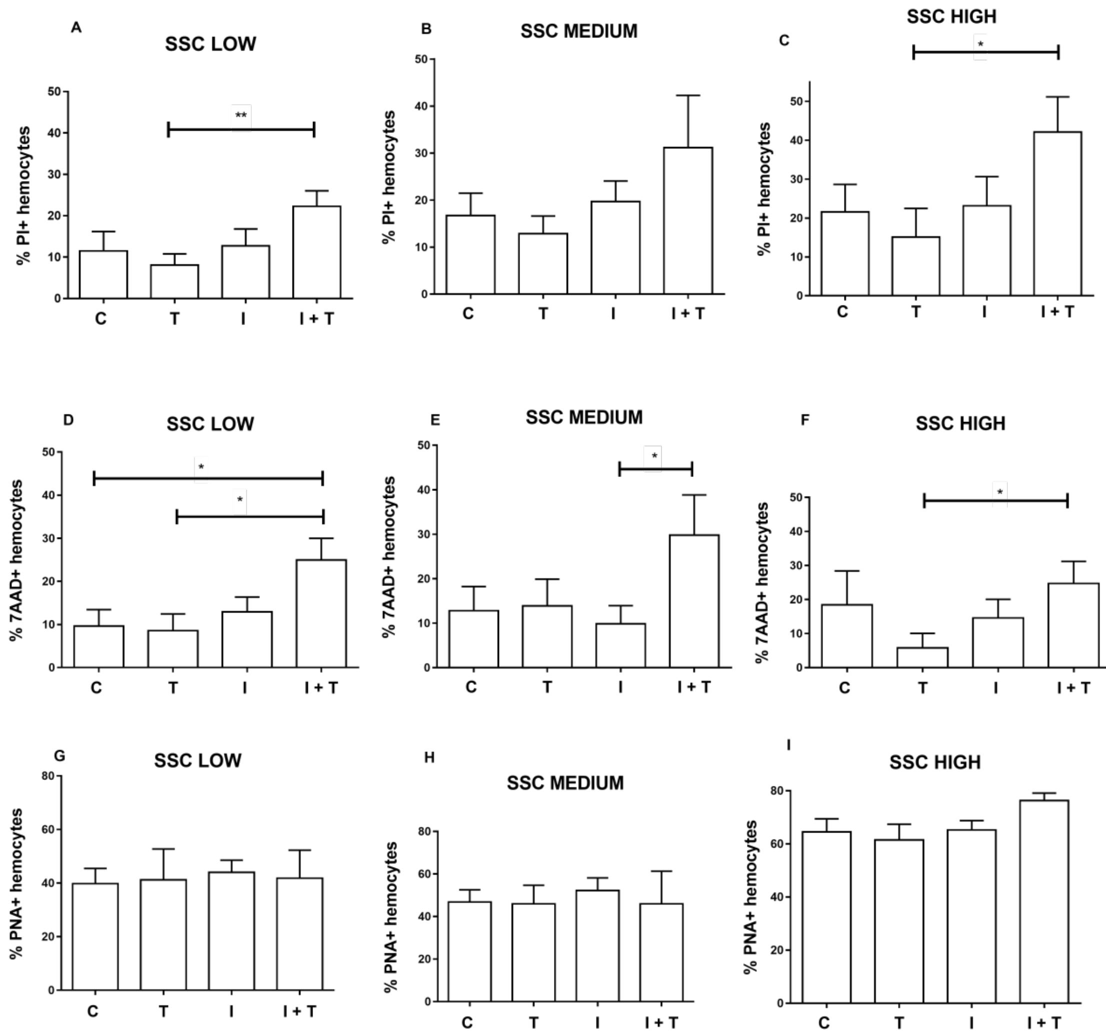

Figure 4: Flow cytometry analysis of three hemocyte subpopulations according to side scatter variations (SSC) – low SSC, medium SSC and high SSC. Each bar represents the percentages (%) of dead hemocytes (positive PI or 7-AAD incorporations) in the four groups studied: Control (C); Treated (T); Infected (I); Infected and Treated (I+T). Data are presented as mean ± standard error of dead hemocyte subpopulations. The significance of differences in the percentages of dead hemocytes between cell subpopulations was measured with the nonparametric Mann-Whitney test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Cell frequencies by flow cytometry

When estimating the absolute number of hemocyte populations with low SSC (blast-like cells), we observed higher cell numbers in the infected+treated (I+T) group and the group that received only treatment (T) when compared to the control group (C) (p<0.0001; p=0.00022, respectively). The same was observed in group I, but with lower significance (IxC, p=0.0130; IxT, p=0.0208, respectively). In populations with intermediate SSC, we observed differences only when comparing the T and I+T groups (p=0.0136). Furthermore, when we analyzed the population with high SSC, group T (p=0.0325) and I+T (p<0.0001) showed significant differences compared to the control group (C) (Figure 5).

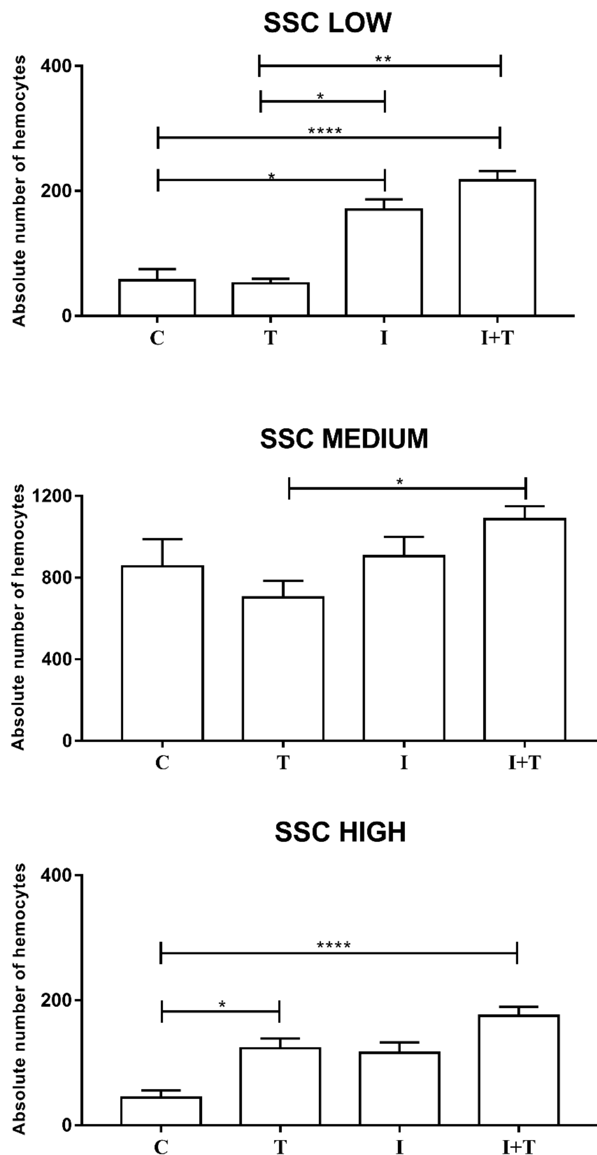

Figure 5: Graphs representing the absolute number of different types of circulating hemocytes from *Biomphalaria glabrata*, according to the SSC characteristics. (A) low SSC; (B) medium SSC; and (C) high SSC. (C) control group; (T) Roundup-treated group; (I) S. Mansoni-infected group; and (I+T) S. Mansoni-infected-Roundup-treated group. Statistical analysis was performed by nonparametric Mann-Whitney test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

DISCUSSION

Mollusks and other invertebrates have a defense system that acts against external factors that can affect these animals, such as environmental pollutants, xenobiotic components, microorganisms and parasites. As part of this defense system, mollusks have cells that protect against exposure to various infectious (Russo& Lagadic, 2000; Martins-Souza et al., 2009). Our data indicate that hemocytes were most abundant in the infected + treated group, a similar result to that of Friani (2017), who analyzed circulating hemocytes of *B. glabrata* through Neubauer chamber counting when infected or not by *S. mansoni* and exposed to the latex of *Euphorbia milii* (synplendens) var. *hislopii* (Euphorbiaceae).

(Mohamed, 2011), analyzing the amount of circulating hemocytes of *B. alexandrina* by Neubauer chamber observation after exposure to Roundup for six hours, one, three and seven days, found similar values between the treated and infected groups and the group treated on the seventh day (39.8 and 37.9, respectively). When analyzed only the infection, they found a hemocyte infection rate of 4.77% in the infected group and 14.25% in the control group. These findings differed from ours, probably due to the fact that those authors did not observe the hemocytes in the patent period, in which the immune system has already been fully activated.

The flow cytometry technique used in this work proved to be more refined due to the number of samples that were processed in a single analysis, including separation of the cell

populations present in the hemolymph. Here, when analyzing cell death through flow cytometry, we observed the highest percentage in the I+T group, using both markers 7AAD and PI. Similarly, the highest percentage of apoptosis in *B. alexandrina* hemocytes was observed in the group infected by *S. mansoni* and exposed to Roundup (88.92%), followed by the treated group (79.1%), the infected group (42.8%) and control group (2.5%) (Mohamed, 2011).

In a recent study, Monte et al. (2019) observed changes in *B. glabrata* hemocytes infected with another species of trematode, *Echinostoma paraensei*, and subsequently exposed to glyphosate-based herbicide. The results indicated that the infected and treated group also showed higher frequencies of dead hemocytes compared with the other experimental groups, in line with our data. These findings suggest that regardless of the trematode species, the association of these two factors can influence the intermediate host-parasite relationship.

Cavalcanti et al. (2012) and Friani et al. (2017) demonstrated that hyalinocytes were the most common cell type in the hemolymph of *B. glabrata*, followed by blast-like cells and granulocytes, which were also found in the present study in *B. glabrata*, where hyalinocytes were the most common cell type in all groups studied, with the greatest phagocytic power (Carvalho et al., 2008), helping to fight infection and also exposure to the herbicide, corroborating previous results (Monte et al., 2019). Those authors reported similar results analyzing hemocyte types of *E. paraensei* infected-*B. glabrata*. The difference between our findings and theirs was the fact that the treated group presented granulocytes as the second most numerous cells, followed by the blast-like cells. This may be related to the particularity of each trematode species used in the studies.

With respect to morphological differentiation, Mohamed (2011) observed on the seventh day after infection that hyalinocytes were the most numerous cells in all groups analyzed. Again, there were some differences in comparison with our results, especially in infected + treated groups. This was probably due to the fact that Mohamed (2011) analyzed snails in the pre-patent period of infection, which includes the period before release of cercariae by the snail. Another difference was the concentration of Roundup used the two studies (0.012% by us and 0.02% by Mohamed. These two factors possibly explain the low presence of hemocytes in the infected group and the high concentration in the treated group.

Martins-Souza et al. (2006) used FITC-conjugated ConA, PNA, SBA and WGA lectins to label hemocytes of *S. mansoni*-infected *B. glabrata* by fluorescence microscopy, followed by Neubauer chamber counting 5 days after infection. They observed that after labeling with PNA lectin in the first 24 hours, there was an increase in the amount of hemocytes, but from 70 hours onward the number of hemocytes remained constant. In this respect, they found that small cells were in greatest quantity, followed by medium cells and large cells, which had the smallest number. The amount of hemocytes found of each cell type corroborates the data observed in this study.

Other authors have analyzed the immune system of mollusks when infected with *S. mansoni*, differentiating mainly the types of hemocytes that are found in the hemolymph of *Biomphalaria* snails. Martins-Souza et al. (2009) analyzed the hemolymph of *B. glabrata* infected and not infected by *S. mansoni* with ethidium bromide dye and acridine orange dye. In snails infected 40 days ago there was a significant decrease in the small hemocytes when compared to the cells of the uninfected ones, and there was an increase in the number of medium and large hemocytes. In our study, we also observed an increase in the medium (hyalinocytes) and large (granulocytes) cells, possibly because hyalinocytes were the cells presenting the greatest phagocytic power against pathogens and external agents.

The control and treated groups presented similar cell death results, demonstrating that herbicide exposure alone causes less damage to the snail defense system. When analyzing the treatment and the infection separately, we observed that the infection caused greater damages to *B. glabrata*.

Herbicides such as the Roundup are strong environmental pollutants, affecting snails and *S. mansoni* infection. Therefore, limits should be established for its use near aquatic ecosystems. Changes in the snail defense system can bring about changes in the trophic chain relationship of these aquatic organisms in the ecosystem. Rohr et al. (2008) demonstrated that herbicides applied to crops can favor an increase in the food resources of snails, allowing population growth. Snails can also present a higher survival rates due to the fact that herbicides affect snail predators such as crayfish, thus allowing a greater increase in the number of snails due to the lack of predators, influencing the composition of aquatic ecosystems and communities (Halstead et al., 2014, 2018).

CONCLUSION

The results obtained in this work showed that *B. glabrata* was more affected when concomitantly infected with *S. mansoni* and exposed to a sublethal concentration of original Roundup®. This herbicide, when applied in or near aquatic environments, can alter the survival of snails by contaminating the environment where snails and other aquatic organisms are present, altering the infection by *S. mansoni* and other parasites. This interaction affects the immunological response of these snails, causing an increase in defense cells, as demonstrated by Neubauer chamber counts through flow cytometry and morphological differentiation of the hemocytes. These immunosuppressive methods enable the interaction of helminth infection with exposure to herbicides, triggering a snail suppressant response.

ACKNOWLEDGMENTS

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AUTHORS' PARTICIPATION

VSM: investigation, methodology, formal analyses, data curation, writing – original draft; **TQC**: flow cytometric data acquisition and analysis, data curation; **RFN**: flow cytometric data acquisition and analysis; **TCBS**: methodology; **MCV:** resources; **TCCM:** methodology, critical analysis, **ALB:** flow cytometric data acquisition and analysis, critical analysis, writing – review and editing; **MJF:** conceptualization, critical analyses, supervision, writing-revision and editing, project administration.

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