BRAZILIAN JOURNAL
BJHBS
OF HEALTH AND BIOMEDICAL SCIENCES

Original article

A high-fat diet and *schistosomiasis mansoni* cause histological changes in mice livers

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Abstract

Introduction: Human and animal studies have shown that schistosomiasis protects against metabolic diseases such as dyslipidemia, atherosclerosis and diabetes. Objectives: However, less is known about the effects of a high-fat diet on hepatic architecture, formation and composition of granulomas in acute *schistosomiasis mansoni*. Methods: Male C57BL/6 mice fed high-fat (60% fat) chow or standard (10% fat) chow for 13 weeks were infected with 80 *Schistosoma mansoni* cercariae. The mice were assigned into four groups: uninfected fed standard (SC), high-fat chow (HFC), infected fed standard (ISC) and high-fat chow (IHFC). Blood lipid concentrations (cholesterol, triglycerides, LDL, VLDL, HDL), oral glucose tolerance test, body mass gain, liver mass and intestinal parasite egg counting (oogram), cellular composition, percentage of inflammatory infiltrates and morphometric features (area and perimeter) of liver granulomas were measured. The volumetric density of hepatocytes, steatosis, sinusoids, necrosis and hepatic fibrosis were estimated by using stereology. Results: IHFC mice showed lower blood lipid levels, biometrics, improved glucose tolerance and less steatosis compared to control mice (HFC). The IHFC group showed larger granulomas, predominance of polymorphonuclear cells and rich inflammatory infiltration, binucleated hepatocytes and histopathological changes more severe than ISC. Conclusion: Although acute *schistosomiasis* reduces the effects of a high-fat diet on the host's metabolism, it induces damage to liver architecture.

Keywords: *Schistosomiasis mansoni*; Liver; Comorbidities; Lipids; Stereology.

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BJHBS, Rio de Janeiro, 2022;21(2):101-110
DOI: 10.12957/bjhbs.2022.71624
Received on 29/04/2021. Approved on 11/07/2022.

Introduction

Schistosomiasis mansoni is a neglected waterborne tropical disease with high morbidity and mortality, which has an impact not only on the quality of life, but also on the productivity of infected human hosts. Intestinal *schistosomiasis* is caused by a blood-dwelling flatworm, *Schistosoma mansoni*.[1,2] In Brazil, *schistosomiasis mansoni* afflicts more than 2 million people and a further 25 million show a potential risk of infection, with the largest number of cases concentrated mainly in the Northeast region of the country.[3]

The adults worms live in the portal and mesenteric veins, where females produce approximately 300 eggs per day, which cross the intestinal wall and are released within the feces into the environment.[4,5] Due to blood flow to the liver, most produced eggs become trapped in the hepatic pre-sinusoidal vessels or are disseminated to adjacent organs.[4,5] *S. mansoni* eggs are metabolically active and highly antigenic, releasing secretions such as Soluble Egg Antigens (SEA), which evoke an inflammatory response capable of activating the definitive host's cellular immune system around trapped eggs in host tissues.[5,6] The granulomatous inflammatory reaction is the hallmark feature in the immunopathology of hepatic, intestinal and splenic *schistosomiasis*.[4,5] Granuloma play a dual role, contributing to the pathogenesis of parasitosis, while also protecting the host against antigens released by the egg, preventing their diffusion into the vascular

medium7. The schistosomal granuloma is a compact, dynamic and organized collection of migrating cells, such as eosinophils, neutrophils, lymphocytes and macrophages, which together with the cells of the affected tissue are arranged in the midst of extracellular matrix components and form a spherical-like structure that surrounds the egg in order to block the hepatotoxic effects of SEA. Although granuloma formation is a beneficial organ response for the host, it can lead to fibrosis with excessive accumulation of collagen and extracellular matrix proteins in the periportal space, triggering severe liver injury in the host.[8]

Several factors can influence the formation and granulomatous modulation, including the parasite strain, the disease phase (acute or chronic), the experimental model[9] and type of diet. [10-12] In *schistosomiasis mansoni*, the nutritional status of the host has been widely reported,[13-15] enabling the establishment of a relationship between the development of pathology and its complications.

Experimental models have been used to understand the role of lipid metabolism in diseases caused by helminths.[13,16,17] Blood-dwelling parasites, such as *S. mansoni*, are known to uptake lipids, including cholesterol, as a host nutrient because they are unable to synthesize long-chain fatty acids or *de novo* cholesterol.[18] It has been demonstrated that hepatosplenic patients show decreased total blood cholesterol, concentrations of phospholipids and triglycerides.[12,13] Likewise, schistosomiasis infection also reduces blood lipids levels in mice,[19] including those fed a high-fat diet.[11-13]

The nutritional profile of the human population has changed in recent decades.[20] As a consequence, a new threat to the survival of modern society has emerged: obesity and its comorbidities.[19,20] These chronic non-communicable diseases (NCDs) are strongly dependent on several factors, including those of social, behavioral, and environmental origin, such as greater availability of high energy density foods and sedentary lifestyle habits. On the other hand, *S. mansoni* ameliorates dyslipidemia by reducing blood cholesterol levels,[21,22] neutralizing the effects of atherosclerosis and reducing the prevalence of diabetes.[23]

Several murine experimental models have been used in studies of metabolic disorders, in which the C57BL/6 strain is the most widely investigated among mice because of its genetic structure for the development of obesity, dyslipidemia, diabetes and syndrome metabolic associated with diet.[13] Although epidemiological investigations of these comorbidities exist, experimental models are important sources of data for the knowledge and understanding of liver alterations triggered by schistosomes and metabolic disorders.[24] Thus, the aim of this study is to investigate the effects of a high-fat diet on liver architecture during acute *schistosomiasis mansoni* infection in C57BL/6 mice.

Materials and methods

Experimental model and diet

Male C57BL/6 mice, two months old, were fed a standard diet (SC, 10g lipids/100g diet) or a high-fat diet (HF, 60g lipids/100g diet) during 13 weeks. The vitamin and mineral content of both diets followed the recommendations of the American Institute of Nutrition (AIN 93M),[25] as shown in Table 1. The animals were maintained in polypropylene boxes (40 x 33 cm) under standardized conditions (12h light/dark cycle, 21±1 °C temperature, and 60±10% relative humidity) and *ad libitum* access to food and water within the vivarium facilities (Department of Microbiology, Immunology and Parasitology, Faculty of Medical Sciences, The University of the State of Rio de Janeiro, UERJ). The experimental procedures were approved by the Animal Experimentation Ethics Committee of the Institute of Biology Roberto Alcantara Gomes (UERJ) under protocol (CEUA/013/2013).

Experimental infection with *Schistosoma mansoni*

Ninety days after starting each diet, mice were infected subcutaneously with approximately 80 *S. mansoni* (BH strain) cercariae obtained from laboratory-bred and experimentally infected *Biomphalaria glabrata* snails at the Laboratory of Malacology (Oswaldo Cruz Foundation, Rio de Janeiro, Brazil). A total of 33 mice were divided into four experimental groups: uninfected control fed standard chow (SC, n=5); uninfected control fed high-fat chow (HFC, n=9), infected fed standard chow (ISC, n=9) and infected fed high-fat chow (IHFC, n=10). At 42 days after infection, all animals were submitted to parasitological examination[26] to assess the effectiveness of the infection. The animals' body mass was evaluated weekly on the same day and time.

Biochemical analysis and euthanasia

Blood glucose and oral glucose tolerance tests (OGTT) were measured after a 6-hour fast one week

Table 1. Composition of experimental diet

Content (g/kg)	Groups	
	SC	HF
Casein	140.0	190.0
L-cysteine	1.8	1.8
Corn starch	620.7	250.69
Sucrose	100.0	100.0
Fibers	50.0	50.0
Soya bean oil	40.0	40.0
Lard	--	320.0
Vitamin mix	10.0	10.0
Mineral mix	35.0	35.0
Hill	2.5	2.5
Antioxidant	0.008	0.008
Protein (%)	14	14
Lipids (%)	10	60
Calories(kcal/g)	3.81	5.00

Legend: SC: standard chow. HF: high-fat chow.
Source: The authors (2021).

before sacrifice.[13] Blood glucose concentrations were measured at 0, 15, 30, 60 and 120 min, after oral gavage of glucose (1g/kg), using a glucometer (Accu-Chek, Roche Diagnostic, Germany) and then area under the curve (AUC) was determined by the trapezoid rule to assess glucose intolerance.[27] The mice were killed by CO2 asphyxiation at week nine of infection (acute infection). The experiment lasted a total of 21 weeks since the beginning of the administration of the diet and the close of the ninth week in case of infection by *S. mansoni*. Blood samples were obtained quickly by cardiac puncture and centrifuged (1200g for 20 minutes) at room temperature and stored at -20°C. Total cholesterol (TC), triglycerides (TG) and HDL were evaluated by a colorimetric kinetic assay (Bioclin 100, Quibasa Química Básica, Belo Horizonte, MG, Brazil). LDL and VLDL were estimated using the Friedewald formula.[28]

Intestinal egg burden (oogram)

The parasite load was estimated by counting eggs in the small intestine by use of digestion in potassium hydroxide at 56 °C and centrifugation (2,000 rpm) for 5 minutes. Five aliquots with 50µL of digested tissue were placed onto histological slides covered with a coverslip and eggs visualized by light microscopy were counted.[29]

Stereological analysis

Stereology is a quantitative technique whose principles take into account geometry and statistical probability.[30] This technique has been widely used as a quantitative tool to evaluate liver lesions in clinical studies and to investigate the nutritional effects of high-fat diets in schistosomiasis-infected mice in recent years.[10] Stereological analysis was performed by light microscopy, using a video microscopy system with a microscope (Olympus BX41, USA) at x200 magnification and a test system with 36 total points (PT) coupled to the computer monitor (Figure 1). The images were analyzed and captured using image analysis software (Image Pro Plus Cybernetics, USA). The volumetric density of hepatocytes, steatosis, sinusoids, necrosis, and hepatic fibrosis were estimated by counting the points that touched such structures: five microscopic fields were randomly analyzed in the liver sections of each animal. Vv[structure]:=PP[structure]/PT (where PP is the number of points that touch the evaluated structure and PT is the

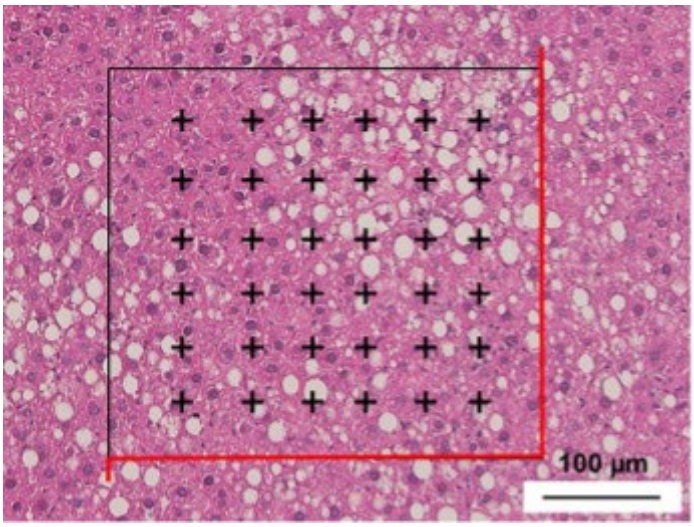

Figure 1. Stereological analysis through the system of 36 total points

Legend: Stereological analysis from a H&E liver section in C57BL/6 fed a high-fat diet. The 36-point count is placed over image.

Source: The authors (2021).

total number of points in the test grid).[31-33] The number of inflammatory infiltrate points was verified by the same method.

Histopathological evaluation

Liver excised from each animal at euthanasia was weighed, fixed in 10% neutral buffered formalin and processed for histopathological analysis. Liver was dehydrated in a series of graded ethanol concentrations (80-100%), diaphanized in xylol and embedded in paraffin. Histological sections (5µm thickness) were taken by means of a microtome (Leica, model RM2125RTS) and stained with Hematoxylin and Eosin (H&E) and Picrosirius. Stained sections were examined under light microscopy (Olympus BX41 microscope, Olympus America, USA) by using x200 magnification for identification of necrosis, inflammatory processes, collagen deposition, changes in cell structure and qualitative and quantitative cellular composition of schistosomal granulomas.

Hepatic granulomas

For morphometric analysis, images were acquired with a digital camera (BEL Photonics do Brasil, Piracicaba, SP, Brasil) mounted on an Olympus BX41 microscope (Olympus America, USA) using Image Pro Plus (Media Cybernetics, USA) software. Ten microscopic fields per animal were randomly examined at x100 magnification. The parameters analyzed were area and perimeter.

Statistical analysis

All statistical analysis was performed using the GraphPad Prism 6 program. The results are reported as mean ± standard error of the mean (SEM). We used the Kruskall Wallis tests followed by Dunn's multiple comparisons or ANOVA analysis followed by the Tukey post-test. Comparisons among pairs of groups were accessed using Student's t-test. Measurements with values of $p \leq 0.05$ were considered significantly different.

Results

Table 2 shows the results of biometric, biochemical, parasitological analyses, morphometry of hepatic schistosomal granulomas and stereology. Infected mice that had been fed high-fat chow (IHFC) had lower final body mass, body mass gain and visceral fat, when compared to their uninfected control with the same diet (SC). Regardless of diet, infected animals showed greater liver mass. IHFC mice showed lower levels of total cholesterol, LDL, triglycerides and VLDL than SC. The IHFC group showed reduced HDL levels when compared to SC, although the difference was not significant.

We found more eggs retained in the small intestine (oogram) in IHFC vs. ISC (p=0.006). Hepatic granulomas in the IHFC group showed larger area (110400±8777 µm² vs. 80880±4209 µm², +36.49%, p=0.0031) and perimeter (1365±71.65µm vs. 1148 42.44µm, +18.90%, p = 0.0104), IHFC and ISC respectively.

Uninfected mice fed a standard chow (SC) showed a higher volume density of hepatocytes (Vv[h]) than HFC, ISC and IHFC groups (+19.58%, p<0.05). A statistically significant difference was not observed between infected groups (ISC and IHFC). As expected, the volumetric density of sinusoids (Vv[s]) was higher in the SC group in comparison with the other study groups (+40.11%, +42% and +40.70%, p≤0.001). The volume density of liver fibrosis Vv[hf] did not show significant difference among infected groups. The IHFC group had a volumetric density of hepatic necrosis Vv[n] greater than ISC (+58%, p<0.0001). The volume of steatosis (Vv [st]) was greater in mice fed high-fat chow (HFC, 9.04±0.69/IHFC, 4.21±0.60), showing a higher density of steatosis compared to those fed standard chow (SC, 2.00 ±0.0/ISC, 0.02±0.02). The HFC group had more (+53%) steatosis than IHFC.

The IHFC group showed reduced glucose tolerance compared with SC (Figure 2), with higher total AUC compared with ISC (+ 160.66%, p< 0.001) and ISC x SC

Table 1. Biometric, biochemical, parasitological analyses, morphometry of schistosomal granulomas and hepatic stereology of C57Bl/6 mice fed high-fat and standard diets (mean ± SEM)

Variables	SC	HFC	ISC	IHFC
Animal biometrics				
Final body mass (g)	33±1.56a	41.3±4.87 a,b	32±2.07	36.68±3.42b
Body mass gain (g)	4.06±1.18a	15.25±5.73a,b	5.75±2.33C	11.17±4.19c
Visceral fat (g)	0.52±0.18a	2.80±0.80ª,b	0.50±0.21C	1.28±0.86b,c
Liver mass (g)	1.42±0.10a	1.51±0.27b	2.10±0.19to	2.13±0.26B
Plasma Lipids (mg/dL)				
HDL	19.9±4.6	12.4±3.9	18.2±5.3	14.4±4.8
LDL	64.9±23.2a	117.5±34.3abc	56.3±13.9b	85.3±45.4b,c
TC	100.2±18.8a	163.8±42.5abc	98.2±37.6c	92.9±10.6b
TG	86.8±14.5a	136.5±41.7abc	86.1±36.8b	91.4±11.4b,c
VLDL	17.36±2.3	27.3±4.1	17.22±2.5	18.28±3.01
Parasitological				
Intestinal oogram	n.a	n.a	1193.0±293.5d	2886.0±447.4d
Morphometry of granulomas				
Area (10^2µm2)	n.a	n.a	808.8±294.6d	1104.1±614.4d
Perimeter (10^2µm)	n.a	n.a	11.5±3.0d	13.7±5.1d
Liver stereology				
Hepatocytes	25.74 ±0.47a ,b,c	21.12±0.57a	20.16 ±1.03b	20.82 ± 0.78c
Sinusoids	10.22 ± 0.50a,b,c	5.84 ± .41a	6.12 ±0.42b	6.06 ± 0.57c
Fibrosis	n.a	n.a	8.62 ±1.12d	9.96 ± 1.00d
Necrosis	n.a	n.a	1.02 ± 0.18d	2.46 ± 0.35d
Steatosis	2.00 ± 0.0a	9.04±0.69a,b,c	0.02 ± 0.02b,d	4.21 ± 0.60c,d

Legend: SC: uninfected control fed standard chow. HFC: uninfected fed high-fat chow. ISC: infected fed standard chow. IHFC: infected fed high-fat chow. HDL: high density lipoprotein. LDL: low density lipoprotein. TC: total cholesterol. TG: triglycerides. VLDL: very low-density lipoprotein. n.a: not available. Significant statistical difference (p≤ 0.05, Kruskal-Wallis test): [a] SC vs HFC; [b] SC vs ISC; [c] SC vs HFC; [d] ISC vs IHFC.

Source: The authors (2021).

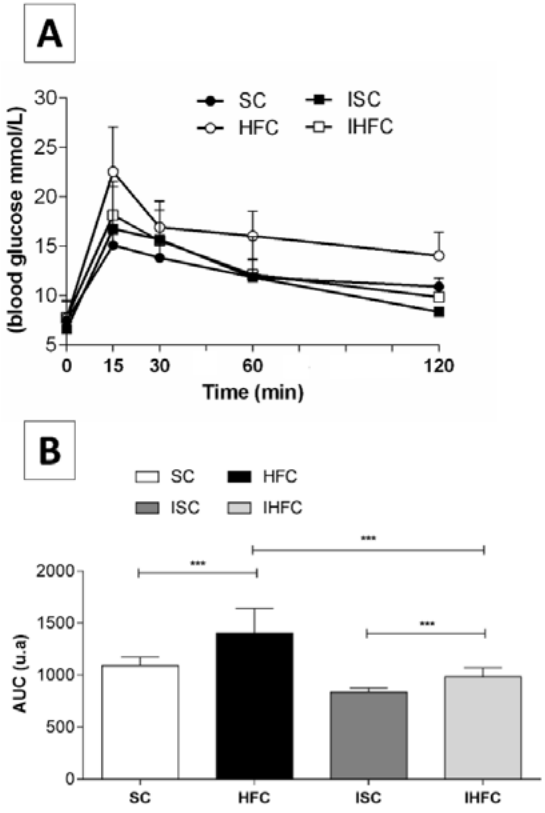

Figure 2. Oral glucose tolerance test and area under curve of C57BL/6 mice (mean ± SEM)

Legend: oral glucose tolerance test (OGTT); B- Area under the curve (AUC) of the experimental groups. SC (uninfected control fed standard chow); HFC (uninfected fed high-fat chow); ISC (infected fed standard chow); IHFC (infected fed high-fat chow). The groups were analyzed using the one-way ANOVA statistical test and Tukey's post-test. [a] SC vs. HFC, [b] SC vs. ISC, [c] HFC vs. IHFC, [d] ISC vs. IHFC.

Source: The authors (2021).

(+ 145.37 %, p<0.001). In the opposite direction, IHFC showed higher glucose tolerance when compared to AUC (-15.93%, p<0.001) and HFC (-31.37%, p<0.001).

Histological examination showed that the SC group with a normal liver architecture presented regularly arranged hepatocyte bundles surrounded by sinusoids with Kupffer cells inside without any evidence of injury (Figure 3A). As a result of the high-fat diet, we verified macrovesicular steatosis inside the hepatocytes in HFC, in which a large fat droplet displaced the hepatocyte nucleus to the cell periphery, and microvesicular steatosis with small fat droplets and nucleus located centrally in the cell (Figure 3B). *S. mansoni* infection led ISC to manifest coagulative necrosis in some hepatocytes, congestion, Kupffer cells associated with inflammatory infiltrate with a predominance of eosinophils and binucleated hepatocytes near schistosomal granuloma in compensatory hyperplasia (Figure 3C). The IHFC group exhibited liver parenchyma with intense inflammatory activity and a predominance of eosinophils, Kupffer cells and coagulative necrosis around the granulomas. The intensity of the aggression revealed hyperplasia of binucleated hepatocytes, but reduced fat deposits in the liver (Figure 3D). Collagen fiber deposits were revealed by picrosirius staining in ISC (Figure 3E) and IHFC (Figure 3F), distributed from larger regions of the hepatic lobule in the IHFC group as a result of proximity to the eggs.

There is not much difference regarding the cellular composition of exudative (E) and exudative-productive (EP) stages in ISC and IHFC groups. However, the exudative stage showed a higher concentration of macrophages and eosinophils in the IHFC group compared to the ISC. As shown in Figure 4, the IHFC

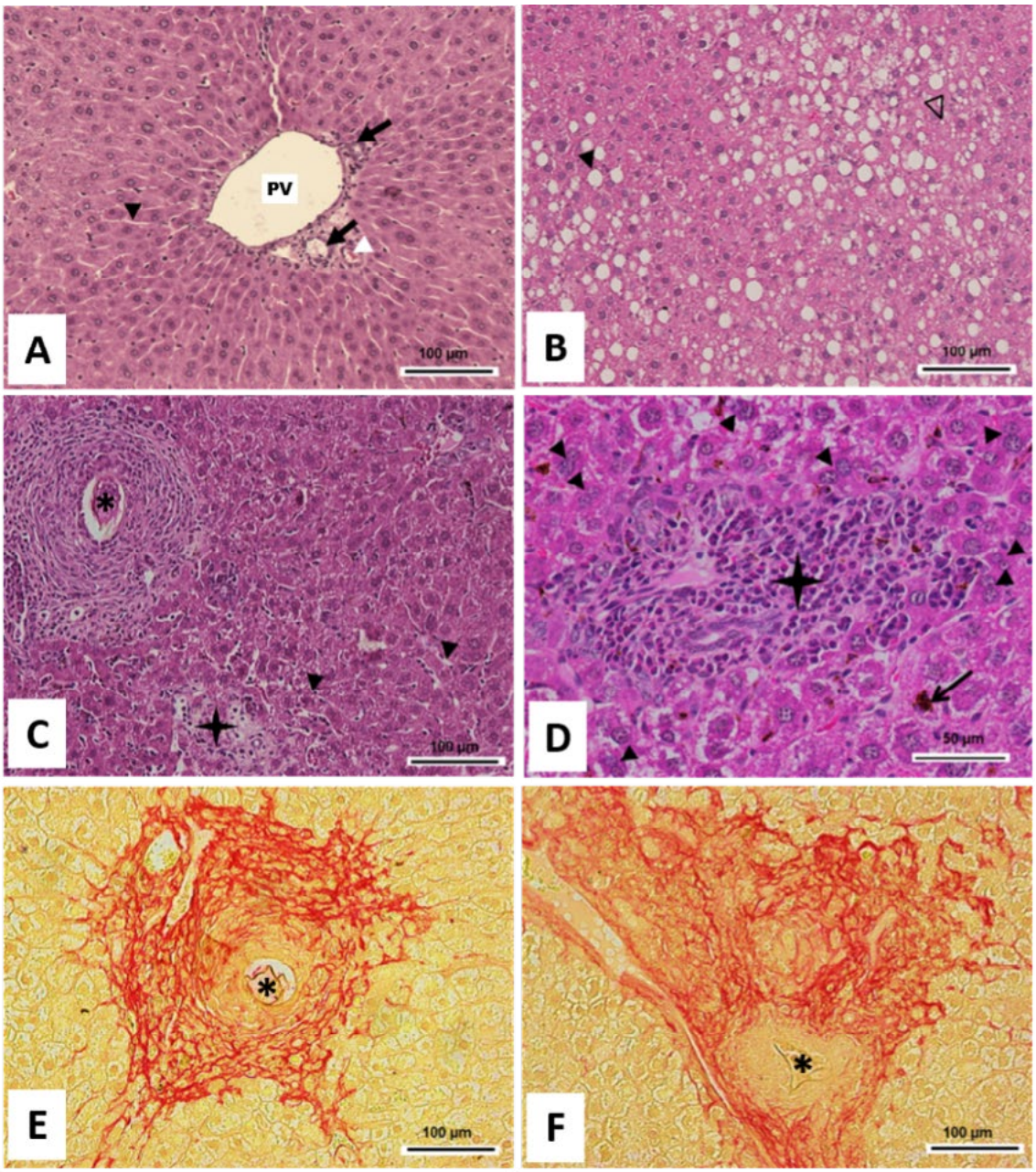

Figure 3. Liver histology of uninfected and *Schistosoma mansoni*-infected C57BL/6 mice fed a standard or high-fat diet

Legend: Mice fed standard chow (A, C and E) or high-fat chow (B, D, and F). **A-** SC with preserved parenchyma, hepatocytes in cords, sinusoids (black arrowheads), portal vein, bile duct (black arrows) and hepatic artery (white arrowhead). **B-** HFC with rich macrovesicular steatosis within hepatocytes (black arrowhead) and microvesicular steatosis (arrowhead with black border). **C-** ISC showing schistosomal granuloma with a central schistosome egg (*), eosinophilic inflammatory infiltrate ( ) close to the area of the granuloma, Kupffer cells (black arrowhead) **D-** IHFC with a rich inflammatory infiltrate composed predominantly of eosinophils, Kupffer cells and proliferation of binucleated hepatocytes is highlighted in an area close to inflammatory infiltration (black arrowhead) and schistosomal pigment (black arrow). **E-** ISC shows collagen deposit around schistosomal granuloma with a central schistosome egg (*). **F-** IHFC with greater collagen fiber distribution in the hepatic parenchyma around schistosomal granuloma. Staining A, B, C and D – H&E; E and F - Picrosirius; SC (uninfected control fed standard chow); HFC (uninfected fed high-fat chow); ISC (infected fed standard chow); IHFC (infected fed high-fat chow).

Source: The authors (2021).

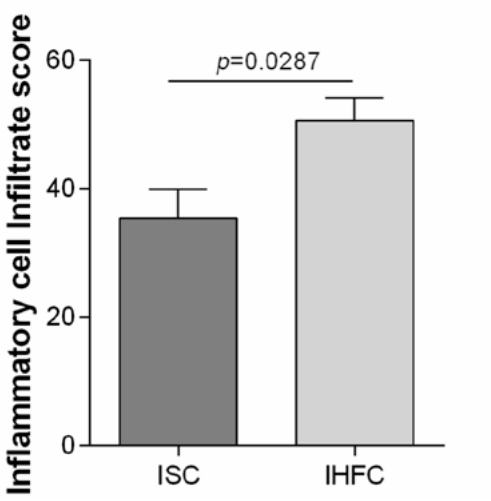

Figure 4. Liver inflammatory infiltrate score in C57BL/6 schistosomiasis-infected mice fed a standard or high-fat diet

Legend: ISC: infected fed standard chow. IHFC: infected fed high-fat chow.
Source: The authors (2021).

group had more liver inflammatory infiltrate points than the ISC (+33.96%, p=0.0287).

Discussion

The association between a high-fat diet and *schistosomiasis mansoni* has recently been highlighted in humans.[22,23] In this study we seek to explore this association based on the C57BL/6 model in the acute phase of *schistosomiasis mansoni*. The role of lipids and their metabolites in host-parasite interaction has become a focus of interest in this study. In this scenario, infected mice fed a high-fat diet show a reduction in body mass gain, visceral fat and serum lipids, suggesting that adult worms capture lipids from their host's bloodstream[17,31] for their benefit.[11] In addition, it seems reasonable to infer that schistosomes can establish beneficial effects for the host not only by acquiring lipids from the bloodstream, but through derived products, which can limit the adipose tissue mass and inflammation, thereby promoting metabolic benefits.[17,35,36] Infection of mice with *S. mansoni* promotes enlargement of the liver, due to the granulomatous reaction around trapped eggs.[12,13] We believe that with the inflammatory process there was an increase in the production of TNF-α, which acts by inhibiting the insulin receptors of the cells, reducing the uptake of their glucose and increasing its the presence in the blood. Given that the C57BL/6 model is already sensitive to metabolic syndrome, this may be an explanation for this result. TNF-α can be produced by visceral fat and acts against insulin receptors. However, the main cytokine produced by the acute inflammatory process, together with IL-1, also explains the results verified in OGTT for HFC and ISC groups.[37]

Morphometric analyses of liver granulomas revealed that the area and perimeter were larger in mice fed high-fat chow than those that received a standard diet. We believe that this difference may be related to the cellular characteristics and functions of adipose tissue. Adipocytes secrete various cytokines and acute-phase proteins that, directly or indirectly, increase the production and circulation of factors related to inflammation.[40] There is evidence demonstrating that the inflammatory state may be due to resistance to the action of insulin and other disorders associated with obesity, such as metabolic syndrome and hyperlipidemia, as studied in the present survey. This suggests that a high-fat diet intensifies the granulomatous reaction, given the fact that white adipose tissue stimulates the production of inflammatory markers mainly by the liver.

Stereological analyses using the D36 technique revealed that both the high-fat diet (HFC group) and infection (ISC and IHFC) caused significant reduction in the volumetric density of hepatocytes and sinusoids, leading to liver injury as described above in the

histological examination. Stereology showed changes in the volumetric density of fibrosis and necrosis among the infected groups, as observed in a previous study;[10] however, we did not identify any statistically significant difference between them.

It is known that non-alcoholic fatty liver disease can cause the deposit of triglycerides in the liver, a condition known as steatosis that was observed in the groups receiving high-fat chow. Interestingly, the volumetric density of steatosis in the infected group (ISC and IHFC) was significantly lower when compared to the uninfected group on the same diet (SC and HFC). The parasite may possibly be using the lipid source for its metabolism and for the regulation of the granulomatous inflammatory response.[11-13] The greater number of inflammatory infiltrate points verified in the IHFC group is due to the double lesion suffered by the tissue. In an attempt to respond to tissue damage, the liver initiates a regeneration process, which is an organic protection mechanism against the loss of liver tissue from injuries of various origins. Although the term regeneration is widely used, the correct biological term for the observed phenomenon is compensatory hyperplasia, since this is a response induced by liver-tissue damage. Lesions of the hepatic parenchyma induced by acute schistosomiasis and non-alcoholic fatty liver disease triggered a regenerative process aimed at tissue restoration. This occurs by compensating cellular hyperplasia of the remaining parenchyma, in a regulated and precise manner.[13]

Conclusions

The results presented here provide evidence that *Schistosoma mansoni* infection maximizes the hepatic inflammatory pathology induced by a high-fat diet, while preventing metabolic disorders, such as dyslipidemias, metabolic syndrome and type 2 diabetes mellitus. We conclude that a high-fat chow and S. mansoni overload hepatic parenchyma, leading to morphological changes in its structure.

Limitations

This study was unable to include the recovery of adult S. mansoni worms. A more detailed analysis of the number of pairs and their relationship with oviposition, added to the use of biochemical and immunological markers of liver inflammation, would allow a more robust discussion about tissue damage from infection and diet.

Funding

This study was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) grant number 470724/2014-5 JRMS. Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. These funding agencies had no role in the study's design, data collection, and analysis, the decision to publish, or the preparation of the manuscript..

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