



Treatment with *Lycopodium clavatum* 200dH Intensifies Kidney and Liver Injury in Mice Infected with *Toxoplasma gondii*

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Abstract

The effects of infection with *Toxoplasma gondii* vary from asymptomatic to the development of alterations in various organs (including the liver and kidneys) which may be irreversible, and lead to the death of the host. Whereas homeopathy is an alternative and effective method for treating various diseases, including those caused by protozoa, we questioned the effect of using *Lycopodium clavatum* in mice infected with *T. gondii*. One hundred male Swiss mice, 60 days old, were divided into four groups ($n=25$ /group): NIC (uninfected and untreated control), IC (infected and treated with un-dynamized 7% alcohol solution [vehicle]), G48 (infected and treated 48 h before infection and treated three more times; at 2, 4, and 6 days post-infection (dpi) with *L. clavatum* 200dH), and G72 (infected and treated for 3 consecutive days before infection with *L. clavatum* 200dH). In this study, physiological, histopathological, and immunological parameters were evaluated. The *L. clavatum* 200dH intensified renal damage in mice infected with *T. gondii* from 7 dpi, causing severe and progressive alterations during this period, such as various degrees of inflammation, edema, atrophy, and tubular cystic dilation, degenerated tubules with intra-cytoplasmic vacuoles and coalescing spots, severe vascular lesions, glomerulonephritis, and peri-glomerular congestion. In the G72 animals, which received *L. clavatum* 200dH, more severe cortex damage was observed (91.66–96.66%) as compared to the IC group (55–80%) and more renal corpuscle, and renal tubule injury was observed (80 ± 5 to $96.7\% \pm 2.89$ of the total area) during all periods, as compared to the IC group ($p < 0.05$). Both groups presented high liver enzyme levels, and the highest values for AST were observable at 60 dpi. We observed significant increases of type I and III collagen, as well as high levels of TGF- β 1 in both organs of the treated animals, the main factor involved in fibrosis in areas damaged by the process. *L. clavatum* 200dH intensifies kidney and liver alterations in mice infected with *T. gondii*. Our results reinforce caution when indicating administration schemes and dosages for ultra-diluted drugs.

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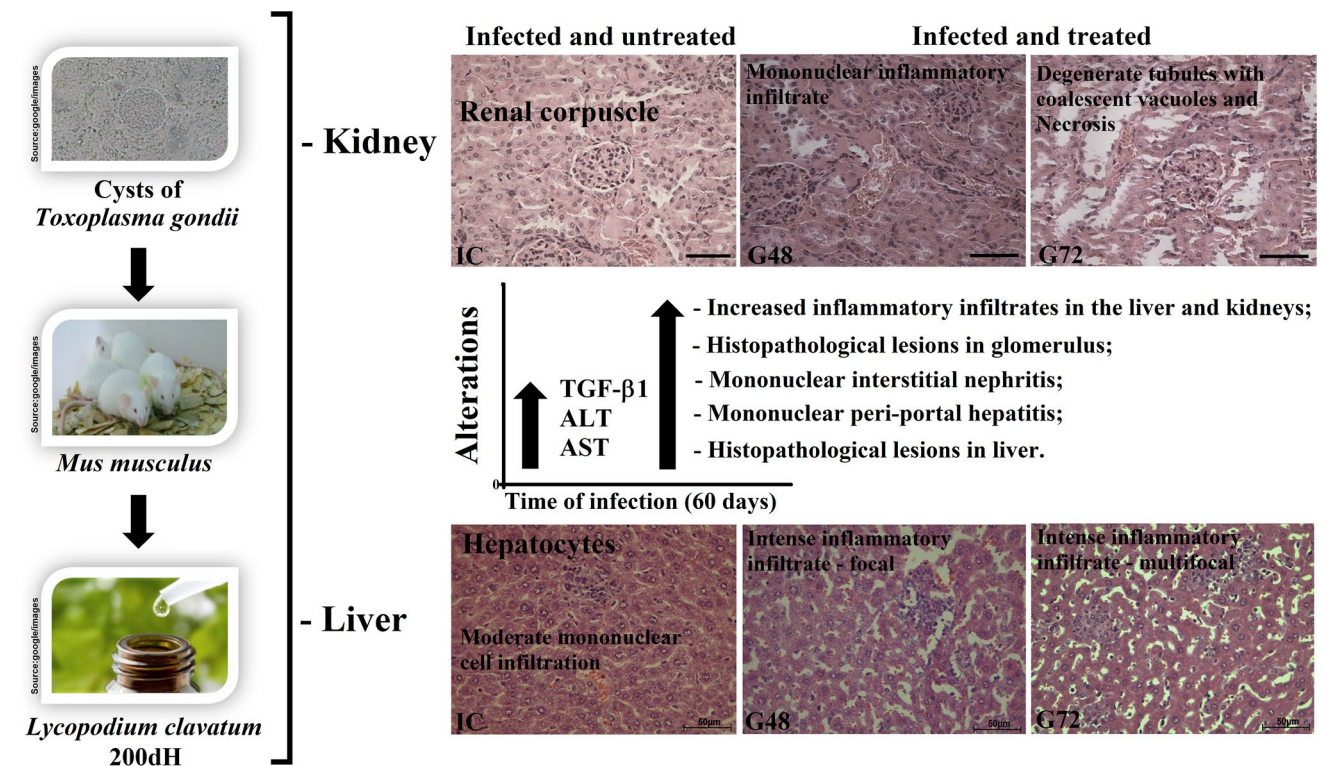
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Graphic Abstract



Keywords Toxoplasmosis · Histopathological · Phytotherapy · Homeopathy · Glomerulonephritis · Hepatitis

Introduction

The parasite *Toxoplasma gondii* can cause severe damage to the host's nervous system, triggering toxoplasmic encephalitis in the immunocompromised and lesions in the lymph nodes, eyes, heart, gastrointestinal tract, kidneys and liver (Alvarado-Esquivel et al. 2011; Góis et al. 2016; Pavanelli et al. 2018; Pereira et al. 2017; Toporovski et al. 2012; Vicentino-Vieira et al. 2015). Further, renal infarcts have also been described including fibro-necrotic lesions, congestion, edema, nephritic syndrome, glomerulonephritis, extra-medullary erythropoiesis, and adrenal necrosis (Toporovski et al. 2012). In the liver, *T. gondii* causes pathological changes such as hepatomegaly, granuloma, hepatic cirrhosis, hepatitis, and hepatic necrosis (Alvarado-Esquivel et al. 2011; Ortego et al. 1990; Ustun et al. 2004).

To halt the damage caused by *T. gondii*, effective alternative medicines with minimal side effects and low costs have been investigated (Braga-Silva et al. 2015; Değerli et al. 2003; El-Zawawy et al. 2015). Whereas homeopathy is an alternative and effective method for treating various diseases, including those caused by the protozoa *T. gondii* and *Trypanosoma cruzi* (Ferraz et al. 2016; Lopes et al. 2016;

Pathak et al. 2003; Pianetti et al. 2011), we questioned what would be the effects of using homeopathic medicine *Lycopodium clavatum* in mice infected with *T. gondii*.

Lycopodium clavatum is the most common species of the moss genus *Lycopodium* of the family Lycopodiaceae (Brasil 2011). It is widely used in popular culture, and widespread in the herbal industry as a diuretic and digestive (Grandi et al. 1989). In homeopathy, *L. clavatum* is dynamized [dilution and succussion (rhythmic agitations)] for the treatment of symptoms related to kidney and bladder inflammation, gallstones, cirrhosis, and liver failure (Boericke 1997; Brasil 2011; Henrique da Silva et al. 2015; Vijnovsky 2012). Its effectiveness has also been demonstrated in mice infected with *T. cruzi*; the authors observed that *L. clavatum*, dynamization 13cH [dilution and succussion (rhythmic agitations)], considerably reduced inflammatory alterations caused by the parasite (Falkowski-Temporini et al. 2016) and presented immunomodulatory effect (Aleixo-Brustolin et al. 2017). However, its mechanism of action against the inflammatory response is still being investigated.

Despite the above reports on the efficacy of homeopathic *L. clavatum* in various inflammatory processes (Pereira et al. 2017), its action in kidney and liver damage resulting from

T. gondii infection is still scarcely reported in the literature. In this sense, studies on its benefits and possible risks are needed. The aim of this study was to evaluate the effects of using *L. clavatum*, dynamization 200dH, administered in differing treatment regimens on parasitological and histopathological findings in kidneys and livers of mice infected with *T. gondii* strain ME49 (genotype II).

Materials and Methods

Ethical Aspects

All experimental procedures in this study were carried out in accordance with recommendations of Brazilian National Law # 11794 for the scientific management of animals, being approved by the Universidade Estadual de Maringá Ethics Committee on Research Involving Animal Experimentation, Registration # 093/2014.

Animals and Infection

The experiment was conducted in duplicate for a blind, controlled, and randomized study. To blind the experiment, the treatments were performed by a researcher who did not participate in the evaluation of parasitological and clinical parameters. Evaluating the parameters, each experimental group received a code such that the researchers were unaware of the treatment that was received by each group.

Swiss mice were divided into groups such that the mean weights of each group were statistically equal and the treatment that each group received was determined by random drawing. One hundred male Swiss mice (*Mus musculus*), 60 days old, with approximately 44.57 ± 1.05 g of body mass were obtained from the central Bioterium at the Universidade Estadual de Maringá. They were divided into four groups ($n=25$ /group): NIC (uninfected and untreated control), IC [infected and treated with 7% alcohol solution (vehicle)], G48 [infected and treated 48 h before infection and treated three more times on days 2, 4 and 6 post-infection (p.i.) with *L. clavatum* 200dH], and G72 (infected and treated for three consecutive days before infection with *L. clavatum* 200dH), to evaluate whether the highly diluted treatment regimen might inhibit visceral damage caused by the parasite after infection.

In this study, a 7% alcohol solution (vehicle) was used to treat the IC group because in a previous study of our group, it was demonstrated that a 7% alcohol solution and water, whether homeopathically succussed or un-succussed, did not cause alterations in the parasitological or clinical parameters in the experimental animal model (Ferraz et al. 2016).

To promote infection, *T. gondii* cysts were isolated from the encephalon of previously infected mice [used for

maintenance of the ME49 strain of *T. gondii* (genotype II)], as described by Dubey (1998a). Infection was performed by oral route in mice for the IC, G48, and G72 groups, which received 20 tissue cysts by gavage. The mice were maintained in the Bioterium sector at a constant temperature of 20 °C, and an alternating 12/12-h light–dark cycle. All the mice received commercial rodent feed (Nuvilab Cr-1® Nuvital®), and autoclaved water ad libitum (Pereira et al. 2017).

Lycopodium clavatum 200dH

Lycopodium clavatum was selected using homeopathic criteria through repertorization based on observations of healthy mice by three homeopathic doctors, one of whom was a veterinarian. We used Lynx Expert System software (Albuquerque, NM, USA) for this evaluation.

According to the repertorization performed by our team, *L. clavatum* tested in healthy mice stood out from the other medications, providing better animal health and was chosen as the constitutional medicine (Lopes et al. 2016; Pereira et al. 2017).

The medication was prepared from the mother tincture using 200dH dynamization [dilution and succussion (rhythmic agitations)], with 70% alcohol solution (Agro-Industrial Tarumã Ltda, São Pedro do Turvo, Brazil). The first 1:10 dilution (v/v) of the *L. clavatum* mother tincture (Deutsche Homöopathie-Union, Germany) was performed in 70% alcohol solution, with 100 succussions, resulting in dynamization 1dH; thus, the 1:10 dilution (v/v) plus 100 succussions of 1dH was used to produce dynamization 2dH. Dynamizations 3dH to 199dH were similarly prepared. The final dynamization, 200dH, was prepared in a 7% alcohol solution, and it was used to treat the animals. Mechanical dynamization was used (AUTIC Dynamizer, Model Denise, Campinas, Brazil). Dynamization 200dH was selected in accordance with the results obtained by Braga-Silva et al. (2015).

Treatment with *L. clavatum* 200dH

The treatment with *L. clavatum* 200dH was performed in the G48 and G72 groups, orally, diluted in the drinking water (1 mL of *L. clavatum* 200dH/100 mL of autoclaved water) in accordance with Aleixo et al. (2012). The treatment remained available for 16 h during the treatment days, starting at 5:00 pm to 9:00 am in both groups (Pereira et al. 2017).

The evaluation of *L. clavatum* 200dH consumption was performed according to Lopes et al. (2016), by measuring the amount of provided liquid to the ingested quantity. The mice were observed for 15 min, checking the frequency of water intake, which ones were going to the water bottle, both

in the morning and at night, the periods in which they have more activity. It was observed that during these periods all of them had access to the bottles. This process was performed for three consecutive days.

Sample Collection

On days 7, 15, 30 and 60 p.i., five mice from each group were euthanized by deep anesthesia intraperitoneally (IP) with ketamine 90 mg/kg + xylazine 13 mg/kg (König SA[®], Avellaneda, Argentina) (Close et al. 1996). Blood was collected by cardiac puncture. After necropsy, the kidneys and liver were removed, washed and fixed in 10% buffered formalin for 24 h.

Enzymatic Markers of Liver Injury

The blood was collected in microtubes containing 50 µL of the anticoagulant ethylenediaminetetraacetic acid (EDTA) (5000 IU/mL) and centrifuged (200 g, 10 min, 4 °C) for plasma separation. The levels of the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were used as hepatocyte damage markers, and determined in plasma by a colorimetric assay using a LABORCLIN diagnostic kit (Bioloquid-Brazil). Results were presented as U/L (Hohmann et al. 2013).

Histopathology

After fixation of the livers and kidneys, the organs were dehydrated in an increasing aqueous ethanol series (70, 80, 90, and 99.6%), diaphanized in xylene and embedded in paraffin. Semi-serial 5-µm sections were obtained by microtome (MAINZ SLEE CUT 5062; Mainz, Germany).

Histopathological analysis was performed on slides with hematoxylin and eosin (HE)-stained kidney and liver sections. To measure inflammatory parameters in the evaluated organs, 30 fields of each cut, in a trinocular light microscope (Olympus BX50; Minato-Ku, Japan) coupled to a video camera (3CCD Pro-series), in high resolution were used.

To evaluate the renal cuts, two renal structures, renal corpuscle and tubules, were analyzed. The total number of clusters, the injured renal corpuscle number, and pathological changes in each cut were counted in each image. The renal corpuscle was considered normal when the capillary loops were open with thin walls, and when the Bowman's capsule was empty (Meyer et al. 2010). The renal corpuscle was considered damaged when presenting glomerular contraction with a closing in of structures inside Bowman's capsule, or with vacuolization of endothelial cells. The degree of renal corpuscle injury was classified according to Meyer et al. (2010) as mild (up to 25% of affected glomeruli), moderate (26–50% of affected glomeruli), high (51–75% of affected

glomeruli), or severe (over 75% of injured glomeruli). Inflammatory liver infiltrates were quantified based on the following classification:

1. Intensity: according to the number of inflammatory cells observed: absent (0–9 cells), discreet (10–25 cells), moderate (26–50 cells), and intense (> 50 cells) (Pereira et al. 2017).
2. Distribution of inflammatory infiltrate: distribution by field: focal: a single infiltration in the visual field; multifocal, more than one infiltrate per visual field, diffuse: inflammatory cells diffusely distributed in the visual field (Pereira et al. 2017).

The sections stained with Picrosirius were used for searching and quantification of type I and III collagen fibers, in the liver and kidney. Four images of the slides stained with Picrosirius, from four sections of each mouse, were analyzed by the Image Pro Plus software (4.5 NIH version, USA—2011). The images were obtained through trinocular light microscope with polarization filter (Olympus BX50; Minato-Ku, Japan) coupled to a high-resolution video camera (3CCD Pro-series).

Immunohistochemistry for Transforming Growth Factor β 1

Tissue sections (5 µm in thickness) were obtained from each mouse kidney and liver and were mounted onto slides for immunohistochemical examination. All immunohistochemical tests were performed using a commercial streptavidin–biotin kit (Invitrogen[™] Histostain[®]—Plus) as described by Li et al. (2015) and Pereira et al. (2017).

To determine the number of immunoreactive cells we used five microscopic fields (Motic) with a 400× objective, which has an area of 0.2488 mm². The renal corpuscle and tubular regions were considered. In the liver, the choice of areas was random. From the values in these fields, an average of the number of immunoreactive cells per tissue fragment of each animal was calculated, and these averages were evaluated for each group (Moreira et al. 2010).

Statistical Analysis

Data were initially submitted to the Shapiro–Wilk or D'Agostino Pearson test to verify the distribution type. Statistical analysis was performed using a one-way analysis of variance (ANOVA). Variables with a normal distribution were analyzed using one-way analysis of variance (ANOVA) followed by the Bonferroni test or Tukey's test. The variables presented non-normal distribution, and the Mann–Whitney test *U* was performed. The results are expressed as mean $\pm$ SD. The data were compared using BioEstat 5.4

software (Mamirauá®), $p < 0.05$ was considered statistically significant.

Results

During the macroscopic kidney analyses, no animal of the evaluated groups presented organ color changes in the period evaluated. In the microscopic analysis, the mice of the IC group presented histological changes consistent with mononuclear interstitial nephritis, diffusely distributed and degenerated tubules with coalescent vacuoles and renal corpuscle changes on days 7, 15, 30 p.i., intensified on day

60 p.i. (Fig. 1a, d). The animals treated with *L. clavatum*, groups G48 and G72, Fig. 1b, c, respectively, presented similar histopathological changes and interstitial mononuclear nephritis characteristics, diffusely distributed, with or without edema, atrophy, and tubular cystic dilation on day 7 p.i. The tubules were degenerated and presented intra-cytoplasmic vacuoles with coalescing spots, and severe vascular lesions, resulting in an intense congestion which remained up to day 60 p.i. Further, we saw a significant intensification of the framework of changes in the cortex of the kidneys in the G72 group (91.66–96.66%) compared to the G48 group (61.67–76.66%), and the IC group (55–80%) in all evaluation periods ($p < 0.05$) (Fig. 1d).

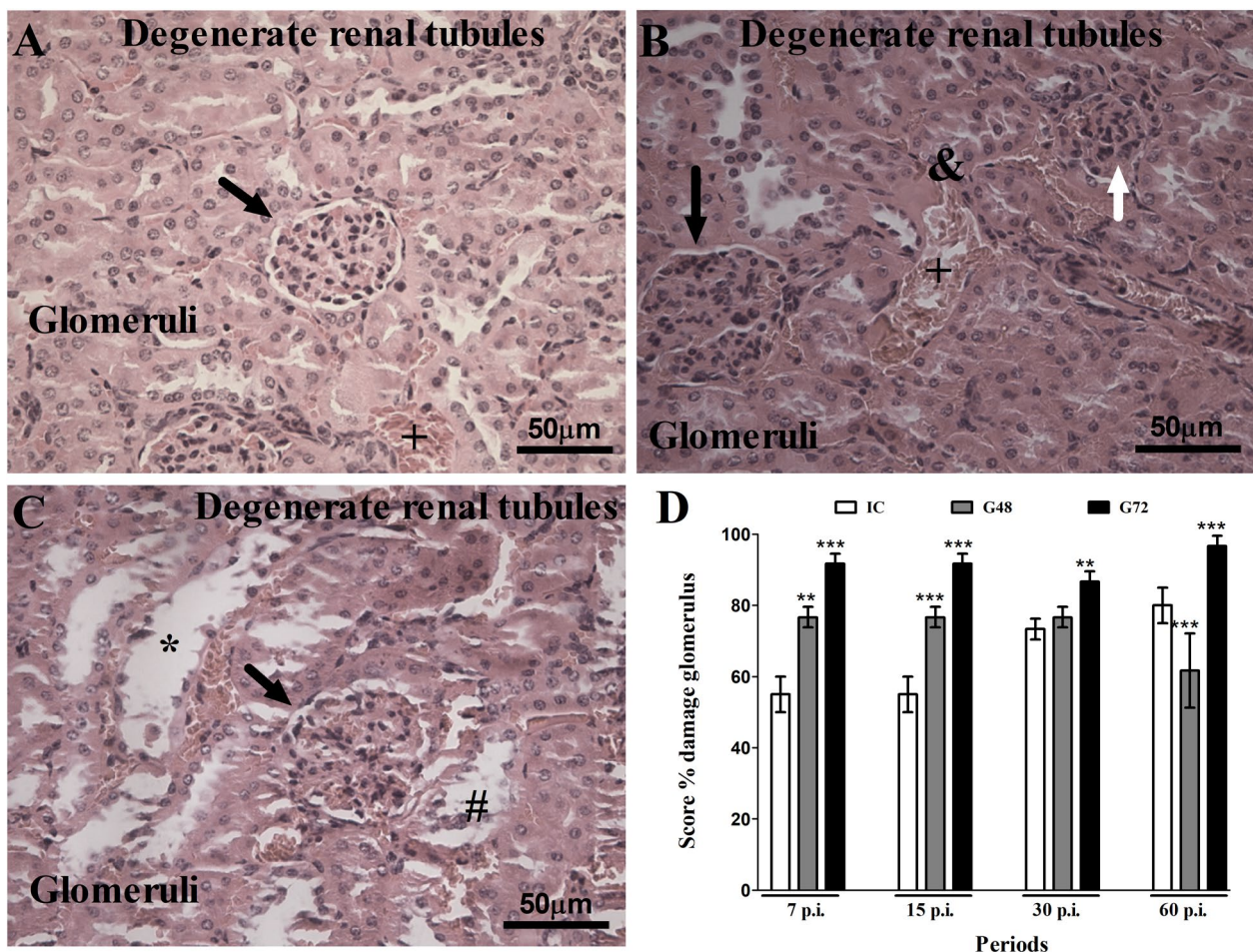


Fig. 1 Kidney changes in mice experimentally infected with 20 tissue cysts of *T. gondii* (ME49 strain, genotype II) and treated with *L. clavatum* 200dH. (a—IC, 60 p.i.) Degenerate tubules with coalescent vacuoles around the renal corpuscle with contraction of the capillary tuft and thickening Bowman's capsule (black arrow), and intense congestion (+). (b—G48) Edema deposition (&), severe congestion (+), degenerated tubules with intra-cytoplasmic vacuoles throughout the field, predominantly moderate lymphocytic (white arrow) mononuclear inflammatory infiltrate, and renal corpuscle with inflammatory mononuclear cell (black arrow) infiltrate. (c—G72) Degenerate

tubules with coalescent vacuoles (*) and contraction of the capillary tuft (black arrow), and necrosis (#). Histological HE stain, magnitude $\times 400$. **d** Percentage of injuries in renal corpuscle in mice. Asterisk (*) indicates a significant difference compared to the IC for each period. ANOVA—one way followed by Bonferroni test; $\alpha = 0.05$. IC [infected and treated with un-dynamized 7% alcohol solution (vehicle)]. G48 (infected and treated 48 h before infection and treated three more times; at 2, 4, and 6 p.i. with *L. clavatum* 200dH). G72 (infected and treated for three consecutive days before infection with *L. clavatum* 200dH)

For the renal corpuscle in the G48, G72, and IC groups, histopathological changes were observed from day 7 (p.i.) including various degrees of inflammation, featuring a glomerulonephritis framework, and peri-glomerular congestion. Thickening of the renal corpuscle, with internal inflammatory cells was observed in all infected groups. The degree of injury, in the renal corpuscle worsened with time of infection. The mice from the IC group presented marked damage lesions on days 7 and 15 p.i. ($55\% \pm 5$), severe on day 30 p.i. ($73.30\% \pm 2.88$), reaching $80\% \pm 5$ of the renal corpuscle injured at 60 days post-infection (dpi) ($p > 0.05$) (Fig. 1d). The mice of the G72 group presented the worst renal corpuscle injury framework, as well as for the renal tubules, with severe damage in the renal corpuscle (80 ± 5 to $96.7\% \pm 2.89$ of the total area) in all periods, significantly differing from the IC group ($p < 0.05$). The animals of group G48 presented severe lesions ($76.7\% \pm 2.9$) from the day 7 p.i. However, the framework remained stable until day 30 p.i., with significantly less degrees of injury on day 60 p.i. ($61.7\% \pm 10.4$) compared with the IC group ($p < 0.05$) (Fig. 1d).

We observed significant differences regarding collagen deposition mainly for the *L. clavatum* treated groups in G72

on day 7 p.i. (4.6 ± 0.5), 15 p.i. (5.8 ± 1.1), 30 p.i. (5.4 ± 1.3), and 60 p.i. (5.7 ± 1.5) compared with the IC (2.8 ± 0.5) group ($p < 0.05$) (Fig. 2a) and transforming growth factor (TGF)- $\beta 1$ expression in the damaged areas, differentiated from uninfected control group ($p < 0.05$) (Fig. 6).

We found that in the period from day 7 p.i., the G48 (12.0 ± 1.7) and IC (19.3 ± 3.0) groups presented higher production of TGF- $\beta 1$ similar to the TGF- $\beta 1$ group as compared to the NIC group (6.6 ± 1.1) ($p < 0.05$) (Fig. 6e). On days 15, 30, and 60 p.i., production of TGF- $\beta 1$ increased similarly in IC (17.6 ± 4.5 ; 23.3 ± 5.7 and 23.0 ± 2.6), G48 (20.7 ± 2.2 ; 23.6 ± 4.0 and 25.0 ± 2.0), and G72 (17.3 ± 0.5 ; 20.3 ± 2.5 and 25.0 ± 1.0) groups, compared to the NIC (6.6 ± 1.1) group ($p < 0.05$) (Fig. 6e).

In the macroscopic examination of the liver, no animal from the evaluated groups presented changes in organ color or texture during the periods. When evaluated microscopically, the animals of the G48, G72, and IC groups presented liver inflammation with increases in intensity and distribution. The IC mouse group presented a progressive framework of liver injury with focal distribution, and some intensity to 7° dpi (18.7 ± 0.6); multifocal lesions with moderate

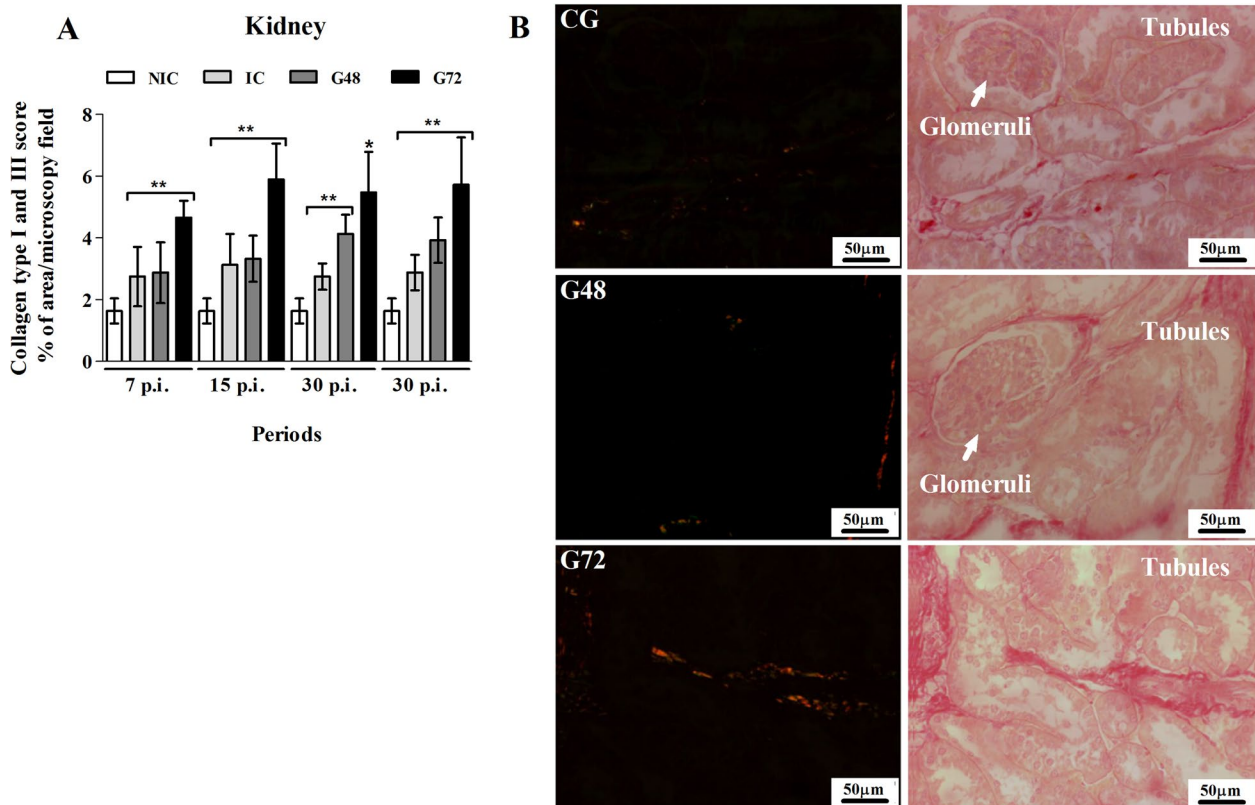


Fig. 2 **a** Quantification of collagen deposition in the kidney sections. The data represent the mean $\pm$ SD of five mice per group. (*) significantly different from control IC ($p < 0.05$). **b** Photomicrographs of kidney sections stained with Picrosirius technique, magnitude $\times 200$. One-way ANOVA followed by Mann–Whitney test U ; $\alpha = 0.05$. NIC

(uninfected and untreated control). IC [infected and treated with undynamized 7% alcohol solution (vehicle)]. G48 (infected and treated 48 h before infection and treated three more times; at 2, 4, and 6 p.i. with *L. clavatum* 200dH). G72 (infected and treated for three consecutive days before infection with *L. clavatum* 200dH)

intensity (27 ± 2) on day 15 p.i., and on day 30 p.i. (25.6 ± 3) ($p > 0.05$) (Fig. 3e).

The G48 group presented on day 7 p.i. a greater number of inflammatory infiltrates with focal and discrete distribution (24.33 ± 1.52), which differed significantly from the G72 (18 ± 1), and IC (18.66 ± 1) groups ($p < 0.05$). This worsened significantly in the G48 group on day 15 p.i., with multifocal infiltrates of moderate intensity (33.33 ± 3.05), different from the IC (27 ± 2) group ($p < 0.05$). On day 30 p.i., the G48 group presented progressive worsening of the inflammatory process (41.33 ± 3) differing from the IC (25.66 ± 3) and G72 (24.33 ± 3) groups ($p < 0.05$). The G72 group presented (on day 7 p.i.) infiltration numbers similar to those observed in the IC group (18.33 ± 1), with a mild to moderate focal distribution, and lower than that in the G48 group (24.33 ± 1.52 ; $p > 0.05$). On day 15 p.i., G72 presented a greater number of inflammatory infiltrates (33.33 ± 2) differing from both G48 (31.66 ± 2), and IC (27 ± 2) groups ($p < 0.05$). On day 30 p.i., the G72 group presented a decrease in the number of inflammatory infiltrates (24.33 ± 3) when compared to G48 (41.33 ± 3), and did not differ ($p > 0.05$) from IC (25.66 ± 3). At 60 p.i., the number of infiltrates increased to G72 (31.6 ± 3.5) and decreased in G48 (31.6 ± 3), differing ($p < 0.05$) from IC (25.6 ± 1.5) (Fig. 3e).

In all groups (IC, G48 and G72), Fig. 3b, c, d, respectively, for all evaluated periods, we observed scattered infiltrates throughout the parenchyma and around the blood vessels, being intense at the portal space, followed by nuclear gigantism and cell hypertrophy in the fields evaluated, characterizing peri-portal mononuclear hepatitis which ranged from moderate to severe for all the groups.

On day 60 p.i., in the liver of IC, G72, and G48 mice groups (Fig. 4), we observed mild diffuse inflammation, which because of the score ratio used did not allow quantification. In all groups, we observed the presence of edema and moderate to severe congestion in the hepatic artery portal space, being more evident in the G48 group. We observed significant differences regarding the collagen deposition mainly for the treated groups *L. clavatum* in G72: on days 7 p.i. (5.2 ± 0.9), 15 p.i. (6.4 ± 0.4), 30 p.i. (7.0 ± 1.2) and 60 p.i. (7.1 ± 0.1) compared with the IC (4.2 ± 0.5) group ($p < 0.05$) (Fig. 4a).

In biochemical analyses, we found that the levels of liver enzymes ALT and AST rose due to *T. gondii* infection in all periods for the IC G48 and G72 groups, compared to the NIC group (Fig. 5a). ALT levels on day 7 p.i. presented no significant difference between the IC, G48, and G72 groups. However, they did show higher levels when compared to the NIC group. On day 15 p.i. the G48 (0.12 ± 0.02) and G72 (0.12 ± 0.04) groups presented higher levels of this enzyme when compared to the NIC (0.03 ± 0.02) and IC groups (0.04 ± 0.02) ($p < 0.05$). By contrast, IC results did

not differ from NIC results in this period. On days 30 and 60 p.i., IC (0.04 ± 0.02), G48 (0.12 ± 0.0), and G72 (0.1 ± 0.0 and 0.1 ± 0.0) groups maintained high enzyme levels in these periods compared to the NIC group (0.03 ± 0.02) ($p < 0.05$).

AST levels (Fig. 5b) on days 7 p.i. [G48 (3.6 ± 1.3)] and [G72 (3.8 ± 2.4)], 15 p.i. [G48 (6.4 ± 0.6)] and [G72 (5.3 ± 0.5)], 30 p.i. [G48 (5.4 ± 1.0)] and [G72 (5.0 ± 0.3)], and in the IC (3.6 ± 0.3) group were significantly higher when compared to the NIC group, the AST levels of groups were especially high from day 60 p.i., compared to the NIC group (0.7 ± 0.6) ($p < 0.05$). However, it is interesting to note that the groups treated with *L. clavatum* G48 (6.2 ± 1) and G72 (5.1 ± 1.3) presented higher levels of this enzyme on day 60 p.i., differing significantly from both the NIC (0.7 ± 0.6) and IC (3.6 ± 0.3) groups ($p < 0.05$).

Knowing that the presence of TGF- β 1 is directly associated with the process of fibrosis and consequent progression of organ dysfunction, we evaluated its presence in the liver and kidney of the studied animals. We found that on day 7 p.i., the G72 (13.1 ± 0.2) group presented similar TGF- β 1 production as compared to the NIC (4.0 ± 1.0) group, while the IC (10.4 ± 0.9) and G48 groups presented an increase of this cytokine compared to the NIC group ($p < 0.05$) (Fig. 6e).

On days 15, 30, and 60 p.i., production of TGF- β 1 (kidney; Fig. 7e) increased similarly to the values seen in the liver for all periods: IC (10.4 ± 0.9 ; 8.3 ± 1.5 ; 13.3 ± 3.2 and $12.3.0 \pm 2.0$); G48 (12.0 ± 1.0 ; 9.0 ± 1.0 ; 14.3 ± 1.5 and 13.0 ± 1.0); G72 (13.1 ± 0.2 ; 8.6 ± 1.1 ; 11.3 ± 0.6 and 10.0 ± 0.6) groups, compared to the NIC (4.0 ± 1.0) group ($p < 0.05$).

Discussion

Taken together, our results showed that *T. gondii* caused severe lesions in the liver and kidneys, and *L. clavatum* treatment did not prevent damage. Pereira et al. (2017) observed pathological changes in mice infected with *T. gondii*; clinical findings such as moderate mononuclear multifocal interstitial encephalon infiltrate and histopathological heart lesions. Likewise, Mattos et al. (2009) observed renal corpuscle lesions and nephritis with perivascular inflammatory infiltrate in mice infected with the ME49 strain. Giraldo et al. (2002) evaluated dogs infected with *T. gondii* and found that 22 or 58% presented chronic meningitis associated with interstitial renal plasma cell infiltrate and membrane-proliferative glomerulonephritis.

Pereira et al. (2017), studying the role of homeopathic medicines in toxoplasmosis, found that they can aggravate symptoms and clinical signs of the disease, leading to intensification when administered in higher doses or dynamizations than indicated, or if administered too often or when therapy was not required.

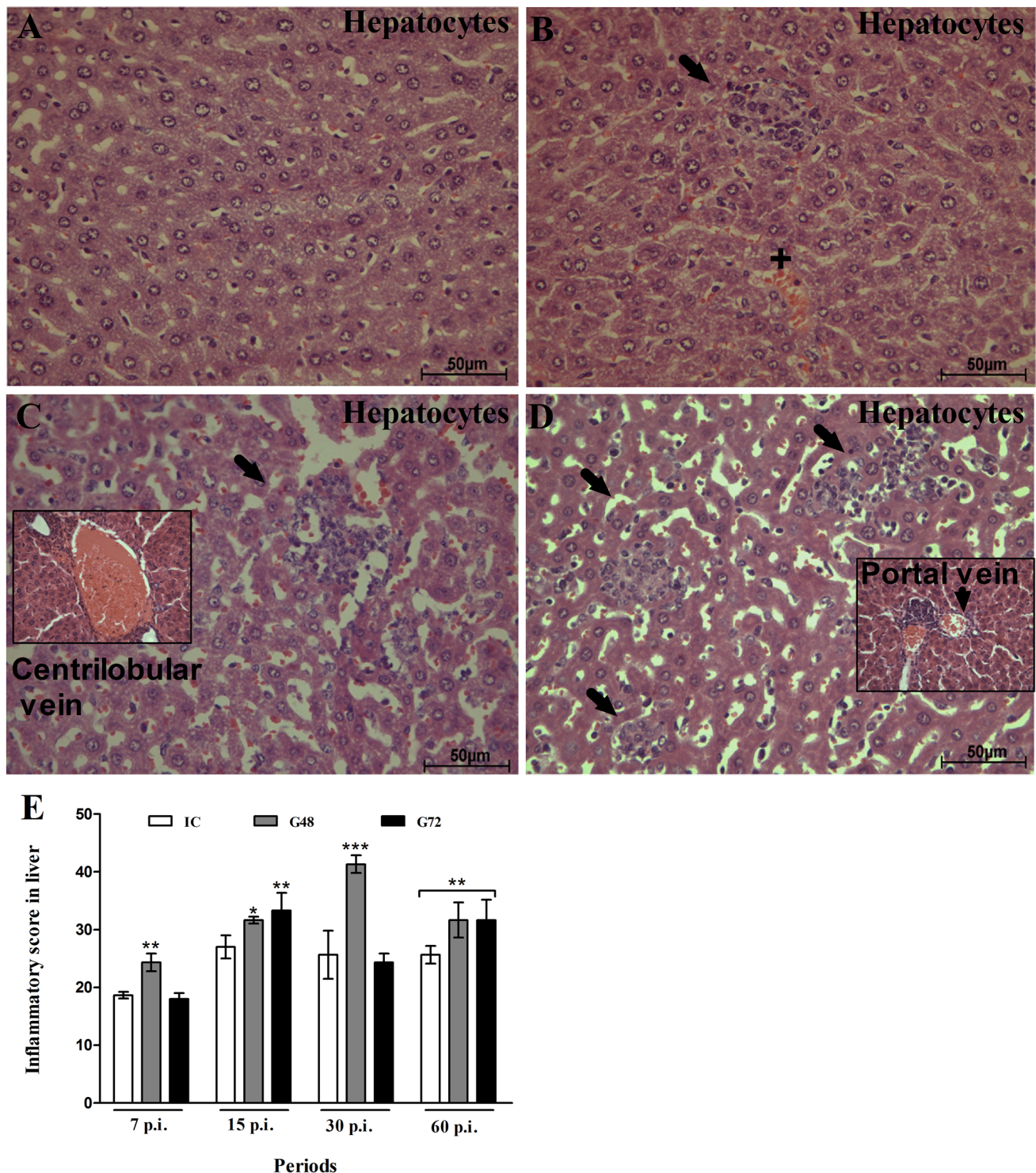


Fig. 3 Hepatic changes in mice, experimentally infected with 20 tissue cysts of *T. gondii* (ME49 strain, genotype II) and treated with *L. clavatum* 200dH, from day 30 p.i. (**a**—NIC) No inflammatory infiltrate. (**b**—IC) Moderate mononuclear cell infiltration (black arrow) and intense congestion (+). (**c**—G48) Three mononuclear cell infiltrates (heavy, moderate and discrete) in the same field (black arrow) and centrilobular vein. (**d**—72) Mononuclear inflammatory infiltrates, intense focal dispersed through the parenchymal (black arrow) and portal vein. Coloration in HE, magnitude $\times 400$. **e** Average number

of inflammatory infiltrates in the liver of mice. Asterisk (*) indicates significant difference compared to the IC group. ANOVA—one-way Bonferroni post-test; $\alpha=0.05$. NIC (uninfected and untreated control). IC [infected and treated with un-dynamized 7% alcohol solution (vehicle)]. G48 (infected and treated 48 h before infection and treated three more times; at 2, 4, and 6 p.i. with *L. clavatum* 200dH). G72 (infected and treated for three consecutive days before infection with *L. clavatum* 200dH)

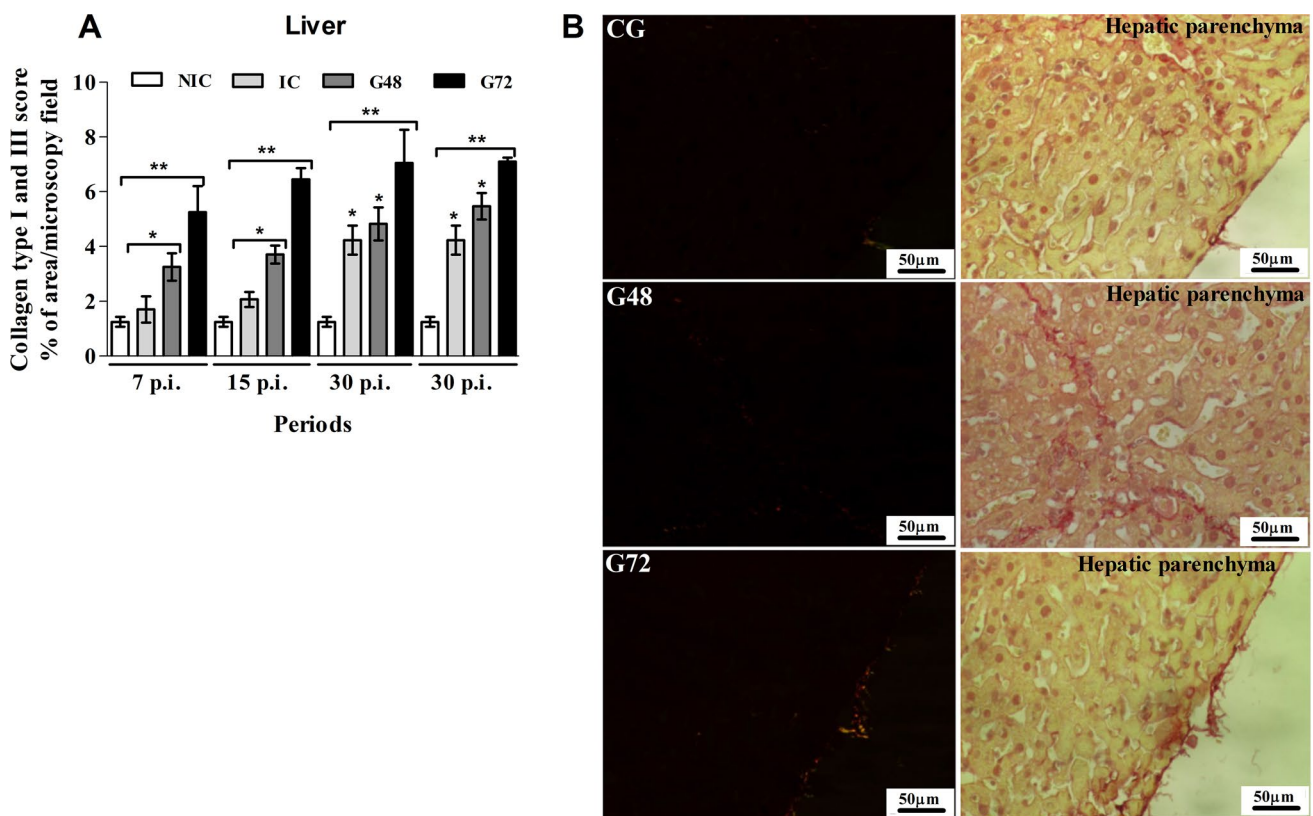


Fig. 4 **a** Quantification of collagen deposition in the liver sections. The data represent the mean $\pm$ SD of five mice per group. *Significantly different from control IC ($p < 0.05$). **b** Photomicrographs of liver sections stained with Picosirius technique, magnitude $\times 200$. One-way ANOVA followed by Mann–Whitney test U ; $\alpha = 0.05$. NIC

(uninfected and untreated control). IC (infected and treated with undynamized 7% alcohol solution [vehicle]). G48 (infected and treated 48 h before infection and treated three more times; at 2, 4, and 6 p.i. with *L. clavatum* 200dH). G72 (infected and treated for three consecutive days before infection with *L. clavatum* 200dH)

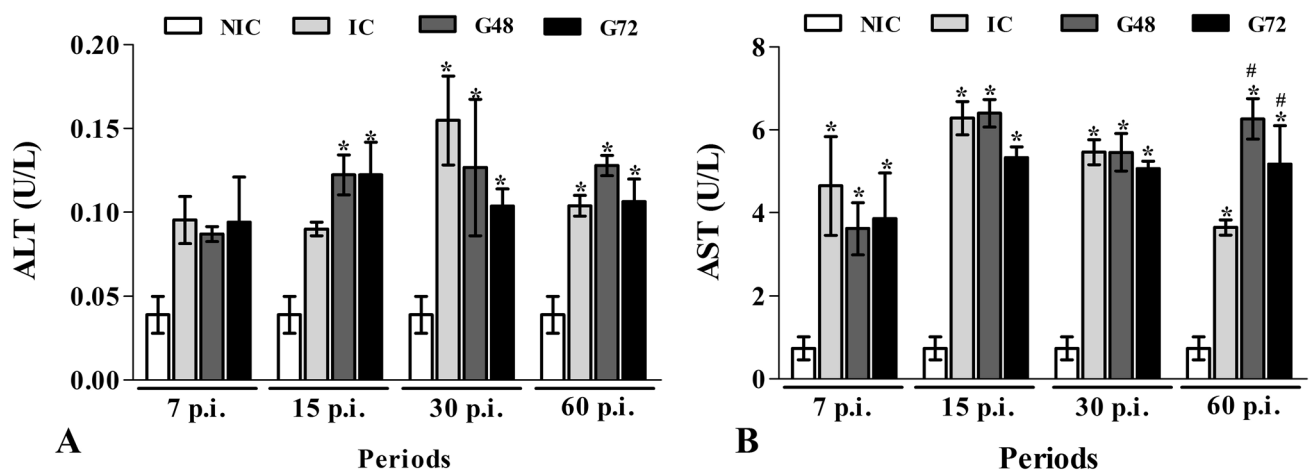


Fig. 5 Kinetics of levels of **a** alanine aminotransferase (ALT) and **b** aspartate aminotransferase (AST) in blood plasma of mice experimentally infected with 20 tissue cysts of *T. gondii* (ME49 strain genotype II) and treated with *L. clavatum* 200dH. Asterisk (*) indicates significant difference compared to the NIC; (#) relative to the IC group. ANOVA—one-way, Bonferroni post-test; $\alpha = 0.05$. NIC

(uninfected and untreated control). IC [infected and treated with undynamized 7% alcohol solution (vehicle)]. G48 (infected and treated 48 h before infection and treated three more times; at 2, 4, and 6 p.i. with *L. clavatum* 200dH). G72 (infected and treated for three consecutive days before infection with *L. clavatum* 200dH)

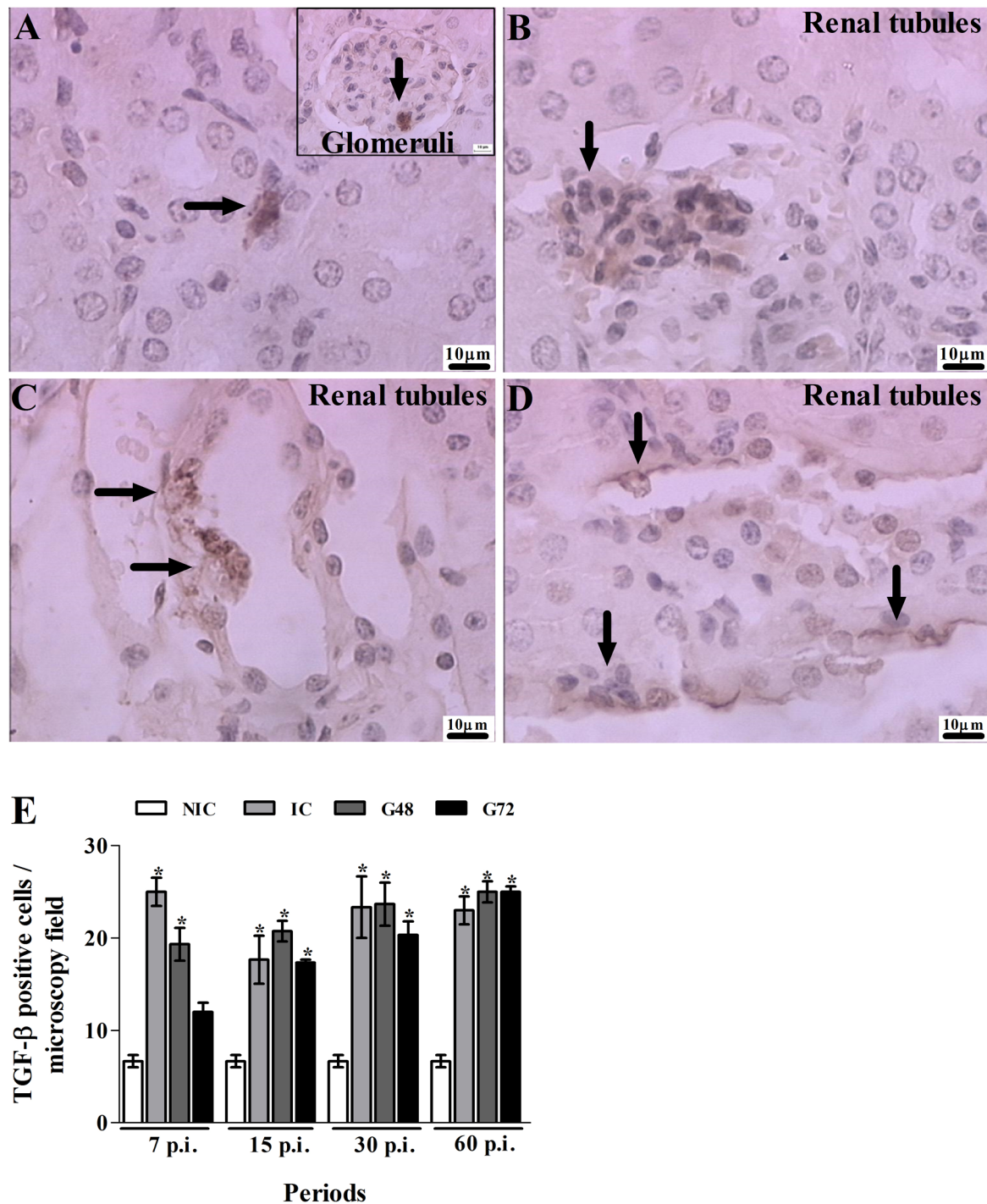


Fig. 6 Immunohistochemical staining for TGF- β 1 in the kidney. (a—NIC; 30 p.i.) Uninfected and untreated control. (b—IC; 30 p.i.) The presence of inflammatory infiltrate denoting mild immunostaining for TGF- β 1 (black arrow). (c—G48; 30 p.i.) Mild immunostaining for TGF- β 1 (black arrow). (d—G72; 30 p.i.) Mild immunostaining for TGF- β 1 (black arrow). e Mean $\pm$ SD involving the pattern of immunostaining for TGF- β 1 in mice kidneys infected with *T. gondii* and treated with *L. clavatum* 200dH. Asterisk (*) indicates signifi-

cant difference compared to the NIC. ANOVA—one-way test Bonferroni post-test; $\alpha=0.05$. NIC (uninfected and untreated control). IC [infected and treated with un-dynamized 7% alcohol solution (vehicle)]. G48 (infected and treated 48 h before infection and treated three more times; at 2, 4, and 6 p.i. with *L. clavatum* 200dH). G72 (infected and treated for three consecutive days before infection with *L. clavatum* 200dH)

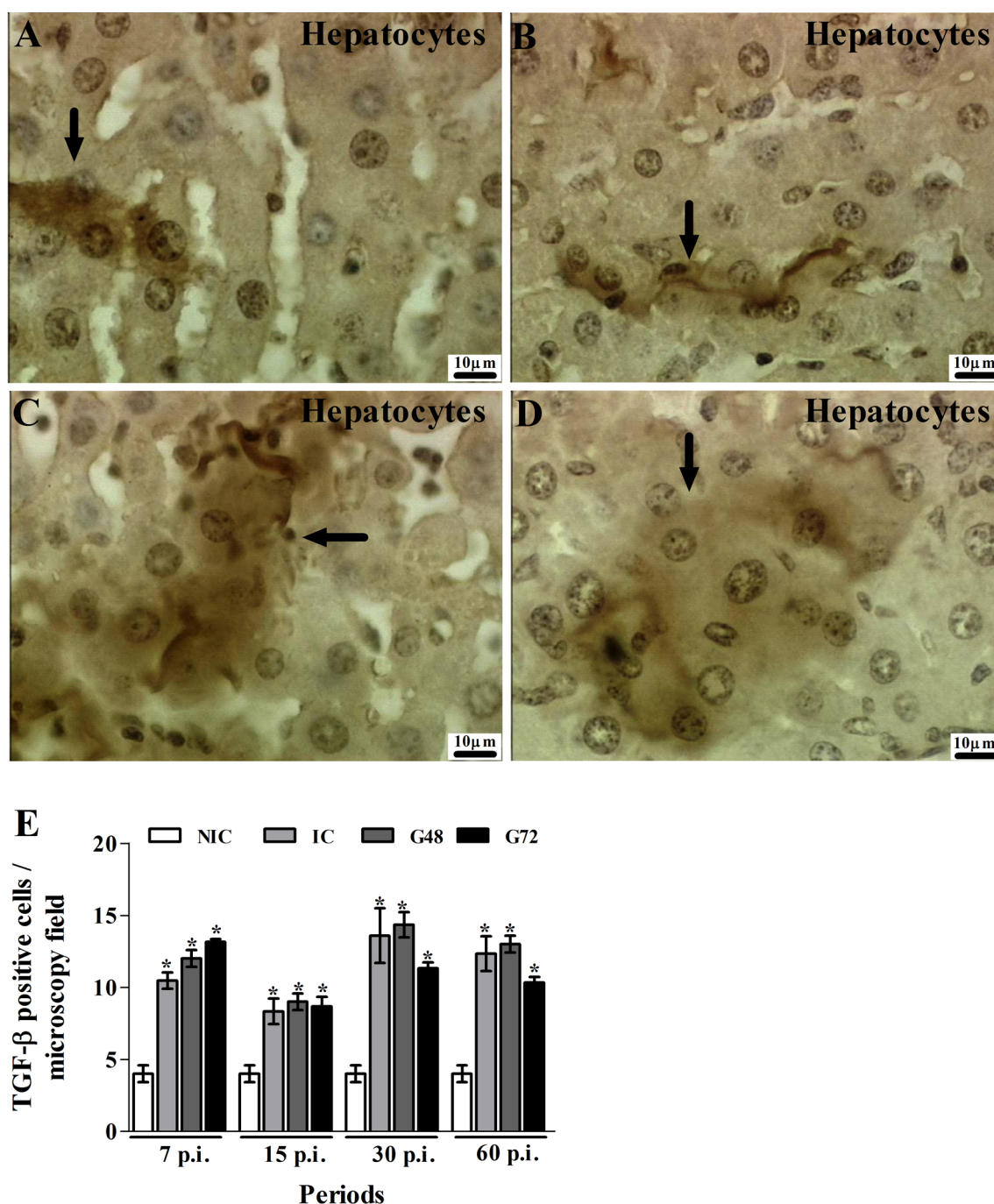


Fig. 7 Immunohistochemical staining for TGF- β 1 in liver. (a—NIC; 30 p.i.) Uninfected and untreated control. (b—IC; 30 p.i.) The presence of inflammatory infiltrate denoting mild immunostaining for TGF- β 1 (black arrow). (c—G48; 30 p.i.) Mild immunostaining for TGF- β 1 (black arrow). (d—G72; 30 p.i.) Mild immunostaining for TGF- β 1 (black arrow). **e** Mean $\pm$ SD involving the pattern of immunostaining for TGF- β 1 in the liver of mice infected with *T. gondii* and treated with *L. clavatum* 200dH. Asterisk (*) indicates signifi-

cant difference compared to the NIC. ANOVA—one-way test Bonferroni post-test; $\alpha=0.05$. NIC (uninfected and untreated control). IC [infected and treated with un-dynamized 7% alcohol solution (vehicle)]. G48 (infected and treated 48 h before infection and treated three more times; at 2, 4, and 6 p.i. with *L. clavatum* 200dH). G72 (infected and treated for three consecutive days before infection with *L. clavatum* 200dH)

In this study, with both the dosing schedule and the high dynamization of *L. clavatum* used, we found intensified harm to the kidneys and liver of the mice infected with 20 tissue cysts of *T. gondii* (ME49 strain, genotype II).

In fact, of the animals treated with *L. clavatum*, the G48 and G72 groups generally presented larger histopathological alteration profiles as compared to the IC group. The mice of the G72 group presented the worst injury profile in the renal corpuscle, as well as in the renal tubules, with severe renal corpuscle damage at all test periods. In the liver, severe damage such as vacuolar degeneration, intense congestion, and edema were seen particularly on day 30 p.i. in the G48 group, in which the mononuclear inflammatory infiltrates were most intense in the portal space.

It is extremely important to inform that in this study, the scheme and dynamization of the treatment caused symptom exacerbation and imbalance of homeostatic mechanisms, exacerbating the inflammatory process caused by the parasite.

We observed significant differences regarding the deposition of collagen in the damaged areas, in both the liver and kidney of animals treated with *L. clavatum*. In the kidneys, the percentage of the damaged area was significantly higher in G72 groups treated with *L. clavatum* on day 15 p.i. ($5.89 \pm 0.58\%$), 30 p.i. ($5.47 \pm 0.65\%$) and 60 p.i. ($5.72 \pm 0.78\%$) of the total area, differing from the IC, which had a percentage of approximately $2.87 \pm 0.28\%$ ($p < 0.05$) in all periods. The percentage of the damaged area was similar between the groups IC and G48 treated with *L. clavatum* of the total area on 15, 30, and 60 dpi and non-infected control, with $1.64 \pm 0.40\%$.

In the liver, the percentage of the damaged area was significantly higher in G72 groups treated with *L. clavatum* on day 15 p.i. ($6.45 \pm 2.20\%$), on 30 dpi ($7.05 \pm 0.60\%$) and 60 dpi ($7.10 \pm 0.14\%$) of the total area, differing from the IC, which had a percentage of $4.23 \pm 0.52\%$ ($p < 0.05$) on day 60 p.i. The deposition of collagen in the damaged areas was higher in G48 ($3.71 \pm 0.32\%$) on day 15 p.i. differing from the IC (2.06 ± 0.27), of the damaged area was similar between the groups IC and G48 treated with *L. clavatum* of the total area on day 30 and 60 p.i. and non-infected control, with $1.25 \pm 0.17\%$.

Yoshioka et al. (1990) studied the distribution of type I and III collagen in normal and diseased renal tissues and observed that damage in renal corpuscle (glomeruli and Bowman's capsule) is accompanied by the appearance of interstitial collagens, and that participation of type I collagen is prominent in crescent organization and global glomerular sclerosis, but is less frequent in mesangial expansion, compared with type III collagen. According to Dubey (1998b), the bradyzoites contained within the cysts have a slow multiplication, in chronic or asymptomatic infections; they have tropism by the brain, retina, skeletal and cardiac muscles,

and to a lesser extent in organs such as the lungs, liver and kidneys, causing damage to these. Normally in the portal spaces in the liver, collagen I and III are found, with type I being abundant (Raso et al. 1983). These authors affirm that, as result of an inflammatory process in the portal spaces, the fibroblasts synthesize and export more collagen, resulting in an increase in collagen I and III.

Quiescent fibroblasts are found in several organs and are involved in the maintenance of the extracellular matrix (Liu 2004). These cells, when undergoing physiological and pathological stimuli, differentiate into myofibroblasts, which are involved in the fibrosis of many organs such as the liver, lungs, and kidneys. According to Albanis et al. (2003) and Kumar et al. (2008), fibrosis is a normal consequence of a single and severe lesion, persistent or repetitive chronic inflammation, and characterized by activation and proliferation of fibroblasts, with large deposition of extracellular matrix, directly interfering with the organ's function. The quantitative analysis performed through staining with Picrosirius revealed an increase in collagen deposition on days 15, 30, and 60 p.i. in the IC group, as well as significant exacerbation evidenced in the groups treated G48 and G72, especially in G72 who received previous treatment with *L. clavatum*. Thus, in accordance with the authors mentioned above, we believe that the fibrosis in the kidney and liver observed in our study was a consequence of the presence of *T. gondii*, which causes lesions in the tissues through which it passes.

In relation to treatment with *L. clavatum*, there were changes in both ALT and AST enzyme levels, confirming a high degree of liver damage. However, to date, the exact mechanisms causing such findings are unknown. It is believed that the lesions occur due to the presence of intense inflammatory responses, rather than to any absence of anti-inflammatory mediator. In addition, the predominance of the Th1 response was demonstrated during treatment with *L. clavatum* (Falkowski-Temporini et al. 2016).

Sylvio et al. (2014) found that infection with *T. gondii* results in liver injury and promotes significant increases in serum ALT and AST and also histopathological changes with inflammatory infiltrates which characterize hepatitis. The data corroborate our histopathological and biochemical findings in which we observed increases in serum ALT and AST, inflammatory infiltration, edema, and moderate congestion in all infected groups.

Although AST may be found in other organs, according González and da Silva (2013), ALT is considered a hepatospecific enzyme, and serum increase in its activity is only observed with hepatocellular degeneration or necrosis. Apart from inducing increases, the treatments maintained such high levels; being observed at all examination times.

Henrique da Silva et al. (2015) showed that pretreatment with *L. clavatum* at 30cH in paracetamol intoxicated mice causes decreases in liver injury from 72 h of administration,

significantly reducing ALT, yet not reducing AST serum levels. The data are similar to findings by Sur et al. (1990), who reported a reduction in the number of cells and necrotic area involved in livers of mice treated with *L. clavatum* at 200cH, having been submitted to experimental poisoning.

According to Kaneko et al. (1997), persistent high ALT values over a prolonged period indicate the establishment of a chronic condition. In our histopathological findings, we discovered several alterations which were suggestive of chronic disease in the animals treated with *L. clavatum*; changes in the liver (hepatitis and necrosis), and the kidneys (glomerulonephritis, interstitial nephritis and glomeruli).

Fibrosis is a common endpoint for various chronic pathologies, characterized by excessive deposition of extracellular matrix that leads to progressive dysfunction of the affected organ (Branton and Kopp 1999; Li et al. 2015). TGF- β 1 overproduction is the key molecular mediator of fibrosis; significant correlations exist between the degree of fibrosis and TGF- β 1 expression, especially in the kidneys and liver. High levels of TGF- β 1 are related to the development of renal disease, including tubular degeneration, glomerulonephritis, glomerulitis (Kumar et al. 2008; Li et al. 2015; Strutz 2001) and hypertensive nephropathy (Branton and Kopp 1999).

We evaluated the presence of this cytokine in organs as an indirect measure of fibrosis and observed in general, that all infected groups presented intense TGF- β 1 markings throughout the tissues in all periods, confirming the histopathological and biochemical findings above.

Contrary to that observed by our group, Braga-Silva et al. (2015) found that treatment with a bio-therapeutic, obtained from *T. gondii* 200dH in mice infected with *T. gondii* (ME49), promoted TGF- β 1 level reductions. The mechanisms involved in immunomodulation by highly diluted drugs have not yet been elucidated (Elia et al. 2007). However, studies show that highly diluted medications demonstrate benefits in treating infections, constituting an alternative for their treatment (Aleixo-Brustolin et al. 2017; Falkowski-Temporini et al. 2016). Treatment with *L. clavatum* altered the immune response and caused increased interferon γ and interleukin (IL)-12, and decreased IL-10 concentration in mice infected with *T. cruzi*. Treatment with *L. clavatum* 13c promoted a beneficial immunomodulatory action, reducing the progression of Chagas' disease (Aleixo-Brustolin et al. 2017), and polarizing the Th1 response (Falkowski-Temporini et al. 2016).

Taken together, our results suggest that *L. clavatum* 200dH administered in an inadequate treatment regimen/dynamization (in the acute phase) results in more severe histopathological damage. This is contrary to its beneficial effect in mice infected with *T. cruzi*, when used at higher dosage frequencies and smaller potencies (Aleixo-Brustolin et al. 2017; Falkowski-Temporini et al. 2016; Lopes et al. 2016).

Lopes et al. (2016) using a more frequently administered *L. clavatum* at 13cH in a more severe acute infection (Swiss mice $\times$ *T. cruzi*) observed both decreased parasitism and inflammatory processes. This is consistent with certain researchers that suggested the use of highly diluted drugs in the acute infection stage requires an increased dosage frequency (Aleixo et al. 2012) to promote homeostatic balance (Bell et al. 2013), as in the chronic phase, less frequent doses are required (Aleixo et al. 2012; Wickramasinghe and Abdalla 2000).

Taken together, our results suggest that *L. clavatum* 200dH intensifies liver and kidney alterations in mice infected with *T. gondii*. Furthermore, it reinforces the importance of new studies on *L. clavatum* 200dH and its effects on the host-parasite relationship, as well as highlighting the need for good planning when indicating dynamizations and dosing schedules for *L. clavatum* in *T. gondii*-induced infection models. Thus, future studies should be conducted to evaluate the effects of the experimental infection of mice with *T. gondii* and treated with *L. clavatum* in different dosing schedules and lower or medium potencies, including 17dH and 30dH.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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