

Experimental infection of Mongolian gerbils with *Toxoplasma gondii*: pathological and immunohistochemical evaluations

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Abstract

Toxoplasma gondii is a protozoan parasite which causes toxoplasmosis both in human and warm-blooded animals. Toxoplasmosis is a worldwide disease and largely threatens human and animal health consequently causing economic losses. Also, it affects the visceral organs in different severity degrees according to the strain of parasite and the host. In this study, experimental toxoplasmosis was performed via intra-peritoneal route in 12 gerbils by administrating 5.00×10^3 tachyzoites of *T. gondii* RH strain. The gerbils were sacrificed 7 days after inoculation. All systemic organs were obtained via necropsy and examined by immunohistochemical and histopathological methods. Lesions infected with *T. gondii* mostly observed in the serosa of abdominal cavity organs including stomach, liver, spleen, intestines, and kidneys. The lesions were most severe in liver. The parasite showed an affinity for the hepatic tissue. To our knowledge, this is the first experimental study of acute *T. gondii* infection in gerbil evaluating macroscopic, microscopic and immunohistochemical findings. It is concluded that Mongolian gerbils can be used as experimental animals to investigate toxoplasmosis. Also, these animals are very suitable hosts to study liver pathology and pathobiology of *T. gondii*-related hepatitis.

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Introduction

Toxoplasma gondii, which causes the toxoplasmosis disease, is a coccidian parasite where all warm-blooded animals are intermediate hosts and cats are the definitive host.¹ As the sexual reproduction in the complex life cycle of the agent occurs only in the small intestine of felines, the asexual reproduction phase can occur in all warm-blooded animals, including humans, cats and poultry.² The *T. gondii* can be seen as one of the major problems in public health. This view expresses the importance of global infection and spread of the parasite. One in every three people in the world is reported to be seropositive for *T. gondii*. Infection occurs when the meat products from the infected animals are consumed under-cooked or raw, or by digesting the *T. gondii* oocyst which is shed in the feces of definitive host.^{3,4} After *T. gondii* is taken into the body, it uses blood circulation to spread to organs in the acute phase of infection. Thus, tachyzoites, according to the virulence of parasite, or strains, can go to the tissues and settle there.⁵ The *T. gondii* can live and multiply in several host tissues

such as liver, brain, spinal cord, kidneys, skeletal striated muscle, heart and eyes as well as immune system cells.⁶ Also, experimental toxoplasmosis can be conducted in various laboratory animal models. Among laboratory animals, Mongolian gerbils (*Meriones unguiculatus*) have been increasingly used in scientific research for various infectious disease models. These infectious diseases include those being induced by parasitic agents such as, *Strongyloides stercoralis*,⁷ *Brugia pahangi*,⁸ *T. gondii*,^{9,10} *Giardia duodenalis*,¹¹ *Giardia lamblia*,¹² *Babesia divergens*,¹³ and *Baylisascaris potosis*.¹⁴ In a previous study, Mongolian gerbils were reported to be susceptible to *T. gondii* RH (as sensitive as mice), Beverley and YH strains (more sensitive than mice). The *T. gondii* infection in gerbils shows greater parasite proliferation in organs than in murine models.¹⁵ The worldwide spread of *T. gondii*, threatening human and animal health to a large extent consequently causing economic losses, has led to studies on this issue. Therefore, experimental studies are carried out, and new models are developed. However, the models used in the toxoplasmosis study do not always reflect the

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dynamics of natural infection. In order to obtain accurate and appropriate scientific data, the most accurate experimental model and the most appropriate laboratory animal should be selected.

To date, there are few studies regarding experimental *T. gondii* infection in Mongolian gerbils. To our knowledge, pathological lesion caused by *T. gondii* infection has not been investigated before in Mongolian gerbils. Hence, in this experimental study, Mongolian gerbils were infected with *T. gondii* to evaluate the tissue tropism of parasite and the lesions caused by parasite using necropsy, histopathology and immunohistochemical techniques.

Materials and Methods

Laboratory animals. Fifteen Mongolian gerbils (*M. unguiculatus*) were used in the study. Three of the gerbils were used in the control group and twelve in the experimental group. The Animal Care Committee of the University of Kirikkale, Kirikkale, Turkiye, approved this study (No. 11; 11.02.2011).

Infection model. To create the acute toxoplasmosis in gerbils, the virulent *T. gondii* RH strain, which is maintained in the Parasitology Laboratory of the Turkish Public Health (thanks to Dr. Cahit Babur) was used. The gerbils were infected with 5.00×10^3 tachyzoites using the intra-peritoneal route. The number of tachyzoites used for infection was adapted from the previous study.¹⁰ At the end of the seventh day, the gerbils were anesthetized with ether and euthanized for a necropsy.

Histopathology. Systematic necropsy was performed in each animal to collect tissues and macroscopic changes were recorded. Tissues samples were fixed in 10.00% buffered formaldehyde solution. After fixation, the tissues were trimmed and washed under tap water for 24 hr in tissue block cassettes. In tissue processors, tissues were processed in alcohol, xylol and xylol-paraffin series in a 14-hr program. After the tissues were embedded in paraffin and then cooled, sections with a thickness of 5.00 – 6.00 μm were taken with microtome (Finesse 325; Shandon, Cambridge, UK) and routinely stained with Hematoxylin and Eosin (H&E). Immunohistochemical staining was also performed. Slides were evaluated under light microscope (Olympus BX51, Tokyo, Japan) and photographed by DP2 camera attachment (Sigma Corporation, Kawasaki, Japan).

Immunohistochemistry. While immunohistochemical staining was performed, negative control staining was performed for all tissues simultaneously. The negative control stage was the same as the protocol applied to all slides and phosphate-buffered saline was used instead of primary antibody. Slides were deparaffinized in xylol (three times for five min), passed through a series of alcohols and washed in distilled water for 5 min. It was boiled in citrate buffer for antigen retrieval process for 15

min and the slides were kept in this buffer until cooled. Thus, non-specific binding was prevented. The tissues were bordered with the hydrophobic slide pen (PAP Pen; BioGenex, Fremont, USA). Hydrogen peroxidase prepared in 0.30% methanol was dropped into the tissues and left for 15 min. So, pseudo-peroxidase activity in red blood cells and peroxidase activity in myeloid cells were eliminated and background staining was reduced. Protein blocking solution (Ultra V Block UltraVision Detection System Large Volume Anti-Polvalent, horseradish per-oxidase (HRP; RTU), ThermoScientific, Waltham, USA REF: TP-125-HL, LOT: PHL130321, Waltham, Massachusetts, USA) was incubated for 10 min. Only protein blocking solution was poured without washing the slides. The *T. gondii* primer antibody (Rabbit Polyclonal Antibody Ab-1, ThermoScientific) was dropped diluted with 1 : 100 distilled water; at room temperature (25.00 °C) for 90 min. After this stage, slides were washed three times for five min every process. Secondary antibody with biotin (goat anti-polyvalent with biotin) was dropped and left for 30 min (UltraVision Detection System Large Volume Anti-Polvalent, HRP (RTU), ThermoScientific, REF: TP-125-HL, LOT: PHL130321). Then, streptavidin peroxidase was dropped on the slides and incubated for 30 min. Thus, conjugates were ligated to biotinylated secondary antibody. The 3,3 'diaminobenzidine (DAB) chromogen (UltraVision Detection System Large Volume DAB Substrate System (RTU), Thermo Scientific, REF: TA-125-HD, LOT: HD25395) was prepared, added to the slides and stained under a microscope. The DAB chromogen binds with streptavidin conjugate to make tissue antigens visible. After the slides were stained with DAB chromogen for 12 min, sufficient staining was observed, and staining was terminated with distilled water. The nuclei were stained for 1.50 min in Hematoxylin and counterstaining was performed. Slides washed in tap water were passed through alcohol series and kept in (three times for five min) xylol. Entellan was dropped onto the slides and covered with coverslip. Stained slides were evaluated under the BX51light microscope (Olympus).

Results

Clinical findings. Mongolian gerbils infected with *T. gondii* RH strain showed severe signs of sickness, including anorexia, unkempt appearance, ruffled fur and shortness of breath (Fig. 1). All gerbils in the control group were healthy.

Gross lesions. As a result of infection with *T. gondii*, no significant macroscopic findings were observed in the lung, heart, spleen, kidney, stomach, intestines and brain. The most significant gross findings were in the liver. The liver was swollen and slightly pale.

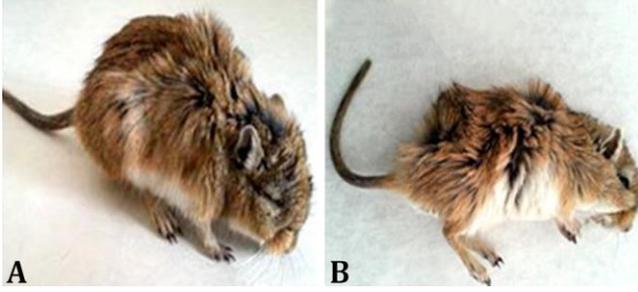


Fig. 1. Clinical and post-mortem appearance of infected Mongolian gerbils. **A)** The gerbils have messy hair, and **B)** they are weak.

Histopathological findings. Lesions due to infection were observed intensively in the liver (Figs. 2A-2D). Multi-focal hepatocellular necrosis was seen in liver. Glisson's capsule was thickened with this necrotic debris being also found between the lobular spaces. Additionally, hyperemia was observed. Similarly, necrotic debris was seen in the spleen capsule. In the follicle and germinal center, cellularity was noted to be decreased. Necrosis was observed just under the capsule. Also, extra-medullary hematopoiesis was seen in the spleen with numerous megakaryocytes along with scattered erythroid and myeloid precursors (Figs. 3A and 3B). Necrotic masses were located at the serosal surfaces of intestines and mononuclear cell infiltrations were noted in lamina propria (Figs. 4E and 4F). In the kidneys, thickened capsule with degenerative changes and hyperemia were observed (Fig. 4A). In the lungs, hyperemia and thickening of the inter-alveolar septum, alveolar edema and focal hemorrhage were noted. Severe hyperemia in inter-alveolar capillaries and foci characterized by mononuclear cell infiltrations in the lungs were observed (Fig. 4C).

Immunohistochemical findings. The intensity of immunohistochemical reaction was high in the liver, spleen and lung. In the liver, strong immunopositive staining along the Glisson's capsule and inter-lobular area with inter-lobular septa and cytoplasmic immunopositivity in hepatocytes just below the Glisson's capsule were observed. Strong immunostained tachyzoites-like ovoid to ellipsoidal granules were observed within necrotic foci in the hepatic parenchyma (Figs. 2E-2H). The *T. gondii* antigen immunoreactivity was detected throughout the lobules. Immunopositive staining and debris were seen in the spleen capsule (Fig. 3C). Immuno-positivity was also observed in the germinal centers and follicles (Fig. 3D). Structures morphologically consistent with tachyzoites were immunopositive stained mostly in the liver and spleen. Immunopositive staining's in lamina propria, intestinal lymphoid tissues, glandular epithelium and serosa were noted. The positive immunolabeling were also seen in gastric glandular epithelium and serosa. In kidney sections, proximal tubule epithelia and cortex near the capsule showed positive immunohistochemical staining (Fig. 4B).

In the lungs with interstitial pneumonia, immunopositivity was present in the thickened alveolar septum (Fig. 4D). Positive immunostainings in necrotic debris, attached to the serosa of intestine and in sub-mucosal lymphoid follicles of intestine were noted (Figs. 4G and 4H).

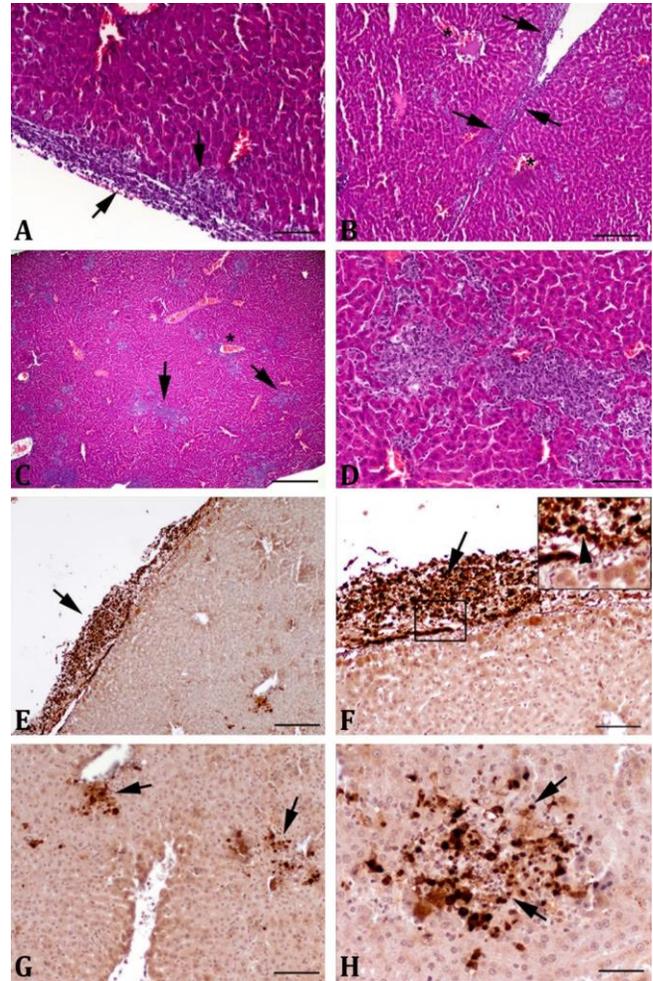


Fig. 2. Hepatic damage, histological changes and immunohistochemical staining results induced by *Toxoplasma gondii* infection. **A)** Necrotic debris at the liver capsule and necrosis below the capsule in hepatic parenchyma (arrows; H&E staining, Bar: 100 μ m). **B)** Necrosis is noted in inter-lobular region and following into the parenchyma. Central veins and sinusoids are distended with blood (asterisks; H&E staining, Bar: 200 μ m). **C)** Multi-focal necrosis (arrows) and hyperemia (asterisk) can be seen throughout the liver (H&E staining, Bar: 500 μ m). **D)** Multi-focal necrosis in liver with hyperemia (H&E staining, Bar: 100 μ m). **E-F)** Immunopositive labeling (arrows) along the Glisson's capsule (anti-*T. gondii* antibody; Contrast staining: Hematoxylin, Bar: 500 μ m in panel E and 100 μ m in panel F). Upper right panel in F: Tachyzoite-like structures at the capsule (arrowhead). **G)** Focal immunopositive staining in the parenchyma (arrows; anti-*T. gondii* antibody; Contrast staining: Hematoxylin, Bar: 100 μ m). **H)** Positive immunolabeling in the focal necrosis composed of karyorrhexis and karyolysis of hepatocytes with tachyzoite-like structures (arrows; anti-*T. gondii* antibody; Contrast staining: Hematoxylin, Bar: 50 μ m).

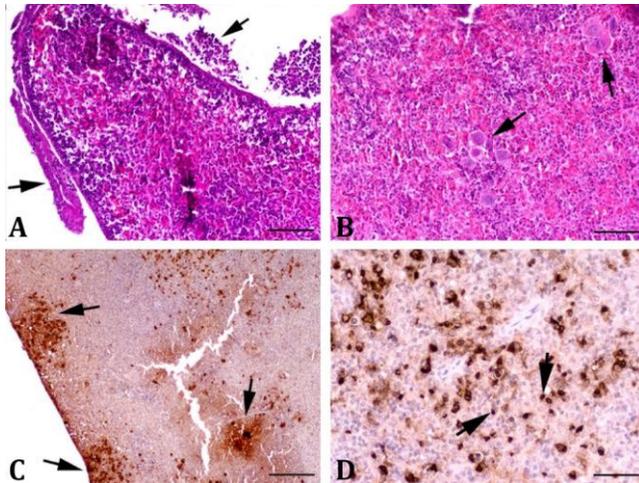


Fig. 3. Pathological and immunohistochemical findings in spleen following *Toxoplasma gondii* infection. **A)** Necrotic debris (arrows) at the spleen capsule (H&E staining, Bar: 200 µm). **B)** Extra-medullary hematopoiesis (arrows) and the normal architecture loss of the splenic lymphoid follicles (H&E staining, Bar: 100 µm). **C)** Focal immunopositive staining in the parenchyma (arrows; anti-*T. gondii* antibody; Contrast staining: Hematoxylin, Bar: 500 µm). **D)** Immunopositive staining of the tachyzoite-like structures (arrows; anti-*T. gondii* antibody; Contrast staining: Hematoxylin, Bar: 50 µm).

Discussion

After the discovery of *T. gondii* parasite by Nicolle and Manceaux in 1908,¹⁶ it was observed that the disease is prevalent in both humans and animals, which led researchers in various scientific disciplines to study it.^{17,18} Research in animals showed that the most resistance to toxoplasmosis was reported to be in rats, guinea pigs, rabbits and mice, respectively.¹⁹ Resistance is thought to be due to the specific innate immunity and fast-developing immunity in species.²⁰ Depending on the model of infection, the humoral response does not occur quickly when the parasite is invaded rapidly. Acute systemic toxoplasmosis and deaths are observed in experimental infections caused by the intra-peritoneal route of parasite.²⁰⁻²² In studies about experimental acute toxoplasmosis in mice, it was reported that mice died 6-8 days following the inoculation.^{23,24}

Intestinal, mesenteric lymph nodes, lungs, liver, spleen and central nervous system are the organs where the lesions are observed intensively.²⁵⁻²⁸ Lesions in the organs are as those observed in natural and experimental *T. gondii* infections, and the common histopathological finding is focal coagulation necrosis.^{25,26,28-31}

In systemic *T. gondii* infections, lungs are commonly affected organs. Hyperemia in the inter-alveolar capillaries, histiocyte and lymphocyte infiltrations in the interstitium, edema in the alveoli, bronchus and bronchiole lumens and focal necrosis have been observed formerly.^{29,32} In this study, no death was found on the 7th

day of infection in gerbils that were infected by the intra-peritoneal route. The gerbils were all euthanized on the 7th day of infection. Although there were no sudden deaths due to the infection in gerbils, infection-related lesions were observed in the liver, lung, kidney, spleen and intestines. The histopathological findings observed in the lungs showed consistency with the previous reports.^{29,32}

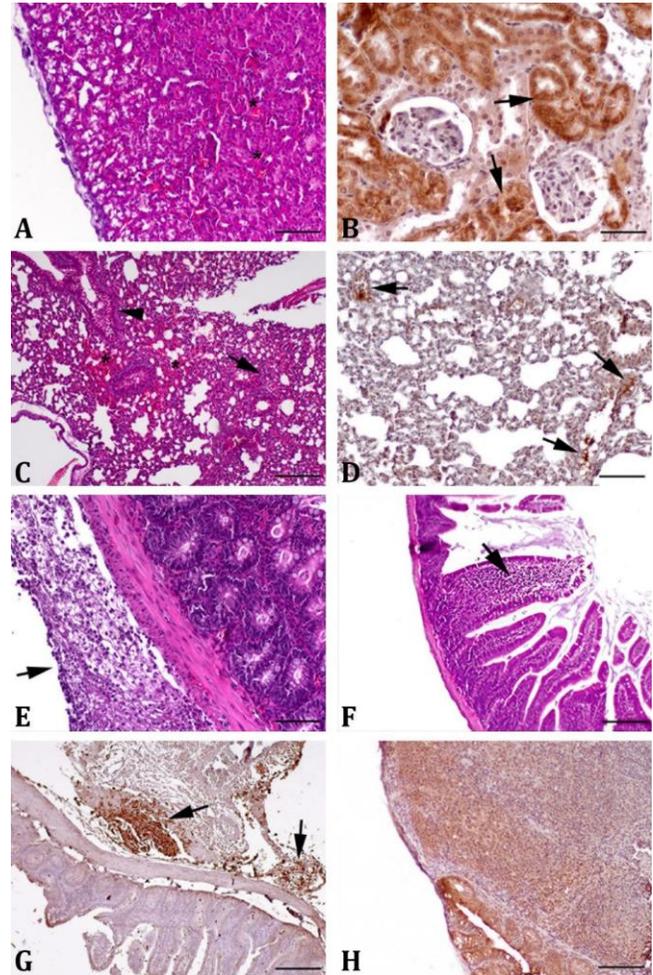


Fig. 4. Pathological and immunohistochemical findings in kidney and lung following *T. gondii* infection. **A)** Hyperemia (asterisk) in kidney (H&E staining, Bar: 100 µm). **B)** Strong immunopositive labeling in tubular epithelium (arrows; anti-*T. gondii* antibody; Contrast staining: Hematoxylin, Bar: 50 µm). **C)** Hyperemia, alveolar edema and hemorrhage (asterisk) as well as inter-alveolar septum thickening (arrow) and foci characterized by mononuclear cell infiltrations (arrowheads; H&E staining, Bar: 200 µm). **D)** Positive immunostaining at alveolar septal thickening (arrows; anti-*T. gondii* antibody; Contrast staining: Hematoxylin, Bar: 100 µm). **E)** Severe necrotic debris (asterisk) at serosa of intestine (arrow; H&E staining, Bar: 100 µm). **F)** Mononuclear cell infiltration in lamina propria (arrow; H&E staining, Bar: 500 µm). **G)** Positive immunostaining in the necrotic debris, attached to serosa of intestine (anti-*T. gondii* antibody; Contrast staining: Hematoxylin, Bar: 500 µm). **H)** Positive immunostaining in sub-mucosal lymphoid follicles of intestine (anti-*T. gondii* antibody; Contrast staining: Hematoxylin, Bar: 200 µm).

According to the reports of many researchers, lung and liver are the most affected organs in mice due to the severe histological changes after intra-peritoneal infections.³³⁻³⁶

Pinheiro *et al.*, have reported that peri-bronchial and peri-vascular infiltrations increase in the septum in the following days after the infection.³⁷ In this study, hyperemia and inter-alveolar septum thickening, alveolar edema and hemorrhage, foci characterized by mononuclear cell infiltrations and the noticeably hyperemic alveolar capillaries were noted. Immunopositivity was observed in the thickening of the inter-alveolar septum and in the interstitial areas. Histopathological and immunohistochemical findings in the lungs indicated that infection occurred systemically, and the agent was transmitted to the lungs through the blood. Atmaca *et al.*, have reported multi-focal hepatocellular necrosis and mononuclear cell and mild lymphocytic and plasma cell infiltrations in the peri-portal area around central veins in the liver of mice infected with *T. gondii* RH strain.³¹ They have demonstrated tachyzoite and clusters clearly in the hepatocytes cytoplasm and karyorrhexis and karyolysis of hepatocytes. Also, they have observed inflammatory lesions on the surface of the liver progressing to the parenchyma during the infection.

In the study, large necrotic foci progressing from the Glisson's capsule to the parenchyma and necrosis spreading inter-lobular starting from the Glisson's capsule were observed. Focal necrosis with karyorrhexis as well as tachyzoite-like structures and cytoplasmic immunopositivity were detected in the liver. The presence of tachyzoites in the cytoplasm of hepatocytes was not clear.

In vivo studies have shown that tachyzoites are morphologically visible in the liver lesions of mice and can be clearly distinguished by light microscopy.³¹ Necrosis foci of hepatocytes with inflammatory cells were more prominent in the liver lesions of gerbils infected with *T. gondii*. Tachyzoites and their forms were not significant in gerbil hepatocytes. Kul and Haziroglu have reported that lesions are located on serosal surfaces of the abdominal organs progressing into the parenchyma of guinea pigs being experimentally infected with *T. gondii*.³⁸

Glatman Zaretsky *et al.*, reported that as a result of experimental infection of C57BL/6J mice with tachyzoites of the Prugniaud strain of *T. gondii*, serious losses in the boundaries between splenic red and white pulps and loss of the normal splenic architecture are observed.³⁹ Also, they have noted extra-medullary hematopoiesis in the spleen. Pinheiro *et al.*, have shown a loss of the normal architecture of the splenic lymphoid follicles in C57BL/6 mice infected with *T. gondii* TgCTBr9 strain.³⁷ Similarly, in this study, cellularity was decreased in splenic germinal centrum and follicles. Necrotic debris with parasites was observed in the spleen capsule. Extra-medullary hematopoiesis was also observed in the spleen. Immunopositive staining showed high intensity in the spleen capsule and

parenchyma and also in the centers of spleen follicles.

Gülbahar *et al.*, conducted an experimental infection study, where Balb/C mice were infected with *T. gondii* RH strain.⁴⁰ Their results showed focal mononuclear cell infiltrations composed of lymphocytes and histiocytes and degenerative changes in the distal and proximal tubules epithelia in kidneys. The authors also stated that these inflammatory changes increased from cortex to medulla. Similarly, hyperemia and degenerative changes under the capsule of kidneys and immunopositive staining in the proximal tubule epithelia were found in the current study. Diffuse mononuclear cell infiltration in the propria mucosa of intestine was reported by Pinheiro *et al.*, and Gülbahar *et al.*, in their studies on C57BL/6 mice infected with *T. gondii* TgCTBr9 strain and Balb/C mice infected with *T. gondii* RH strain, respectively.^{37,40} In this study, necrotic mass in intestinal serosa and mononuclear cell infiltrations in lamina propria of intestinal mucosa were noted. Immunopositive staining was observed in lamina propria, gland epithelium, serosa and intestinal lymphoid tissues.

In this study, the liver, lung, spleen, kidney and intestine were the most affected tissues, respectively. The parasite showed an affinity for the hepatic tissue.

In conclusion, this study was the first to demonstrate macroscopic and microscopic lesions in gerbils due to the infection caused by the *T. gondii* RH strain. Our findings were similar to those of other studies infecting other laboratory animals with experimental toxoplasmosis. The *T. gondii* infection spreads very quickly in animals without any preferential site. However, it causes the highest inflammatory reactions and necrosis in the liver. Additionally, Mongolian gerbils have been shown to be a suitable host to study liver pathology and pathobiology of *T. gondii* infection.

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Conflict of interest

There are no conflicts of interest to be declared.

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