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EXPERIMENTAL TOXOPLASMOSIS: EVALUATION OF THE HEPATIC DAMAGE IN MURINES

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ABSTRACT

Toxoplasma gondii is the most common protozoan found in animals and humans and has been found in several of the host's organs, including the liver. This study aimed to evaluate hepatic injury in experimental toxoplasmosis caused by two strains of *T. gondii* (RH and Me-49 strains). Biochemical and histopathological analyses were performed. It was possible to detect significant increases in serum levels of AST, ALT and LDH in both infections. The histopathological analysis showed inflammatory infiltration in the Me-49 strain infection and hyperemia and vasodilation in the RH strain infection. The acute infection (RH strain) induced hepatic failure and the death of the host. The chronic infection (ME-49 strain) caused liver damage but not enough to kill the host. Therefore this study validates the importance of biochemical concentrations for the evaluation of the infection, showing the importance of rigorous clinical assessment of *T. gondii* infected individuals.

KEY WORDS: *Toxoplasma gondii;* experimental; enzymes; aminotransferase; lactate dehydrogenase.

RESUMO

Toxoplasmose experimental: avaliação das lesões hepáticas em murinos

Toxoplasma gondii é um dos protozoários mais ubíquos encontrado parasitando tanto animais quanto humanos sendo encontrado em diversos órgãos de seus hospedeiros, inclusive o fígado. Este estudo avaliou as lesões hepáticas causadas pela toxoplasmose experimental em duas cepas de *T. gondii*

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(RH e ME-49). Análises bioquímicas e histopatológicas foram realizadas. Foi possível detectas um aumento significativo nos níveis séricos de AST, ALT e LDH em ambas as infecções. A análise histopatológica demonstrou infiltrado inflamatório na infecção experimental com a cepa ME-49 e hiperemia e vasodilatação na infecção com a cepa RH. A infecção aguda (cepa RH) induziu falência hepática e morte do hospedeiro. A infecção crônica (cepa ME-49) levou a lesão hepática, mas não à morte do hospedeiro. Portanto este estudo valida a importância das análises bioquímicas para avaliação da infecção ressaltando o rigoroso exame clinico em indivíduos infectados com *T. gondii.*

DESCRITORES: *Toxoplasma gondii;* experimental; enzimas; aminotransferase; lactato desidrogenase.

INTRODUCTION.

Toxoplasma gondii is a protozoan parasite which presents a facultative heteroxenic life cycle and infects all species of homeothermic animals including mammals, birds and humans. It is a cosmopolitan parasite and presents great veterinary and medical importance as it may cause abortions and congenital diseases in several species of intermediate hosts and also life-threatening disease in immunocompromised individuals (Tenter et al. 2000; Swisher et al. 1994; Luft et al. 1993). The toxoplasmosis infection is divided into two phases: acute and chronic. The acute phase is characterized by the dissemination of the parasite throughout the host's organs. The chronic phase is characterized by the formation of cysts in several organs including the brain (Darcy and Santoro, 1994).

In its chronic phase, *T. gondii* generally produces a cellular destruction of the host's different organs, including the liver, brain and lungs (Dubey and Beattie, 1988; Djurkovic-Djakovic et al., 2012). In spite of this massive destruction the symptoms are not characteristic and the majority of cases are asymptomatic (Dubey and Beattie, 1988).

The kinetics of *T. gondii* evolution in several different hosts has been thoroughly studied (Zenner et al., 1998; Djurkovic-Djakovic and Milenkovic, 2001; Djurkovic-Djakovic et al., 2012; Jokelainen and Nylund, 2012) describing the acute and chronic phases of infection and also the prevalence of the parasite in different organs of the host. However, there is scarce literature describing whether the parasite is capable of causing tissue injuries in those different locations.

The presence of *T. gondii* tachyzoites in the liver has been demonstrated by several authors in experimental models (Milovanovic et al., 2009; Djurkovic-Djakovic et al., 2012; Zheng et al., 2012) however there is not a description of the injuries caused by this parasite on this vital organ.

Hepatic injuries may be detected by the alteration of some biochemical parameters such as the serum concentrations of aminotransferases (AST and ALT), alkaline phosphatase (AP), gamma-glutamyl transpeptidase (GGT) and lactate dehydrogenase (LDH). The concentration of aminotransferases (ALT and AST) in the sera indicates the degree of hepatocyte injury and their release shows cellular injury and death. The concentration of ALT in the sera is used to indicate

the cellular integrity of the liver. An alteration in alkaline phosphatase (AP) is directly related to biliary obstruction with intra or extra-hepatic causes and/or to hepatocellular injury, which could be considered the best indication for the diagnosis of cholestasis (Couto et al. 2008). The gamma-glutamyl transpeptidase (GGT) is involved in the transportation of amino acids and peptides through cellular membranes, in protein synthesis and in the regulation of the levels of tissue glutathione. This enzyme may be found in the liver, biliary ducts, kidney, intestine, prostate, lungs, brain and heart (Emanuelli et al. 2008). Although GGT is an enzyme that is found at greater concentrations in renal tissue, it is of clinical importance in liver and biliary duct injuries mainly cholestasis and toxic and inflammatory hepatic injuries (Araujo et al. 2005). Lactate dehydrogenase (LDH) is an enzyme present in the cytoplasm of all cells but it is found in higher concentrations in the myocardium, liver, striated muscles, kidneys and in red blood cells. An increase in serum levels of this enzyme does not indicate damage, but other cellular injuries such as hemolysis, myocardial infarction, exudates or transudates and meningitis (Astegiano et al. 2004).

Therefore, the aim of this study was to evaluate the liver injuries in BALB/c mice caused by a non cystogenic strain of *T. gondii* with high virulence (RH) and by a cystogenic strain with low virulence (Me-49), through serum concentrations of aminotransferases (ALT and AST), alkaline phosphatase (AP), gamma-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH) and histopathological analysis of hepatic infected tissue.

MATERIALS AND METHODS

Mice

Thirty, one month old, male and female BALB/c mice, were used in this study and were provided by the animal facilities from IPTSP/UFG. They were maintained under standardized humidity, temperature and luminosity with unrestricted access to 5% acetic acid acidified water and Nuvilav® ration. The ethical principles for animal experimentation set out by the Brazilian Society of Laboratory Animal Sciences (Sociedade Brasileira de Ciência em Animais de Laboratório/SBCAL) were followed and this study was authorized by the Committee for Ethical Research of the Federal University of Goiás (CoEp/UFG) (registration number 035/08). The mice were divided into groups of 5 animals per cage to ensure greater comfort.

Parasites

The following strains of *T. gondii* were used: RH strain, type I, non cystogenic with high virulence (Sabin, 1941) and Me-49 strain, type II, cystogenic

with low virulence (Lunde and Jacobs, 1983). These strains were maintained through serial intraperitoneal passages and cryopreservation.

Experimental infection and biochemical analysis

For the RH strain, groups of five mice (total of 25 animals) were inoculated intraperitoneally with approximately 1,000 parasites, which were counted with a Neubauer chamber and diluted into 0.2 mL of sterile saline solution. The infection was confirmed by the presence of parasites in peritoneal exudates. One group of five animals was euthanized daily until the fifth day. The blood of the animals was collected through cardiac puncture with the use of heparin to prevent blood clotting. The blood was centrifuged at 3,000 rpm for 15 minutes and the serum was collected and frozen for the biochemical analysis of AST, ALT, AP, GGT and LDH by the enzymatic colorimetric automatic method A25 (*Random Access Analyzer Clinical Chemistry-Turbidimetry)*.

For the ME-49 strain, 20 mice were inoculated intraperitoneally with 0.2 mL of brain maceration containing approximately 10 *T. gondii* cysts. These infected animals were euthanized at 30, 60, 90 and 120 days after the infection (five animals at each time), and the blood was collected and analyzed as described for the RH strain. As the ME-49 strain presents a chronic evolution and therefore it is not possible to find parasites in the peritoneal exudate, the infection was confirmed by the detection of IgG antibodies through indirect immunofluorescence (Camargo 1964).

As a control group to compare with the infection caused by the RH strain, groups of five mice with no infection were used; one euthanized each day from day one to day five. As a control group to compare with the infection caused by ME-49 strain, groups of fifteen animals with no infection were used and at 30, 60, 90 and 120 days three animals were euthanized. These mice were submitted to inoculation stress with sterile saline solution, and were maintained under the same conditions as the infected groups.

Histopathological analysis

For the histopathological analysis, after euthanizing all animals, their livers were removed and fixed in 10% formaline buffer, processed and blocked into paraffin and then sectioned into 5 micrometer sections and stained with hematoxylin & eosin (HE) and Giemsa. Subsequently, five histological sections from each liver were selected and the slides were analyzed with the use of an optic microscope.

Statistical analysis

All experiments were performed in triplicate. The results were submitted to statistical analysis through the Sigma Stat 2.3 programme. All variables were

tested as to a normal distribution and homogeneous variation. The significance level was of $p<0.05$.

RESULTS

Non cystogenic (RH) strain of *T. gondii*

The non-cystogenic strain infection demonstrated a quick evolution within the host and was capable of killing infected mice in up to seven days. The histopathological analysis showed hyperemia and vasodilatation on the 5th day post infection in the animals infected with the RH strain (Figure 1A). The concentrations of the enzymes detected during the analysis of hepatic alterations in mice infected with the RH strain are described in Table 1. It was apparent that the aminotransferases (AST and ALT) showed significant ($p<0.05$) increase in the infected group when compared to the control group, from the first to the fifth day after the infection. It is important to highlight the considerable increase in those enzymes, when compared to the control group, detected on the 4th and 5th days post-infection, which may indicate mitochondrial injury of the hepatic cells. The GGT and AP enzyme concentrations did not alter from the 1st to the 5th day after infection when compared to the control group (Table 1).

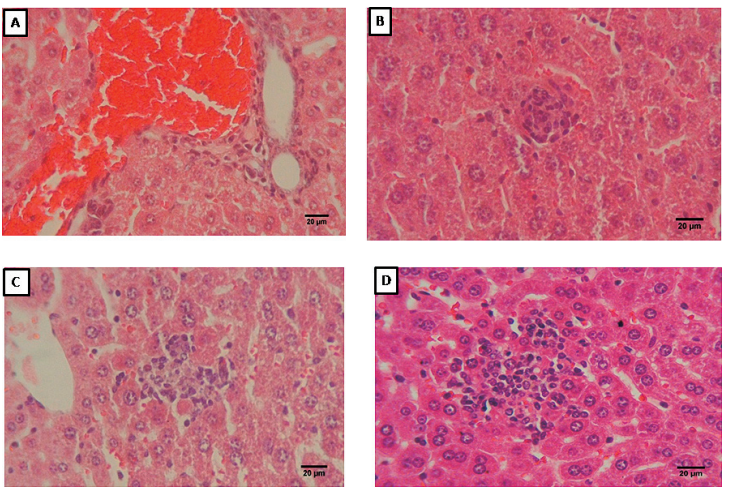

Figure 1. Photomicrographs of mouse liver experimentally infected with RH and Me-49 strains of *Toxoplasma gondii*. (A) hyperemia and vasodilation in an RH strain infected animal; (B), (C) and (D) focal and discrete inflammatory infiltration with the predominance of mononuclear cells at 60, 90 and 120 days post infection, respectively, with the Me-49 strain (H&E, scale = 20 µm).

It was possible to observe a gradual increase in the LDH concentrations in the serum from the infected animals when compared to the control group ($p<0.001$). This enzyme may be found in several tissues, but when associated with elevated levels of AST and ALT as occurred in this study, the high levels of LDH indicate hepatic injury. Furthermore, we may not discount the clinical conditions that the infected mice presented which were characteristic of acute experimental toxoplasmosis induced by the RH strain, causing injuries in several organs leading to a condition of multiple organ failure, as was demonstrated by the high levels of ALT, AST and LDH enzymes especially on the 4th and 5th days after the infection. It is important to highlight that the animals from the control group did not present any clinical alterations or alterations in the hepatic enzyme seric levels.

Table 1. Seric concentrations of AST, ALT, GGT, AF and LDH from BALB/c mice infected with RH strain of *Toxoplasma gondii*.

Enzymes	1st DAI		2nd DAI		3rd DAI		4th DAI		5th DAI	
	I	C	I	C	I	C	I	C	I	C
AST UI/L	170.0* (±4.2)	116.7 (± 3.9)	473.0* (± 10.0)	323.3 (± 12.4)	377.0* (± 7.16)	363.3 (± 9.2)	835.0* (± 5.9)	346.7 (± 8.6)	6210.0* (± 12.8)	291.0 (± 3.2)
ALT UI/L	136.7* (± 5.7)	93.3 (± 5.7)	450.0* (± 5.5)	196.7 (± 12.7)	312.0* (± 3.2)	190.0 (± 6.0)	417.0* (± 5.0)	196.7 (± 9.6)	753.0* (± 10.5)	184.0 (± 11.2)
GGT U/L	11.7 (± 1.7)	20.0 (± 3.2)	10.0 (± 5.0)	20.0 (± 4.2)	10.0 (± 0.0)	36.7 (± 5.5)	10.0 (± 0.0)	20.0 (± 3.2)	15.0 (± 2.2)	16.7 (± 2.4)
AP UI/L	133.3 (± 9.5)	190.0 (± 6.0)	153.3 (± 10.7)	220.0 (± 6.6)	243.3 (± 7.6)	256.0 (± 9.1)	236.7 (± 12.0)	340.0 (± 13.3)	283.3 (± 11.1)	220.0 (± 8.7)
LDH U/L	1293.3* (±10.0)	184.3 (± 11.1)	3103.3* (± 28.2)	276.0 (± 15.0)	4496.0* (± 31.1)	363.0 (± 14.1)	5960.0* (± 33.5)	390.0 (± 13.5)	10416.0* (± 13.9)	456.0 (± 12.8)

*$p\leq0.005$; ± standard deviation; DAI: day after infection, AST: aspartate aminotransferases, ALT: alanine aminotransferases , GGT: gamma-glutamyl transpeptidase, AP: alkaline phosphatase, LDH: lactate dehydrogenase, I: infected group, C: control group

Cystogenic (Me-49) strain of *Toxoplasma gondii*

The histopathological analysis detected no alteration in the liver at 30 days post infection, however at 60, 90 and 120 days post infection it was possible to observe a focal and discrete inflammatory infiltration composed of mononuclear cells in the hepatic parenchyma (Figures 1B, 1C and 1D).

The Me-49 strain, type II, which is cystogenic and of low virulence was not capable of killing the animals during the experimental period of 120 days. When the animals were inoculated they presented discrete symptomatology of lethargy, which disappeared after the first week and remained asymptomatic during the whole period. The infection in those animals was confirmed by the indirect

immunofluorescence technique (IFI) for IgG detection (Table 2). The serology of the animals from the control group was negative.

All the analyzed enzyme levels (AST, ALT, LDH, AP and GGT) presented alterations when compared to the control group (Table 2) but only the concentrations of AST, ALT and LDH presented a significant increase. It was possible to observe that as the period of infection increased the greater were the alterations in the enzymatic levels.

Table 2. Seric concentrations of AST, ALT, GGT, AF and LDH from BALB/c mice infected with Me-49 strain of *Toxoplasma gondii*.

Enzymes	30 DAI		60 DAI		90 DAI		120 DAI	
	I	C	I	C	I	C	I	C
AST UI/L	390.7* (±3.9)	117.0 (±4.9)	505.0* (±13.4)	105.0 (±13.6)	975.0* (±18.7)	94.7 (±4.7)	300..0* (±3.7a)	106.2 (±13.3)
ALT UI/L	43.0* (±3.8)	82.2 (±2.6)	382.5* (±9.6)	86.5 (±2.1)	750.0* (±15.8)	82.5 (±2.5)	292.5* (±4.1)	82.7 (±2.7)
GGT U/L	21.5 (±1.0)	28.2 (±2.40)	25.0 (±3.20)	24.7 (±2.0)	25.0 (2±.40)	28.0 (±2.02)	32.5 (±3.55)	26.7 (±2.14)
AP UI/L	127.75 (±2.1)	145.7 (±6.0)	174.2 (±13.9)	126 (±2.1)	282.5 (±6.8)	126 (±1.7)	342.5 (±9.8)	142.7 (±5.0)
LDH U/L	730.2* (±12.3)	157.5 (±9.5)	4927.0* (±26.5)	84.2 (±7.5)	4390.0* (±23.3)	139.2 (±8.7)	3772.5* (±34.9)	98.0 (±6.0)

*p≤0.005; ± standard deviation; DAI: day after infection, AST: aspartate aminotransferases, ALT: alanine aminotransferases , GGT: gamma-glutamyl transpeptidase, AP: alkaline phosphatase, LDH: lactate dehydrogenase, I: infected group, C: control group

DISCUSSION AND CONCLUSIONS

This study demonstrated the hepatic damage caused by *T. gondii* infection, whether it is caused by a non-cystogenic (RH) or by a cystogenic (Me-49) strain, differentiating the alterations found in an acute versus a chronic infection. The alterations in the hepatic enzymes AST and ALT may be explained by the parasite's reproduction inside the hepatic cells, which leads to great cellular injury and consequent high levels of AST and ALT in serum. These data are in accordance with those of Djurkovic-Djakovic et al. (2012) which demonstrated the kinetics of the parasite burden of two strains of *T. gondii* with the presence of tachyzoites in the liver from the first day of infection with the RH strain.

The concentrations in the sera of GGT and AP are used to detect alterations in the liver, especially in the biliary ducts, in other words, alterations that represent cholestasis in its chronic phase (Couto et al., 2008). The hepatic alteration demonstrated by the high levels of AST and ALT indicates an acute injury of the cells and added to the fact that the RH strain is capable of killing the mice very quickly, approximately 5 to 7 days post infection, there would be no time for the chronic alteration of the biliary ducts to present, and therefore the

alteration of GGT and AP concentrations were not yet demonstrated. These data are in accordance with the description of the kinetics of the parasite burden in experimental murine toxoplasmosis caused by the RH strain made by Djurkovic-Djakovic et al. (2012).

The histopathological alterations observed in the chronic infection caused by the cystogenic (Me-49) strain are in accordance with Aquino et al. (2002) who showed centrilobular degeneration and periportal mononuclear infiltration in dogs experimentally infected by *Trypanosoma evansi.* These results show that systemic protozoans such as *T. gondii* and *T. evansi* are capable of tissue damage through the infection within their hosts.

We believe that the chronic alterations caused by the ME-49 strain infection are related to the inflammatory process demonstrated by the histopathological analysis, which showed that in chronic toxoplasmosis there is cyst formation throughout the mouse which may burst and cause a local inflammatory process (Coutinho and Vergara, 2005; Djurkovic-Djakovic et al., 2012). In spite of the low parasite burden in the liver described only until the 42nd day after infection by Djurkovic-Djakovic et al., (2012) in experimental murine chronic infections caused by the Me-49 strain, our study shows that the hepatic injury persists until 120 days after the infection. Zheng et al. (2012) demonstrated that it is possible to cause experimental toxoplasmosis in mice infected with cryopreserved liver tissues, which indicates the presence of the parasite in this organ during long chronic infections such as 120 days.

A report made by Milovanovic et al. (2009) demonstrated that the experimental chronic infection such as the Me-49 strain of *T. gondii* causes alterations in other biochemical parameters of murine serum. These authors detected alterations of the lipid metabolism caused by a low virulence strain (BGD-1) of *T. gondii* which indicates that this parasite alters the equilibrium of the host's metabolism.

Also other protozoans have been reported to alter the hepatic function of their hosts in experimental infections. As described by Aquino et al. (2002), infection by *Trypanosoma evansi*, a protozoan which forms nests in the tissues in its chronic phase, also induced an increase of AST and ALT in experimentally infected dogs. Herrera et al. (2002) detected that the infection of American coati with *T. evansi* also induced an increase in the AST and ALT levels, remaining increasedfrom the acute phase and throughout the chronic phase. Both studies from Aquino et al. (2002) and Herrera et al. (2002) are in accordance with our results.

In conclusion, the mice infected with the RH strain presented significant alterations in the levels of AST, ALT and LDH in the sera which strongly suggest a hepatic injury. The mice infected with the Me-49 strain presented significant alterations in the levels of AST, ALT and LDH in the sera in all the analyzed samples during the experimental period. The increase of these enzymes in the serum of *T. gondii* infected BALB/c mice added to the histopathological analysis

showed how these parameters may be useful for the clinical evaluation of the course of the infection. Therefore this study validates the importance of assaying biochemical concentrations for the evaluation of damage caused by the parasite in the host tissues. The biochemical parameters may be another option for monitoring cellular injuries caused by *T. gondii*. These results show that lesions may be found in different phases of the infection even when the host does not show clinical signs, highlighting the importance of a rigorous clinical evaluation of chronically *T. gondii* infected individuals.

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