

Special Article - Herbicides

Changes in Hepatic Metabolism of *Rattus norvegicus* Infected to *Angiostrongylus cantonensis* (Nematoda) and Exposed to Glyphosate-Based Herbicide

Braga BV^{1,2}, Garcia JS^{2*}, Simões RO³, Silva JSP⁴, Castro LS⁴, Silva CH⁵ and Maldonado Júnior A²

¹Programa de Pós-Graduação em Biologia Parasitária, Instituto Oswaldo Cruz-FIOCRUZ, Rio de Janeiro, RJ, Brazil

²Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios, Instituto Oswaldo Cruz-FIOCRUZ, Rio de Janeiro, RJ, Brazil

³Departamento de Parasitologia Animal, Universidade Federal Rural do Rio de Janeiro, RJ, Brazil

⁴Laboratório de Fisiologia das Relações Parasitárias, Departamento de Ciências Fisiológicas, Instituto de Ciências Biológicas e da Saúde da Universidade Federal Rural do Rio de Janeiro, RJ, Brazil

⁵Instituto de Ciência e Tecnologia em Biomodelos-FIOCRUZ, Rio de Janeiro, RJ, Brazil

*Corresponding author: Gracia JS, Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatório, Instituto Oswaldo Cruz-FIOCRUZ, Av. Brazil 4365, 21040-900, Rio de Janeiro, RJ, Brazil

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Abbreviations

ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; ALP: Alkaline Phosphatase; TB: Total Bilirubin; TP: Total Protein; L₃: Third-Stage Larvae; LABPMR: Laboratory of Biology and Parasitology of Wild Mammal Reservoirs; IOC-FIOCRUZ: Oswaldo Cruz Institute-FIOCRUZ; C: Control Group; AI: Acute Infection; CI: Chronic Infection; pi: Post-Infection; IHE: Immediate Herbicide Effect, DHE: Delayed Herbicide Effect; I + E; E+I; CEUA-IOC: Ethics Committee on the Use of Animals of Oswaldo Cruz Institute; ANOVA: Analysis Of Variance

Introduction

The active ingredient glyphosate (N-(phosphonomethyl) glycine) is one of the most used herbicides in the world, in several commercial formulations [1-4]. Glyphosate-based products have been tested in conventional experimental models, such as rats and mice, and the results have confirmed their toxic potential and ability to cause metabolic changes [5-9]. Several helminth species have been used as experimental models in herbicide tests to check their effect on parasite biology and influence on the parasite-host relationship [10-13].

Angiostrongylus cantonensis (Chen, 1935) is an endemic nematode in South and Southeast Asia and the Pacific Islands [14-15], and it is now observed in North and South America, the Caribbean, Africa, and Australia [16]. This wide distribution is particularly due to the presence in these locations of several species of gastropod molluscs

Abstract

Helminth infection associated with exposure to pesticides has received little attention regarding its effect on the human population and on farm and wild animals. The aim of this study was to evaluate the effects a glyphosate-based herbicide on the hepatic and glycemic metabolism of *Rattus norvegicus* (Wistar) infected by *Angiostrongylus cantonensis*. Experimental groups were orally infected with 50 L₃ larvae of *A. cantonensis* and exposed to the herbicide after and before the infection. Biochemical serum analyses were carried out to determine the levels of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Total Bilirubin (TB), total protein, albumin, urea, creatinine, uric acid, glucose and hepatic glycogen. All exposed groups showed an increase in the concentration of glycogen, AST, ALT and TB, the last ones suggesting liver tissue damage. Exposure to the herbicide caused hyperalbuminemia as an antioxidant response to the herbicide. These findings contribute to a better understanding of how glyphosate-based herbicides can change the hepatic metabolism of the vertebrate and to influence the parasite-host relationship.

Keywords: Biochemical hepatic parameters; Experimental Infection; Helminth; Roundup®

susceptible to infection and that act as intermediate hosts [17-19], along with the presence of synanthropic rodents (*Rattus rattus* Linnaeus, 1758, and *Rattus norvegicus* Berkenhout, 1769) as definitive host. Under experimental conditions, infection by *A. cantonensis* can compromise the health of animals, depending mainly on the parasite burden and the animals' immunological condition [20-22].

Humans become infected through ingestion of raw or undercooked foods contaminated with third-stage larvae (L₃) [16,23,24]. However, they are considered accidental hosts because helminths do not develop to the adult stage and are retained in the meninges. This parasite causes eosinophilic neuromeningitis in humans, the main clinical symptom of the infection, which can lead to death if not properly treated. It is thus considered a public health problem in endemic areas [16,25,26].

Based on data on the toxic effect of glyphosate on rodents and helminths, Kelly et al. [27] suggested that animals exposed to this herbicide can become more susceptible to infection due to their lower resistance. To test this hypothesis, we evaluated the effects of the commercial formulation Roundup® on biochemical parameters of Wistar rats (*R. norvegicus*) infected by *A. cantonensis*.

Materials and Methods

Parasites and experimental infection

The strain of *A. cantonensis* used in experimental infections was isolated from the specimens of the snail *Achatina fulica* collected

in the municipality of São Gonçalo (state of Rio de Janeiro, Brazil) (22°49'37"S, 43°03'14"W) in 2014. Since then, the biological cycle has been maintained under experimental conditions at the Laboratory of Biology and Parasitology of Wild Mammal Reservoirs (LABPMR) of Oswaldo Cruz Institute (IOC/FIOCRUZ), using specimens of *R. norvegicus* (Wistar) and *Biomphalaria glabrata* as definitive and intermediate hosts, respectively. For the experimental infection, individual 3-month-old female rodents weighing 260g were infected by orogastric gavage with 50 third-stage larvae (L₃) of *A. cantonensis*, previously recovered from *B. glabrata* specimens, using the sedimentation technique of Baermann & Moraes [28].

Roundup® concentration and rodent exposure

The glyphosate-based herbicide used in the assay was the commercial formulation Roundup® (480g/L isopropylamine salt N-(phosphonomethyl) glycine; 360g/L equivalent acid N-(phosphonomethyl) glycine; 684g/L inert ingredients) produced by Monsanto do Brasil Ltda. The animals were exposed daily, by orogastric gavage, to 500mg/kg body weight, for 15 days [7]. This concentration is considered not to cause adverse effects [29].

Experimental groups

Seven groups of 10 *R. norvegicus* (Wistar) females were formed: 1-control (C): not infected and not exposed, treated with 500µl of dechlorinated water; 2 and 3- Acute Infection (AI) and Chronic Infection (CI) both groups orally infected with 50 L₃ of *A. cantonensis*, in a volume of 500µl of dechlorinated water, and analyzed 15 and 50 days Post-Infection (pi), respectively; 4 and 5- groups exposed daily to the herbicide for 15 days and analyzed those same 15 days (Immediate Herbicide Effect-IHE) and 65 days later (Delayed Herbicide Effect-DHE); 6-infected with 50 L₃ of *A. cantonensis* in 500µL and exposed daily to the herbicide for 15 days after infection (I+E); 7- exposed to the herbicide daily for 15 days and 24 hours after the last administration, infected with 50 L₃ of *A. cantonensis* in 500

µL of dechlorinated water (E+I). These last two groups (E+I and I+E) were analyzed 50 pi (Figure 1).

All animals were fed with autoclaved Nuvilab CR-1 and water ad libitum. They were maintained under controlled conditions of temperature (23 ± 2 °C) and lighting (12h photophase) [30]. At the end of each experiment, rodents were euthanized by an overdose of anesthetics, inoculated intramuscularly on the outside of the quadriceps femoris muscle, using ketamine hydrochloride (200mg/Kg) and xylazine hydrochloride (10mg/Kg) as pre-anesthetics and sodium pentobarbital (250mg/Kg) as anesthetic. All the procedures involving the use of laboratory animals were in accordance with license no. L-004/2019, granted by the Ethics Committee on the Use of Animals of Oswaldo Cruz Institute (CEUA-IOC).

Biochemical analysis

The blood samples were collected by cardiac puncture just after the euthanasia of all animals and were stored in gel and clot activator tubes for serum separation. A dry biochemical analyzer (Vitros 250 system - Ortho-Clinical/Johnson & Johnson) was used for the subsequent biochemical analyses: Aspartate Aminotransferase (AST - EC 2.6.1.1) and Alanine Aminotransferase (ALT - EC 2.6.1.2) activities; serum levels of Alkaline Phosphatase (ALP - EC 3.1.3.1); Total Bilirubin (TB) and Total Proteins (TP), including albumin; kidney function parameters (urea, uric acid, and creatinine); and blood sugar level. In addition, the hepatic glycogen concentration was evaluated at the Laboratory of Physiology of Parasite Relations of Federal University of Rio de Janeiro, following the protocol presented by Garcia et al. [20].

Statistical analysis

The results obtained were expressed as mean ± standard deviation. After confirmation of normal distribution, the data were submitted to one-way Analysis of Variance (ANOVA) followed by the Kruskal-Wallis test to investigate if there were significant differences among

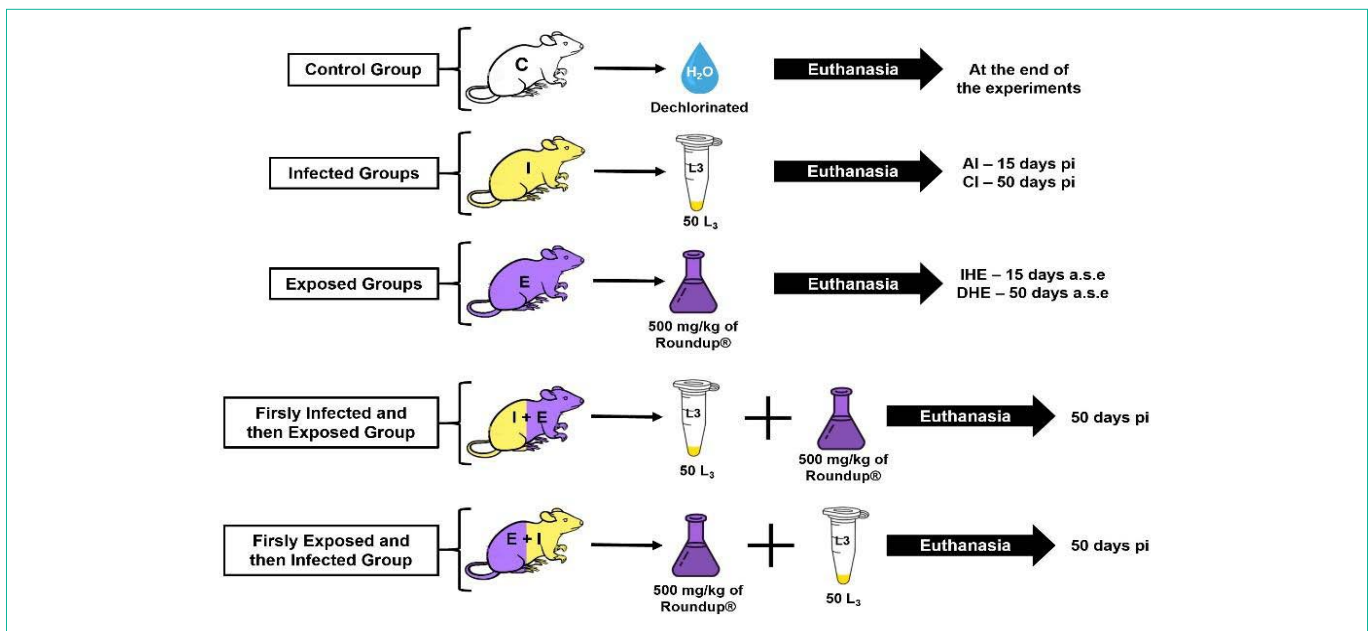


Figure 1: Experimental design-C (Control group); I (Infected groups split into two subsets - AI (Acute Infection) and CI (Chronic Infection)); E (Exposed groups split into two subsets - IHE (Immediate Herbicide Effect) and DHE (Delayed herbicide effect)); I+E (Firstly infected and then exposed group); and E+I (Firstly exposed and then infected group); L₃ = third-stage larvae; pi = post-infection; a.s.e = after the start of exposure; H₂O = water.

the groups. The Tukey-Kramer and Dunn *post hoc* tests were utilized to reveal which groups differed from each other. The Past 3.25 software [31] was used for these analyses and values $P < 0.05$ were considered significant.

Results

Hepatic function

The activity of AST increased significantly in all experimental groups ($P < 0.005$), except for Acute Infection (AI) when compared to the control group (C) (Table 1).

Chronic Infection (CI) produced a significant increase in the activity of ALT in relation to the control group ($P = 0.0001$) and the other experimental groups ($P < 0.005$). The activity of ALT also increased in the Acute Infection (AI) group ($P = 0.01$), immediate and delayed herbicide exposure groups ($P < 0.005$) and infected and exposed groups (I+E: $P = 0.0001$; E+I: $P = 0.01$) in comparison with the control group (Table 1).

The Chronic Infection (CI) promoted a significant increase of ALP serum activity compared to the control group ($P = 0.01$) and the Acute Infection (AI) ($P = 0.004$) group. In contrast, exposure to the herbicide significantly decreased the ALP serum activity as a delayed effect (DHE group) ($P = 0.001$), when compared to the control group ($P = 0.03$) and to IHE group ($P = 0.003$). A decrease in the serum activity of ALP was also observed in the I+E group in comparison with the chronic infection group ($P = 0.02$). The same was observed for the E+I group when compared to the control ($P = 0.03$), chronic infection ($P = 0.0002$) and IHE groups ($P = 0.002$).

The concentration of total bilirubin increased significantly in acute and chronic infection group ($p = 0.01$), in animals only exposed to the herbicide Roundup[®] (IHE and DHE) ($p = 0.02$ and $p = 0.001$), and in the I+E group ($p = 0.003$), when these groups were compared to the control group (C) (Table 1).

Exposure to Roundup[®] promoted hyperalbuminemia in the IHE and DHE groups, compared to the control ($P = 0.03$), while the other experimental groups did not show any significant change in this parameter (Table 1).

The serum concentration of total proteins showed an increase in the CI group compared to the AI ($P = 0.002$) and control ($P = 0.0001$) groups. The same as observed in the DHE group compared to the control group ($P = 0.02$). The I+E and E+I groups presented an increase in the concentration of TP in comparison with the control ($P = 0.001$ and $P = 0.02$, respectively) (Table 1).

The rodents belonging to the CI group presented hypoglycemia when compared to rodents belonging to the AI ($P = 0.02$) and control groups ($P = 0.03$). Both infected and exposed (I+E and E+I) groups presented hypoglycemia when compared to the control ($P < 0.05$) and AI ($P < 0.05$) groups. In contrast, the DHE group presented hyperglycemia when compared to the IHE group ($P < 0.05$) (Table 1).

There were no changes in the parameters used to evaluate renal function (urea, creatinine, and uric acid) (Table 1).

Discussion

In clinical biochemistry, the measurement of AST and ALT enzymes in serum is used to indicate liver tissue damage, since they are found mainly in hepatocytes. The aminotransferases overflow into the blood when there is damage in these cells, increasing the respective serum levels [32]. Both infection by *A. cantonensis* and oral exposure to the herbicide Roundup[®] promoted alterations in the hepatic metabolism of *R. norvegicus*. Garcia et al. [20] observed similar results in Wistar rats infected by *A. cantonensis*, possibly associated with the excretion or secretion products of the larval stages and adult helminths, which can increase the permeability of the hepatocyte membrane and consequently promote the outflow of enzymes or liver-specific cells in the blood [33-35]. Likewise, oral exposure to different concentrations of glyphosate-based herbicides

Table 1: Biochemical analyses (AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; ALP: Alkaline Phosphatase; total bilirubin; albumin; total protein; glucose; Glycogen (liver) - GLY; Blood urea nitrogen - urea BUN; uric acid; and Creatinine), expressed as mean \pm standard deviation ($X \pm SD$) of *Rattus norvegicus* infected to *Angiostrongylus cantonensis* (Nematoda) and exposed to glyphosate-based herbicide.

Parameters	Experimental Groups						
	C	AI	CI	IHE	DHE	I+E	E+I
	$X \pm SD$	$X \pm SD$	$X \pm SD$	$X \pm SD$	$X \pm SD$	$X \pm SD$	$X \pm SD$
AST (U/L)	96.2 \pm 8.7 ^a	103.3 \pm 12 ^{ab}	140.1 \pm 16.2 ^b	124.7 \pm 12.2 ^b	130.2 \pm 14.7 ^b	142.7 \pm 17.8 ^b	125 \pm 14.7 ^b
ALT (U/L)	53.5 \pm 3.7 ^a	63.4 \pm 6.2 ^b	81.4 \pm 6.3 ^c	69.2 \pm 6.9 ^b	71.8 \pm 13.7 ^b	74 \pm 9.3 ^b	65.4 \pm 9.1 ^b
ALP (U/L)	60.6 \pm 8.2 ^a	58.2 \pm 12.8 ^{ac}	74.8 \pm 8.2 ^{bd}	73.8 \pm 20.2 ^{ab}	50.3 \pm 22.4 ^{cd}	57.7 \pm 9.6 ^{ac}	46.2 \pm 15.9 ^c
Total bilirubin (mg/dL)	0.5 \pm 0.1 ^a	0.6 \pm 0.1 ^b	0.6 \pm 0.1 ^b	0.6 \pm 0.1 ^b	0.7 \pm 0.2 ^b	0.7 \pm 0.1 ^b	0.6 \pm 0.2 ^{ab}
Albumin (g/dL)	3.5 \pm 0.4 ^a	3.6 \pm 0.5 ^{ab}	3.6 \pm 0.5 ^{ab}	4.0 \pm 0.9 ^b	4.0 \pm 0.6 ^b	3.8 \pm 0.4 ^{ab}	3.6 \pm 0.3 ^{ab}
Total proteins (g/dL)	6.1 \pm 0.6 ^a	6.5 \pm 0.7 ^{ac}	7.8 \pm 0.6 ^b	6.6 \pm 1.1 ^{ab}	7.5 \pm 0.7 ^{bc}	8.2 \pm 0.4 ^{bc}	8.0 \pm 0.5 ^{bc}
Glucose (mg/dL)	264.1 \pm 25.4 ^{ac}	268.6 \pm 27.1 ^{ac}	228 \pm 23.9 ^{bc}	250.8 \pm 23.1 ^{ab}	291.8 \pm 25.2 ^{cd}	219.7 \pm 27.5 ^{bd}	212.1 \pm 23.4 ^{bd}
GLY (mg/g of tissue)	4.2 \pm 0.6 ^a	6.4 \pm 0.9 ^b	3.4 \pm 1.4 ^{abcd}	2.4 \pm 0.9 ^{cd}	5.8 \pm 0.7 ^{bc}	4.3 \pm 2 ^{abc}	1.5 \pm 0.6 ^d
Urea (BUN) (mg/dL)	42.6 \pm 3.6 ^a	40.4 \pm 4.2 ^a	43.9 \pm 5.8 ^a	39.6 \pm 7.8 ^a	42.9 \pm 4.9 ^a	47.3 \pm 4.9 ^a	41.3 \pm 3.1 ^a
Uric acid (mg/dL)	3.2 \pm 1.9 ^a	3 \pm 1.9 ^a	3.5 \pm 1.9 ^a	3.8 \pm 2.4 ^a	3.4 \pm 2.4 ^a	2.4 \pm 1.3 ^a	2.3 \pm 1.4 ^a
Creatinine (mg/dL)	0.4 \pm 0.1 ^a	0.4 \pm 0.1 ^a	0.4 \pm 0 ^a	0.4 \pm 0.1 ^a	0.4 \pm 0.1 ^a	0.4 \pm 0.1 ^a	0.4 \pm 0.1 ^a

Different letters, in the same row (a-e), indicate a significant difference among the groups. P-Values less than 0.05 were considered significant. Groups: C: Control; AI: Acute Infection; CI: Chronic Infection; IHE: Immediate Herbicide Effect; DHE: Delayed Herbicide Effect; I+E: Firstly infected by *Angiostrongylus cantonensis* and then exposed to the herbicide; E+I: Firstly exposed to the herbicide and then infected by *A. cantonensis*.

can significantly increase the serum activity of ALT and AST in *Mus musculus* mice and *R. norvegicus* Wistar rats [7,36]. This could be a consequence of an increase in the lipoperoxidation process due to the strong capacity of the herbicide to stimulate the production of reactive oxygen species [5,7]. Interestingly, the infection by *A. cantonensis* followed by exposure to herbicide promoted an increase in ALT and AST when evaluated separately. However, the results observed for the infected and exposed group (I+E) and exposed and then infected group (E+I) suggest that association does not have a synergistic effect.

Chronic Infection (CI) caused by *A. cantonensis* increased the activity of ALP in the blood of rodents, suggesting cholestasis. Garcia et al. [20] proposed that infection by *A. cantonensis* impairs bile flow and solubilization of this enzyme in hepatocyte membranes, and consequently increases serum availability [32,37]. Furthermore, it has been postulated that liver tissue damage caused by helminth infection may result in cytolysis of hepatocytes, resulting from cell membrane damage and enzyme release into the bloodstream [20]. In contrast, exposure to the herbicide Roundup[®] promoted a decrease in the enzymatic activity of ALP. It has been verified that this change is a frequent characteristic of human pesticide poisoning, and also may be associated with malnutrition [38,39]. Our data corroborate the weight loss observed in rats exposed to the herbicide (data not shown). The animals were not deprived of food, suggesting that the weight loss and decreased enzyme level could be resulted from the intoxication of the exposure to glyphosate, which could lead to a hyporexia and consequently causing malnutrition. Some mineral salts are related to ALP activity, such as zinc (Zn). Deficiency of these elements, due to malnutrition, can lead to decreased serum activity of this enzyme [40,41]. It has also been proposed that the decrease of ALP activity results from bone tissue impairment [42], since glyphosate accumulates in this tissue, leading to changes in ALP levels in osteoblasts [43]. The association of infection by *A. cantonensis* and oral exposure to the herbicide Roundup[®] (groups I+E and E+I) promoted a reduction in serum ALP activity, showing that exposure to glyphosate produced an opposite effect to helminth infection.

Serum levels of total bilirubin increase when hepatic metabolic capacity is compromised [44]. We observed a similar significant increase in all experimental groups after oral exposure to Roundup[®] and *A. cantonensis* infection. Owagboriaye et al. [45] observed the same result in rats exposed to concentrations of 50.4 mg/kg and 284.4 mg/kg of the same herbicide. This change can have three explanations: (1) increased activity of the heme oxygenase enzyme induced by the herbicide, which results in further degradation of the heme prosthetic group, releasing the heme group, which is converted into bilirubin, consequently increasing its serum levels; (2) inactivation of bilirubin conjugation in the liver, due to the hepatotoxicity promoted by Roundup[®] [46]; and (3) hepatotoxic effect of the surfactant Polyoxyethyleneamine (POEA), which may have influenced the hepatic metabolism of bilirubin [36]. Although separately the infection by *A. cantonensis* and the exposure to Roundup increased the bilirubin serum levels, when administrated concomitantly (I+E and E+I groups), the animals did not present an enhanced effect.

Changes in serum levels of total proteins are associated with variations in albumin levels in blood plasma, since this protein accounts for more than 50% of the total proteins present in the blood

[47,48]. In the present study, the CI group presented an increased serum level of total proteins, but the albumin levels of the animals belonging to this group remained unchanged. Similar results were observed in animals infected by species of the genus *Angiostrongylus* [20,49], including *A. cantonensis*. The authors suggested that this change could be associated with the immune response against the respective infections, with an increase in gamma globulins, among other types of proteins, such as immunoglobulins, which are related to inflammatory processes [50,51].

Oral exposure to the herbicide caused hyperalbuminemia in rodents from the IHE and DHE groups, demonstrating that the increase in albumin concentration did not only occur soon after exposure, but remained until the end of the experiment. This change may have been a reaction to the herbicide's ability to produce reactive oxygen species [5,6], since albumin is an antioxidant agent, among other functions [52]. Another late effect of exposure observed in rodents (DHE group) was the increase of total proteins, which is a consequence of hyperalbuminemia [47]. It has been shown that the effects of glyphosate-based herbicides on total serum protein levels can vary in rodent models, with or without change depending on the concentration of herbicide used in the trials [53,54].

The animals infected by *A. cantonensis* and exposed to Roundup[®] (I+E and E+I groups) did not present alteration in the level of albumin, as observed in the equivalent exposure control group (DHE). This suggests that the infection acted due to the effects of the herbicide. In fact, June-Der et al. [55] demonstrated that infection by *A. cantonensis* in C57BL/6 mice promoted the switch of albumin present in the blood to the Cerebrospinal Fluid (CSF). This change caused hyperalbuminemia in the CSF and a decrease in the albumin levels in the blood. These groups also presented an increase in total protein levels. Although the infection and the exposure, separately, promoted the same effect in TP, association did not have a synergistic effect.

The animals in the Immediate Herbicide Exposure (IHE) and Chronic Infection (CI) groups presented a decrease in the concentration of hepatic glycogen as a continuous effect at the end of exposure. The same was observed in the E+I group, with no change in the effect of the herbicide. Salbego et al. [56] suggest that the liver undergoes changes in carbohydrate metabolism, promoting greater degradation of glycogen in an attempt to detoxify the body. This could be a delayed effect of exposure to reestablish homeostasis with physiological induction of the gluconeogenesis process, to counteract the decrease of this energy reserve, as explained above. In addition, another delayed effect observed was hyperglycemia, due to glycogenolysis promoted soon after the end of exposure [57].

There were no changes in the renal function parameters, indicating that infection by *A. cantonensis*, oral exposure to the herbicide Roundup[®] and the association of these two agents were not detected in the kidneys of Wistar rats under the conditions applied in the present work. Similarly, in dogs experimentally infected with *Angiostrongylus vasorum*, no changes were observed [49]. Nevertheless, results involving herbicides can vary from unchanged creatinine levels [58] to decreased levels, while the concentrations of urea and uric acid in blood plasma increased in Wistar rats intraperitoneally exposed to Roundup[®] [53].

The results of this study indicate that the association between infection by *A. cantonensis* and exposure to Roundup® can promote alteration in the hepatic physiology, based on the increase of the AST and ALT serum activities. In addition, the association affected the carbohydrate metabolism. These findings contribute to a better understanding of how glyphosate-based herbicides can influence the parasite-host relationship.

Declaration

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