HISTOPATHOLOGICAL ASSESSMENT OF THE LIVER DURING EXPERIMENTAL INFECTION WITH *Leishmania (Leishmania) chagasi* IN IMMUNOSUPPRESSED BALB/C MICE

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ABSTRACT

BALB/c mice were experimentally infected with *L. (L.) chagasi* and immunosuppressed for the study of the histopathological changes present in their liver. The 96 studied mice were subdivided into four groups: control (I) – no treatment; immunosuppressed (II) – treatment with dexamethasone (DXM) and pentoxifylline (PTX); infected (III) – infection with *L. (L.) chagasi*; and infected and immunosuppressed (IV) - infection with *L. (L.) chagasi* and treatment with DXM and PTX. The day of infection was considered the day zero and immunosuppression occurred at 60 days P.I. Samples were obtained at distinct moments, 15, 30, 60, 75 and 90 days P.I., by collecting liver fragments for histopathological assessment. There was progressive and constant inflammatory reaction of inflammatory infiltrate and complete granulomas, which was renewed in all observation phases. For inoculated groups, the presence of amastigotes was confirmed by means of immunohistochemistry. The immunosuppressed group showed weak inflammatory reaction and rare incomplete granulomas. Immunosuppression influenced the immune response of the liver, which is capable of controlling murine visceral leishmaniasis.

Keywords: visceral leishmaniasis, BALB/c, immunosuppression, immunohistochemistry, liver.

AVALIAÇÃO HISTOPATOLÓGICA DO FÍGADO DURANTE A INFECÇÃO EXPERIMENTAL POR *Leishmania chagasi (Leishmania)* EM CAMUNDONGOS BALB / C IMUNOSSUPRIMIDOS

RESUMO

Camundongos BALB / c foram infectados experimentalmente com L. (L.) chagasi e imunossuprimidos para estudar as alterações histopatológicas no fígado presente. Os 96 ratos estudados foram divididos em quatro grupos: controle (I) - sem tratamento; imunossuprimidos (II) - O tratamento com dexametasona (DXM) e pentoxifilina (PTX); infectado (III) - infecção por L. chagasi (L.); e infectados e imunodeprimidos (IV) - infecção por L. chagasi (L.) e tratamento com DXM e PTX. O dia da infecção foi considerado o dia zero e imunossupressão ocorreu 60 dias P.I. As amostras foram obtidas em vários momentos, 15, 30, 60, 75 e 90 dias

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Corrêa APFL, Luvizotto MCR, Oliveira SL, Oliveira GC, Langoni H. Histopathological assessment of the liver during experimental infection with *Leishmania (Leishmania) chagasi* in immunosuppressed BALB/c mice. Vet. e Zootec. 2017 Jun.; 24(2): 384-397.

P.I., por coleta de fragmentos de fígado para avaliação histopatológica. Houve constante reação inflamatória e infiltrado inflamatório progressivo e granulomas completos, que foi renovado em todas as fases de observação. Para os grupos inoculados, a presença de amastigotas foi confirmada por imuno-histoquímica. O grupo de imunodeprimidos apresentou uma resposta inflamatória fraca e granulomas raros e incompletos. Imunossupressão influenciou a resposta imune do fígado, que é capaz de controlar a leishmaniose visceral murina.

Palavras-chave: leishmaniose visceral, BALB / c, imunossupressão, imuno-histoquímica, fígado.

EVALUACIÓN HISTOPATOLÓGICO DEL HÍGADO DURANTE LA INFECCIÓN EXPERIMENTAL POR *Leishmania chagasi (Leishmania)* EN RATONES BALB / C CON INMUNOSUPRESIÓN

RESUMEN

Ratones BALB / c se infectaron experimentalmente con L. chagasi (L.) y inmunosuprimidos para el estudio de los cambios histopatológicos presentes en su hígado. Los 96 ratones estudiados se dividieron en cuatro grupos: control (I) - sin tratamiento; inmunosuprimidos (II) - El tratamiento con dexametasona (DXM) y pentoxifilina (PTX); infectada (III) - la infección con L. chagasi (L.); y infectada y inmunosuprimidos (IV) - infección con L. chagasi (L.) y el tratamiento con DXM y PTX. El día de la infección fue considerado como el día cero y la inmunosupresión se produjo a 60 días P.I. Las muestras se obtuvieron en momentos distintos, 15, 30, 60, 75 y 90 días P.I., mediante la recopilación de fragmentos de hígado para la evaluación histopatológica. Hubo reacción inflamatoria progresiva y constante de infiltrado inflamatorio y granulomas completos, que fue renovado en todas las fases de observación. Para los grupos inoculados, la presencia de amastigotes se confirmó por medio de inmunohistoquímica. El grupo inmunosuprimidos mostró una reacción inflamatoria débil y granulomas incompletas raras. Inmunosupresión influyó en la respuesta inmune del hígado, que es capaz de controlar la leishmaniosis visceral murino.

Palabras clave: la leishmaniasis visceral, BALB / c, inmunosupresión, inmunohistoquímica, el hígado.

INTRODUCTION

Leishmanias are intracellular parasites of monocytes and macrophages of lymphoid organs like the spleen, the lymph nodes, the bone marrow and the liver. Visceral leishmaniasis (VL) is the most severe manifestation of this disease, which can be fatal, and is among the six endemic diseases considered priority in the world (1). An estimated 500 thousand new cases occur every year in the world (2), while in Brazil at least 3,000 confirmed cases are annually recorded (3).

Co-infection with human immune deficiency virus/acquired immune deficiency syndrome (HIV/aids) is possible, and the World Health Organization (WHO) estimates that of the 30 million people infected by HIV in the world, one-third lives in endemic areas for leishmaniasis (4). This association decreases the life expectancy of patients since HIV/aids causes more rapid visceralization of the infection and human VL shortens the virus latency period (5).

Immunosuppressive therapy modifies the immune response during infection with *Leishmania* spp. Dexamethasone (DXM) has anti-inflammatory and immunosuppressive action

(6), inhibiting the transcription of genes that involve innate immunity proteins (7). It also acts as a potent regulator of the adaptive immune response, suppressing maturation, differentiation and proliferation of T and B cells (8). Pentoxifylline (PTX) has immunomodulatory, anti-inflammatory and antitumorigenic action, inhibiting the production of tumor necrosis factor-alpha (TNF-α) *in vivo* and *in vitro* (9), besides interfering with the synthesis of other cytokines like IL-1, IL-2, IL-6, IL-8, IL-12 and interferon-gamma (INF-γ) (9,10,11). On the other hand, it stimulates the production of IL-4, IL-10 and IL-13 (12).

Both DMX and PTX act by determining changes in the immune response of Th1 to Th2 profile, mimicking the stress system activation with the release of glucocorticoids, which in turn prevent the release of pro-inflammatory cytokines and their products. Thus, they activate macrophages and, consequently, tissue injury (12). Either isolated or in association, they alter the course of toxoplasmosis and leishmaniasis in murine models (13,14).

Murine models have been widely used in infection with *Leishmania* spp. to study the parasite life cycle, the infection pathogenesis and the parasite-host relationship. It must be emphasized that, besides the genetic characteristics of mice, which define the infection progression or resolution patterns, additional factors may influence the disease evolution such as the inoculum, the inoculation site, and the parasite strain and its origin or isolation source (15).

Development of Th1 response by resistant mouse strains induce the production of interferon-gamma (INF-γ) and the development of minor lesions and low parasitemia. Conversely, susceptible mice induce an inappropriate immune response, developing larger lesions and high parasitemia, and are not capable of controlling the disease, dying due to the infection (16).

During VL, the multiplication of amastigotes in mononuclear phagocytic cells of organs like the spleen, the liver and the bone marrow (17) causes hyperplasia and hypertrophy of lymphoid tissue cells. As the infection progresses, other organs like the lungs and the kidneys are also affected (18). In experimental VL, caused by *L. (L.) donovani* (19), *L. (L.) infantum* or *L. (L.) chagasi* (20,21) the hepatic infection is generally self-limiting and the immune response represents a good example of predominance of mononuclear cells in the granulomatous inflammatory response, involving Kupffer cells, monocytes and TCD4+ and TCD8+ cells.

Molecular and cellular interactions, necessary for the efficient formation of hepatic granuloma, are important in the release of *Leishmania* spp. (22). The cytokines involved in the immune response are INF-γ, IL-12, IL-4 and TNF, and moderate TNF levels are important for the host's hepatic protection by means of local TNF production in the hepatic granuloma (23). Administration of TNF-α causes neovascular necrosis, related to subsequent development of fibrosis (24), recruiting and activating macrophages for the injured tissue and subsequently releasing fibrogenic cytokines (25). The hepatic reaction resolves but at the same time amastigote multiplication in the spleen is out of control (26).

For BALB/c mice experimentally infected with *L. (L.) donovani*, the histopathological changes are granulomas which, in the liver, are shown as mature granulomas and in the spleen and in the bone marrow as immature granulomas. Gutierrez et al. (27) studied the dynamics of their formation, collagen deposition and resolution in the liver, noting the formation of countless granulomas in the first four weeks of infection, which slowly reduced until the 20th week. In the beginning, granulomas are immature, of relatively constant sizes, with amastigotes, and are capable of evading the release mechanisms. The granuloma formation dynamics occurs after parasitemia, when the agent is sequestered by macrophages of the spleen and the liver. In the latter organ, Kupffer cells phagocytize the agent but, although they have endocytic and secretory properties, there is a failure in the production of reactive oxygen intermediates, one of their main antimicrobial mechanisms (28,29). Consequently, the amastigote forms multiply and there is concomitant aggregation of Kupffer cells to form the nucleus of granulomas.

The second step of granuloma formation occurs near or in the hepatic parenchyma and depends on chemotactic factors that induce the migration of inflammatory cells (granulocytes, lymphocytes and monocytes). The rapid migration and early preponderance of T-helper cells may trigger a series of events. First, there is production of IFN-γ to maintain the reactive oxygen intermediate production by monocytes (30), which in turn are activated, becoming capable of degrading and inactivating phagocytized amastigotes (31). T-helper cells can produce cytokines like IL-2, IL-3 and IL-4, and each of them has its specific roles in the cell-mediated immunity.

Bradley and Kirkley (32) described the course of *L. (L.) donovani* infection for seven mouse strains, mentioning the formation of granulomas in the liver of six of them. Mathias et al. (33), studying infected hamsters to detect total IgG in their lungs and liver, observed that the lesions were progressive in different organs, changing their characteristics along the infection evolution. In the liver, the inflammatory infiltrate of mononuclear cells became more prominent than the hyperplasia of Kupffer cells at the last study moments, while in the lungs, the cell population in the interstitial infiltrate modified during the infection course. Also in the liver, hyperplasia and hypertrophy of Kupffer cells progressively increased from the 7th to the 80th day post-infection, when hyperplasia became less pronounced. Foci of mononuclear cells were observed since the 7th day post-infection, starting in the periportal and centrilobular spaces.

The aim of this study was to analyze the morphological changes in the liver of BALB/c mice that were infected with *L. (L.) chagasi* and immunosuppressed, assessing the organ-specific immune response during infection.

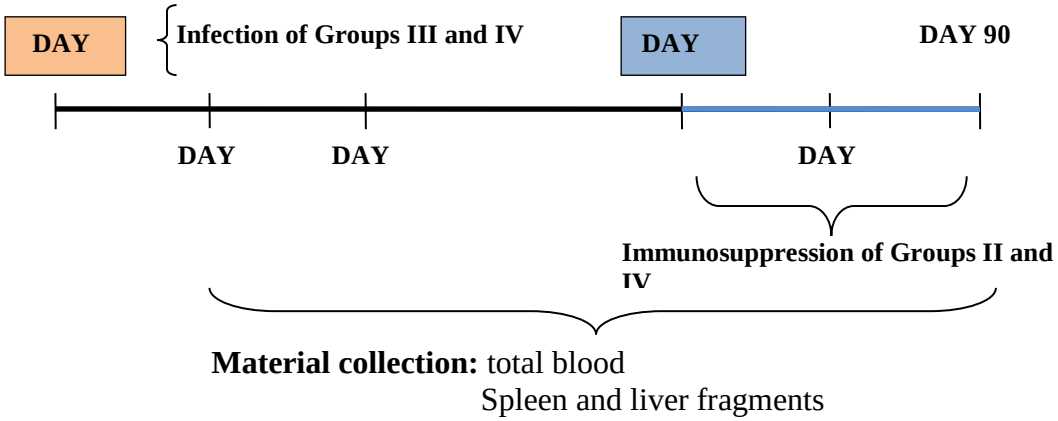
MATERIAL AND METHODS

This experiment was approved by the Ethics Committee on Animal Experimentation of the School of Veterinary Medicine and Animal Science (FMVZ), UNESP - Univ Estadual Paulista, Botucatu Campus, São Paulo State, Brazil, protocol no. 124/2008-CEEA, and conducted at the Zoonosis Research Center (NUPEZO), Department of Veterinary Hygiene and Public Health, FMVZ, UNESP, Botucatu Campus, São Paulo State, Brazil.

The study included 96 male mice of the isogenic strain BALB/c, aged seven weeks, from the Multidisciplinary Center for Biological Investigation - CEMIB, University of Campinas. The animals were kept in polypropylene boxes allocated to a ventilated shelf (Alesco Ind. & Com. Ltda, Monte Mor - Brazil), receiving commercial animal food specific for their species (Nutrilabor® - Guabi, Campinas, Brazil) and water *ad libitum*.

Four groups of 24 mice each were formed, as follows: **Group I:** 24 BALB/c mice **not infected** and **not immunosuppressed** (**control**). **Group II:** 24 BALB/c mice immunosuppressed with pentoxifylline and dexamethasone. **Group III:** 24 BALB/c mice **infected** with *L. (L.) chagasi*. **Group IV:** 24 BALB/c mice **infected** with *L. (L.) chagasi* and **immunosuppressed** with pentoxifylline and dexamethasone.

The experimental design followed the scheme:



Mice of Groups **III** and **IV** were infected by the retro-orbital venous plexus with 10^7 promastigotes/mL saline solution of *L. (L.) chagasi* strain M6445, provided by the Laboratory of Protozoology – Institute of Tropical Medicine, University of São Paulo - IMTSP. For mice of Groups **I** and **II**, the same volume of water was used for injection. **The inoculation day was considered the day zero of infection.**

Animals belonging to Groups **II** and **IV** were immunosuppressed from 60 to 90 days post-infection (P.I.), totaling 30 days. **Dexamethasone** disodium phosphate was used at the dose of **15 mg/kg/day** in a volume of 200 µL/day, by the intraperitoneal route, and **pentoxifylline 150 mg/kg/day** in a volume of 300 µL/day, by the subcutaneous route, in the scapular region (13). Water inoculation for animals of Groups **I** and **III** was done by adopting the same volume and the same inoculation route.

Four mice from each group, at 15, 30, 60, 75 and 90 P.I., were subjected to euthanasia in an acrylic chamber by means of administration of isoflurane (5V%) diluted in oxygen (5 L/min), remaining there until cardiac arrest for the collection of liver fragments, which were fixed in formalin solution at 10% and embedded in paraffin for histopathological sections.

The histopathological processing was carried out at the Veterinary Pathology Service of the Department of Veterinary Clinics, FMVZ - UNESP, Botucatu Campus, São Paulo State, Brazil. Liver fragments were cleaved and stored in plastic cassettes which were identified and processed according to the routine methods that involve dehydration, diaphanization and embedding in paraffin. Three-µm thick histological sections were prepared and stained according to the method of hematoxylin-eosin (HE) for examination under light microscope.

Immunohistochemistry reaction was carried out for 3-µm liver sections by following the routine methods, which are deparaffinization, hydration, antigen recovery by heat, endogenous peroxidase inhibition and unspecific binding inhibition. Anti-leishmania primary polyclonal antibody at the dilution of 1:800, produced in mice, was kindly supplied by the Laboratory of the Faculty of Medicine of University of São Paulo – FMUSP, LIM 500. Secondary monoclonal antibody bound to commercial biotin, avidin/biotin/peroxidase solution and chromogen 3-3-diaminobenzidine (DAB) were employed according to the instructions of the manufacturer of kit Novolink (Leica® Biosystems Newcastle Ltd., United Kingdon)

RESULTS

The changes evidenced in the histopathological exam, according to the moments and groups, were: Animals of Group I (control) had preserved hepatic parenchyma structure, showing hepatocytes with a central nucleus, acidophilic cytoplasm, Kupffer cells, as well as vascular and biliary excretion structures. The hepatic tissue of Group II (immunosuppressed), subjected to immunosuppression for 15 and 30 days, kept the same histological characteristic of Group I.

At 15 days P.I., histological analysis evidenced that the liver of animals of Groups III (infected) and IV (infected and immunosuppressed) had hepatic inflammatory reaction translated by perivascular and multifocal lymphohistiocytic infiltrate, especially surrounding the centrilobular vein and the portal tract. The grouping layout of inflammatory cells mimicked the initial formation of granulomas. Amastigote forms were identified in the cytoplasm of macrophages in inflammation foci, as well as in Kupffer cells that were hyperplastic (Fig. 1).

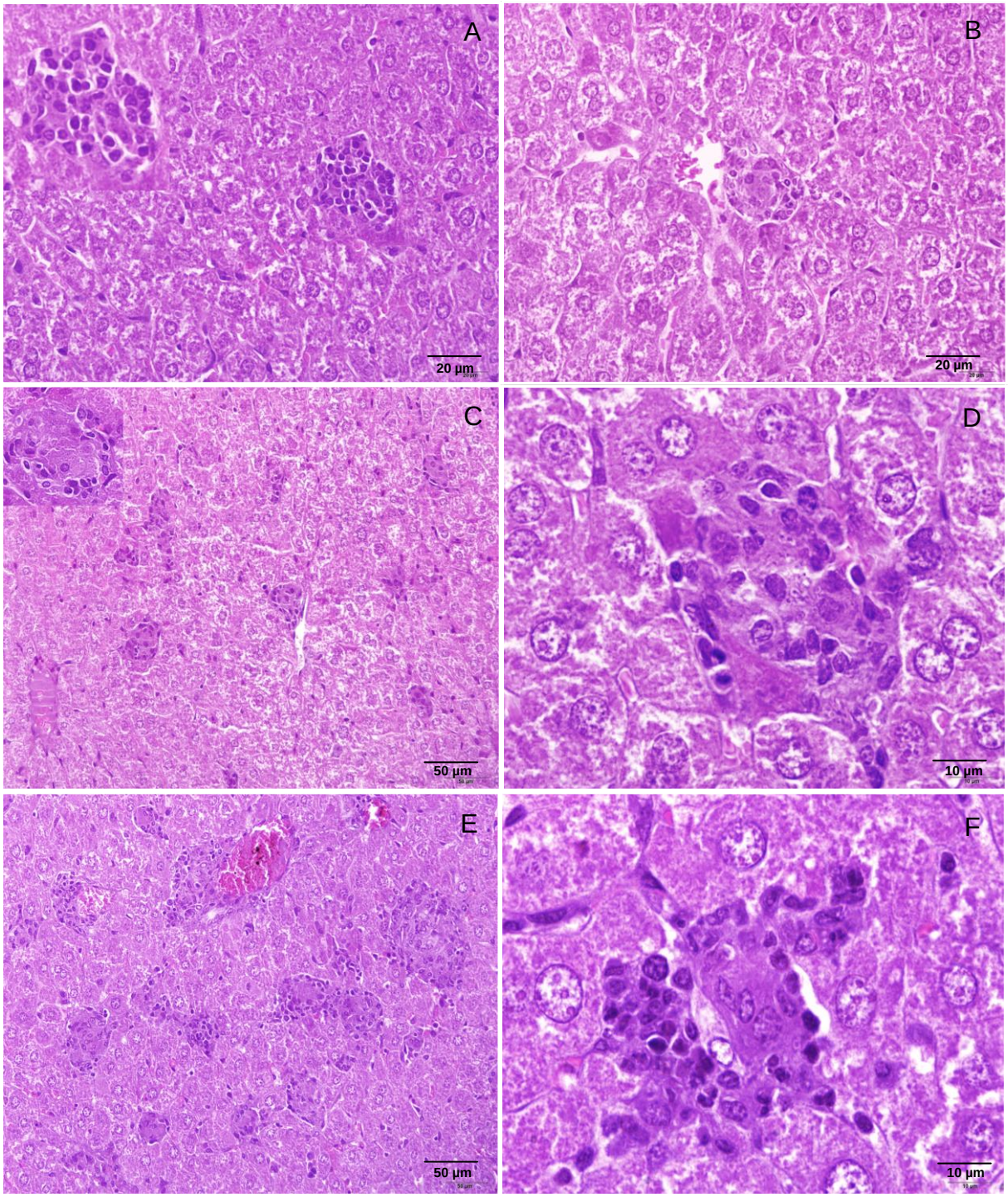


Figure 1. Histopathological analysis of BALB/c mouse liver sections stained with HE. (A-B) 15 days post-infection. (A) Granulomatous reaction; in detail, presence of intracellular amastigotes. (B) Perivascular granulomatous reaction. (C-D) 30 days post-infection. (C) Disseminated granulomas; in detail, intracellular amastigotes. (D) Granulomas with intracellular amastigotes. (E-F) 60 days post-infection. (E) Multifocal granulomas. (F) Cells morphologically similar to the giant cell with intracellular amastigotes.

At 30 days P.I., Groups III and IV had inflammatory reaction of greater amplitude which revealed, in association with the lymphohistiocytic infiltrate, the presence of neutrophils obtaining conformation of incomplete granulomas diffusely distributed in the hepatic parenchyma. Such granulomas showed groups of macrophages full of amastigotes among neutrophils and were surrounded by lymphocytes and epithelioid cells. At this studied moment,

there was infiltration of densely parasitized lymphocytes and macrophages around the vascular routes and the portal space (Fig. 1).

At 60 days, Groups III and IV had inflammatory foci with marked characteristics of granulomas and greater organization and extension, which highly compromised the hepatic parenchyma in the form of complete and incomplete granulomas, always evidencing the amastigote forms of the agent. Cells morphologically similar to giant cells were also observed. Mononuclear cells with characteristics of plasmocytes were evidenced in the composition of granulomas, in addition to fibroblast proliferation (Fig. 1).

At 75 days P.I., Group III evidenced the same characteristics shown on day 60 P.I., differencing only for greater compromising of the hepatic parenchyma, probably due to the coalescence of both granulomas and neoformed granulomas (Fig. 2).

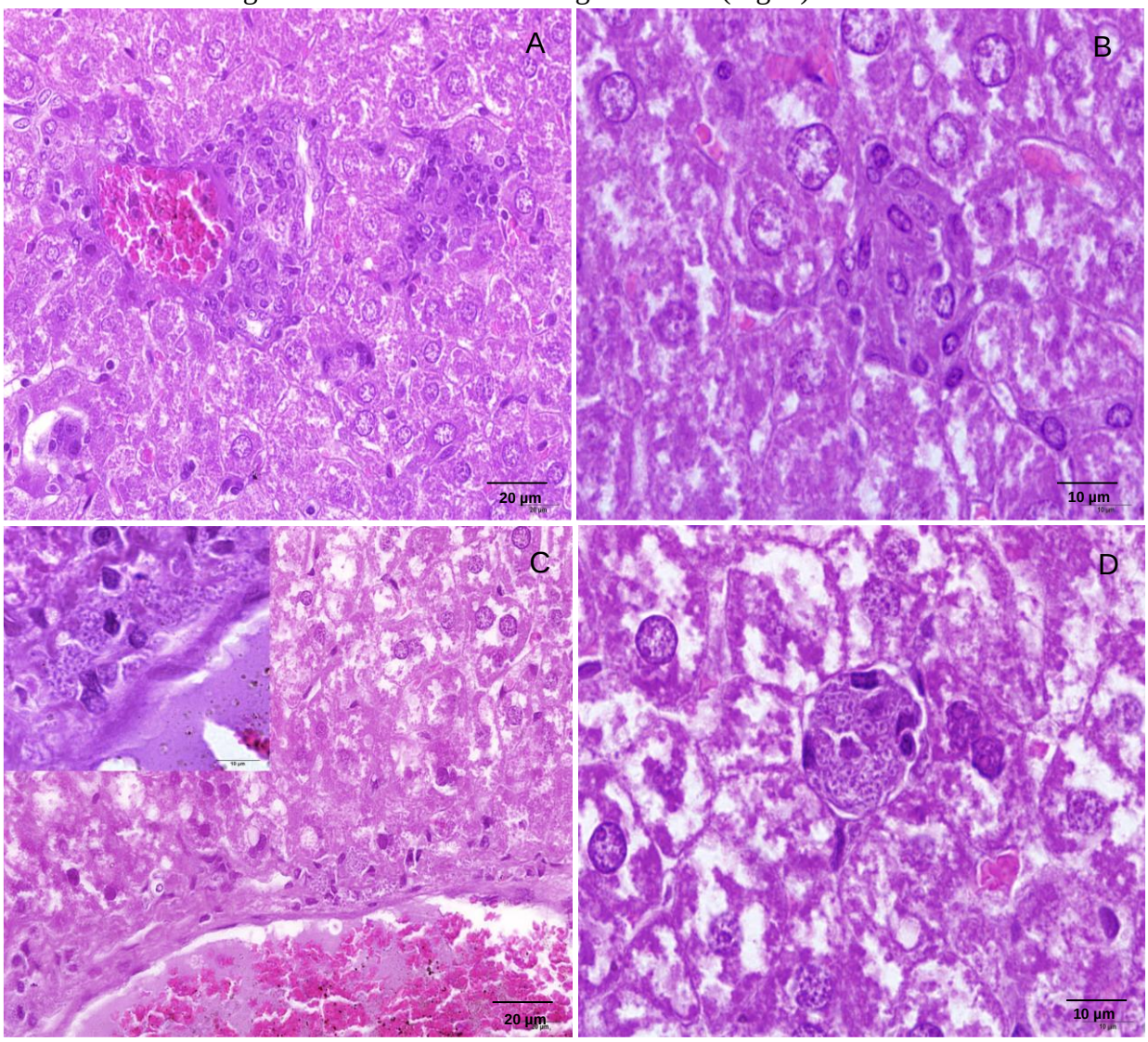


Figure 2. Histopathological analysis of BALB/c mouse liver sections, at 75 days post-infection, stained with HE. (A-B) Group III (infected). (A) Perivascular granuloma. (B) Granuloma with intracellular amastigotes. (C-D) Group IV (infected and immunosuppressed). (C) Minimal perivascular inflammatory reaction; in detail, amastigote forms. (D) Granulomas with intracellular amastigotes.

For Group IV, at 75 days, there was diffuse vacuolization of hepatocytes, minimal focal inflammatory reaction translated by groupings of parasitized lymphocytes and macrophages frequently associated with polymorphonuclear cells. Amastigotes were predominant in Kupffer cells (Fig. 2). There was similar inflammatory reaction around the vessels and the portal space.

For this same group, at 90 days, histopathological changes were discreet, compared to those at 75 days, and a multifocal inflammatory reaction of discreet intensity persisted; however, a more evident fibroblastic reaction could be detected as perivascular, portal and capsular, in association with intense and specially portal parasitism (Fig. 3).

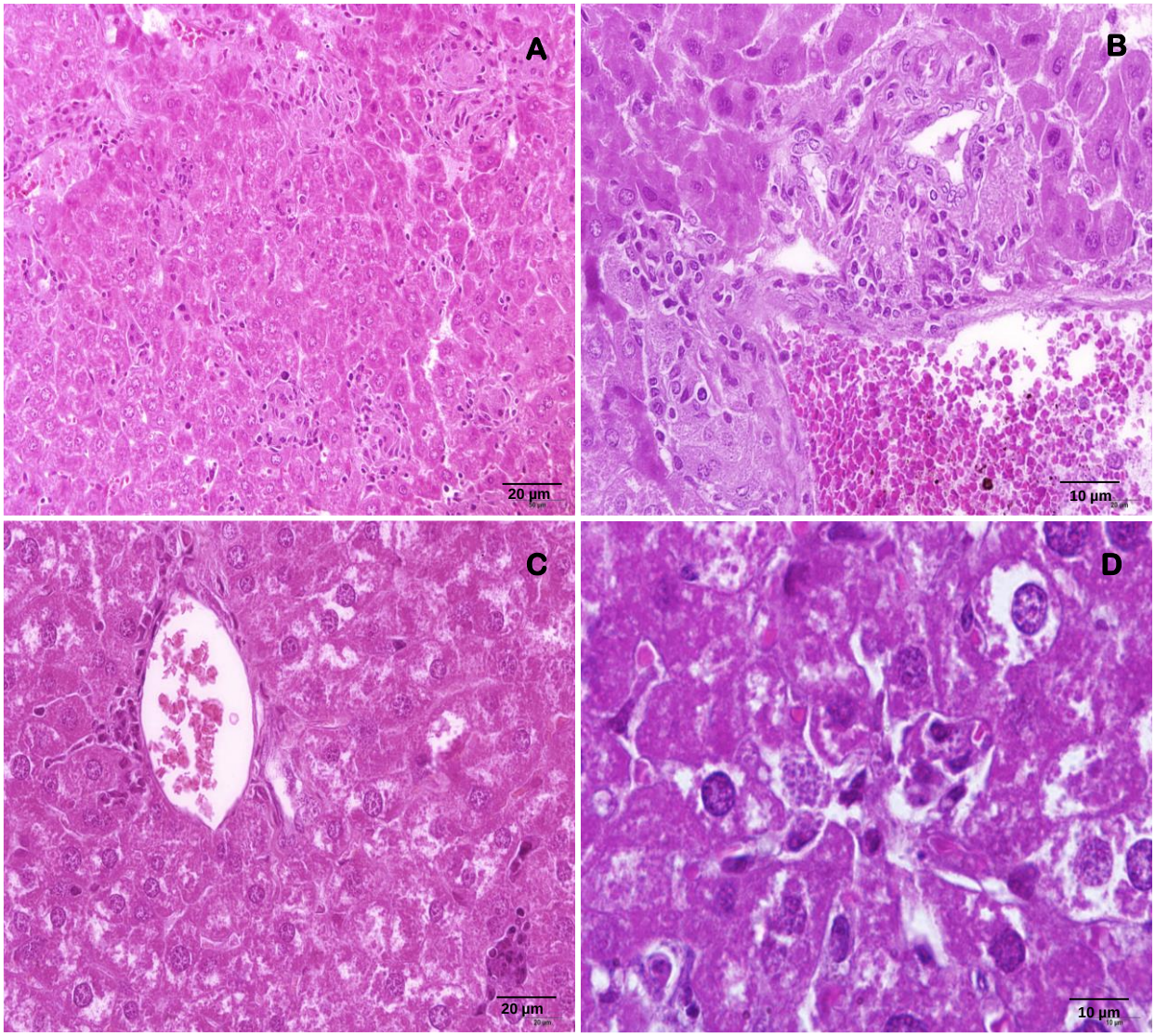


Figure 3. Histopathological analysis of BALB/c mouse liver sections, at 90 days post-infection, stained with HE. (A-B) Group III (infected). (A) Multifocal granuloma. (B) Perivascular granuloma with intracellular amastigotes. (C-D) Group IV (infected and immunosuppressed). (C) Minimal perivascular inflammatory reaction. (D) Minimal inflammatory reaction with intracellular amastigotes.

At 90 days, Group III had disseminated compromising of the hepatic parenchyma caused mostly by organized granulomas, but there were others with initial formation morphology translated by groupings of neutrophils surrounded by macrophages, lymphocytes and plasmocytes, besides epithelioid cells. Amastigotes were a constant in disseminated granulomatous inflammation foci. Fibroblastic proliferation was more marked in the composition of perivascular granulomas, spreading throughout the parenchyma and hepatic capsule of fibrotic aspect (Fig. 3).

Light microscopy of tissue sections subjected to immunohistochemistry reaction indicated positive labeling for amastigote forms of *L. (L.) chagasi* in the inoculated groups and analyzed moments (Fig. 4 and Fig. 5).

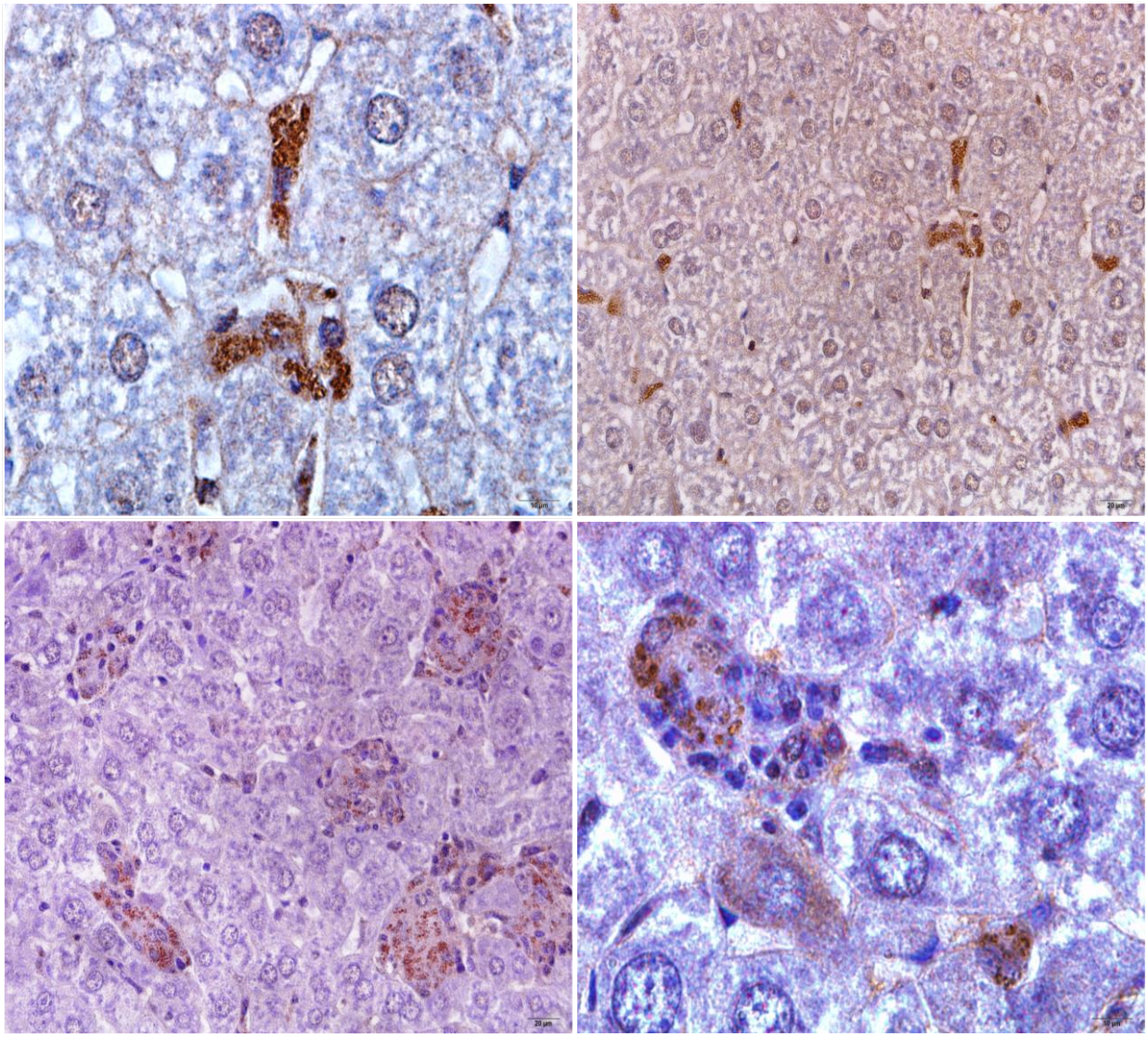


Figure 4. BALB/c mouse liver sections subjected to immunohistochemistry reaction. Detection of amastigote forms of *Leishmania (Leishmania) chagasi* immunolabeled in the cytoplasm of macrophages and Kupffer cells. (A-B) 15 days post-infection. (C) 30 days post-infection. (D) 60 days post-infection.

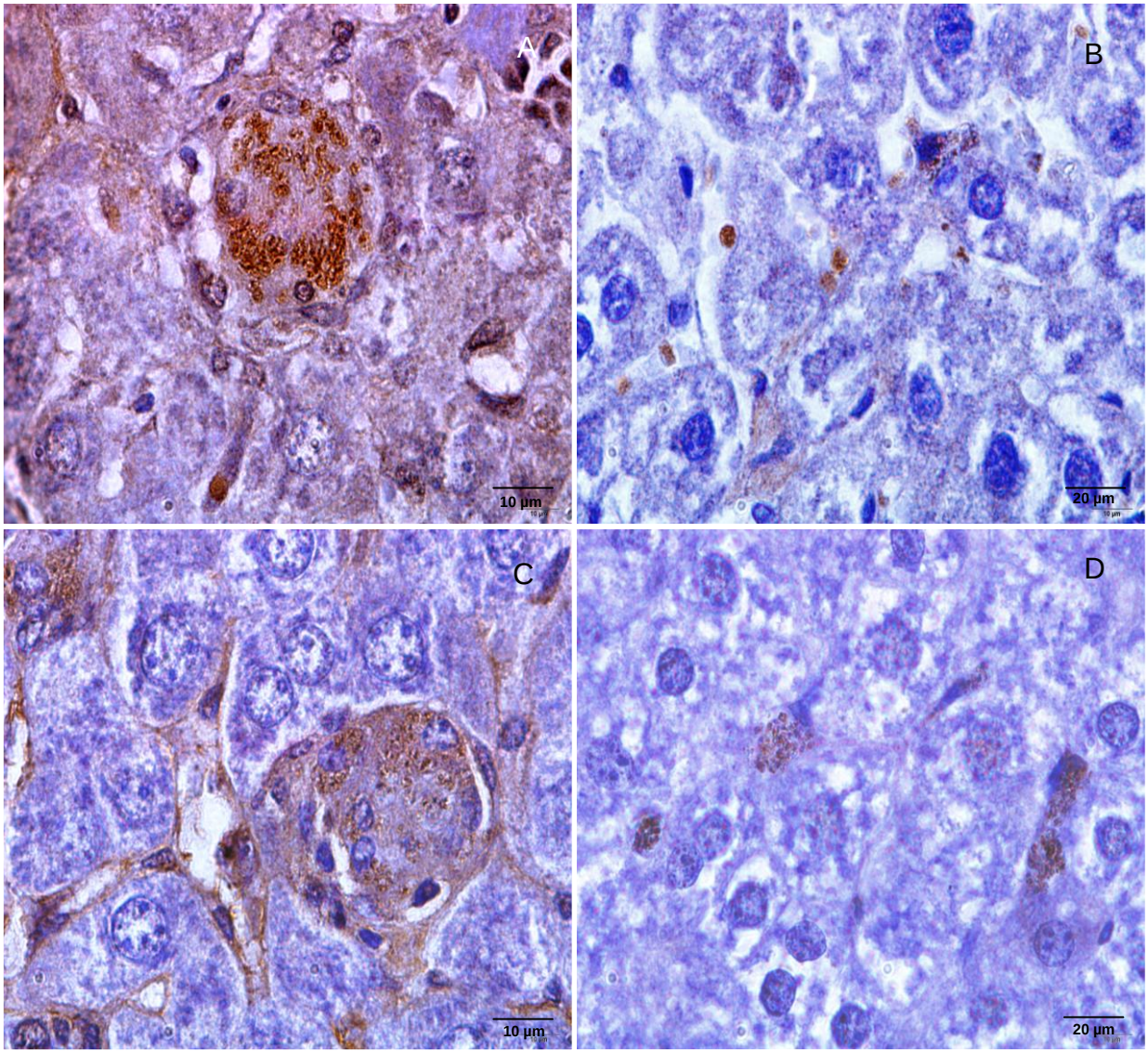


Figure 5. BALB/c mouse liver sections subjected to immunohistochemistry reaction. Detection of amastigote forms of *Leishmania (Leishmania) chagasi* immunolabeled in the cytoplasm of macrophages and Kupffer cells. (A-B) Groups III and IV (respectively), 75 days post-infection. (C-D) Groups III and IV, 90 days post-infection.

DISCUSSION AND CONCLUSION

All groups were analyzed at all moments, although Groups I and II were not infected and served as control of the organ structures, compared to Groups III and IV. Inflammatory reaction was observed for Groups III and IV at all moments, but for Group III it was intense and progressive until the last moment, while for Group IV it was weak on the 75th day P.I. and rare, but not absent, on the 90th day P.I. This could possibly be detected if the assessment had occurred later.

For infected animals, the inflammatory reaction was progressive, showing different characteristics, and the infection became chronic from the beginning of the focal inflammatory infiltrate to the complete formation of granulomas. This aspect was constant, corroborating Gutierrez et al. (27), who studied the dynamics of hepatic granuloma formation. Hyperplasia and hypertrophy of Kupffer cells, at every analyzed moment, is characteristic of systemic diseases, according to Mathias et al. (33). Macrokaryotic hepatocytes from 60 to 90 days P.I.

indicate regeneration of hepatic cells during infection with *L. (L.) chagasi*, characteristic of this organ.

Epithelioid cells were noted from 30 days P.I. to the end of the experiment, demonstrating the beginning of granuloma formation, when macrophages lose their phagocytic role and start to produce cytokines. Necrosis of hepatocytes in association with hepatic fibrosis may occur due to the action of TNF-α, produced in the hepatic granuloma (23) as well as to the release of fibrogenic cytokines (25).

The parasite densely populating macrophages and Kupffer cells was also a constant for Groups III and IV. At 90 days P.I., for Group IV, the parasite was in the light of large blood vessels, which probably explains the stimulus and the characteristics of the intense immune reaction in this organ. There was not morphological expression of diffuse hepatic fibrosis characteristic of the infection resolution in the liver, possibly due to the study period.

On the other hand, for the immunosuppressed group, we can consider that the observed changes were expected. Vacuolized cytoplasm is characteristic of the use of glucocorticoids; it occurs due to liquid accumulation and was also found for mice of Group II. Weak or rare inflammatory reaction, showing rare complete granulomas, is due to the anti-inflammatory action of DXM and PTX.

ACKNOWLEDGEMENTS

To CAPES for the doctoral scholarship.

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Recebido em: 11/07/2016
Aceito em: 11/04/2017