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Hepatic Tissue Changes in Rats Due to Chronic Invasion of *Babesia microti**

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The etiological agents of babesiosis are intraerythrocytic parasites of the genus *Babesia,* which are transmitted by ticks. The course of disease is characterized by variable severity. The risk of a complicated course of babesiosis occurs in premature infants, the elderly, splenectomized patients and other immunocompromised patients. Severe cases of this disease can lead to multiple organ dysfunction. The study focuses on the impact assessment of chronic *Babesia microti* invasion on the morphology and ultrastructure of rat liver. The analyzed material was comprised of liver samples collected from *Wistar* rats infected with a reference strain of *B. microti* (ATCC 30221). None of the livers collected from rats with babesiosis was enlarged. The histopathological analyses showed signs of intensive inflammatory processes, especially in the perivascular areas. The hepatic mononuclear phagocyte system was characterized by increased activity. The ultrastructral analyses confirmed disintegration of hepatocytes with vacuolization in the perivascular areas. In addition, the perisinusoidal space (space of Disse) had irregular structure. In some areas, the space of Disse was enlarged or compressed. The morphological and ultrastructural analyses of rat liver with chronic babesiosis caused by *B. microti* showed significant pathological changes in perivascular areas which may be the cause of hepatic dysfunction.

Key words: *Babesia microti*, liver pathology, rats, histological examination, transmission electron microscopy.

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Babesiosis is an infectious disease transmitted by ticks. The etiological agents are intraerythrocytic parasites of the genus *Babesia* (phylum: *Apicomplexa*, order: *Piroplasmida*) (KJEMTRUP & CONRAD 2000; YOSHINARI *et al*. 2003; HILDEBRANDT *et al.* 2013). Some protozoan species (*B. microti* and *B. equi*) are able to invade lymphocytes. These blood cells may be damaged during the life cycle of parasites (SONENSHINE 1993; KJEMTRUP & CONRAD 2000; SCHUSTER 2002). Protozoa are capable of attacking a wide range of vertebrates: rodents, birds, dogs, cats, cattle, horses and humans (SCHUSTER 2002; HILDEBRANDT *et al.* 2010; LEIBY 2011; MOSQUEDA *et al*. 2012; CAPLIGINA *et al.* 2014). *Babesia* spp. is the second most common hemoparasite of mammals after trypanosomes (HUNFELD *et al*. 2008; SCHNITTGER *et al.* 2012).

Recent observations indicate that ticks are becoming frequent carriers of babesial parasites.

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This has led to an increase in morbidity rate among humans and animals. Therefore piroplasmosis has been categorized as an emerging infectious and invasive disease (LEIBY 2011; GRAY *et al.* 2002; HOMER *et al.* 2003; SKOTARCZAK 2008). Human babesiosis can be caused by several *Babesia* species characterized by different geographical distributions. However, *B. microti* is the most common etiological agent of this disease in the world (TEAL *et al.* 2011).

The course of babesiosis is characterized by variable severity: from subclinical to severe manifestation with multiorgan failure which can lead to death (ZAIDI & SINGER 2002). Clinical symptoms of disease resemble those of malaria. Among them, the most frequently mentioned are high fever (above 40°C), chills, fatigue, malaise, pain in muscles, nausea, vomiting, night sweats and weight loss. In the blood parameters, hemolytic anemia, which leads to hyperbilirubinuria and hemoglobinuria, is observed. Among biochemical changes, high levels of transaminases, alkaline phosphatase (ALP), unconjugated bilirubin and lactate dehydrogenase (LDH) in blood serum are identified (HOMER *et al.* 2000; LODES *et al.* 2000; GRAY *et al.* 2002; YOSHINARI *et al.* 2003; HUNFELD *et al.* 2008; GRAY *et al.* 2010; LOBO *et al.* 2010; MOSQUEDA *et al.* 2012).

Increased enzymatic parameters suggest damage to parenchymal organs (e.g. liver, spleen, kidney), which can become enlarged in the course of babesiosis (HOMER *et al.* 2000; ABRAMOWICZ 2007). The severity of symptoms directly depends on the level of parasitemia in the blood and the general health condition of the patient (HOMER *et al.* 2000). The risk of a complicated course of disease is increased in immunocompromised patients, i.e. premature infants, the elderly, splenectomized patients and those with diseases causing a decrease of immunity (KJEMTRUP & CONRAD 2000; TORRES-VÉLEZ *et al.* 2003; KUWAYAMA & BRIONES 2008; HILDEBRANDT *et al.* 2013). The acute phase of babesiosis can progress to chronic disease. In wild and domestic animals, chronic parasitemia may persist for many years. In rodents, this period can be up to 2 years. Usually, symptom exacerbations appear at the end of chronic disease (HOMER *et al.* 2000). Human asymptomatic invasion might persist even over a year. Babesiosis relapses are rarely observed in the case of a properly functioning immune system. Symptomatic persistent babesiosis appears in immunocompromised patients and can cause multiorgan damage (MINTZ *et al.* 1991; KRAUSE *et al.* 1998; KRAUSE *et al.* 2008).

The present study focuses on the impact assessment of chronic *B. microti* invasion on the morphology and ultrastructure of rat liver.

Material and Methods

The analyzed materials were liver samples collected from 20 *Wistar* rats during post-mortem examination. Earlier, the animals were infected with a reference strain of *B. microti* (ATCC 30221). The pathogen culture, obtained from American Type Culture Collection, was cultivated *in vivo*. The procedure involved injecting intraperitoneally (i.p.) 0.5 ml of protozoan culture into two six-week-old rats. The remaining rodents were infected by i.p. injection of 0.5 ml blood obtained from rats with confirmed parasitemia. The animals were housed in standardized conditions (temperature: 20-22°C, humidity: 50-60%, lighting cycles: 12 hours light and 12 hours darkness). Breeding was conducted in cages with pine sawdust which was autoclaved before use. The animals had unlimited access to water and fodder. Control material was comprised of livers collected from 2 healthy rats. During the experimental steps, 0.5 ml of sodium chloride physiological solution for intravenous injection (Inj. Natrii Chlorati Isotonica Polpharma, 9 mg/ml, Polpharma, Starogard Gdański, Poland) was injected intraperitoneally into the control rats. The control rats were housed in the same conditions as animals infected with *B. microti*. The use of animals in this study was approved by the Local Ethics Committee for Animal Experiments in Katowice, Poland (Resolution number: 32/2011 dated May 23, 2011). The study was carried out according to protocol based on European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

Parasitemia monitoring

Rat parasitemia was monitored by microscopic observation of blood smears which were performed at 2-week intervals. The smears were fixed in methyl alcohol (POCh, Gliwice, Poland) and stained by May-Grünwald-Giemsa (MGG) according to the manufacturer's instructions (Aqua-Med ZPAM, Cracow, Poland). Microscopic analysis was conducted using an Olympus BX60 microscope according to Schilling's method. Typically, 100 leukocytes were counted using microscopy. During this process, the morphological features of other blood cells (shape, size, color and intraerythrocytic inlusions) were analyzed.

Histological and ultrastructural analyses of livers

Six months after inoculation, the animals were euthanized by the use of inhaled anesthetics with isoflurane (Forane®-Baxter, Deerfield, IL, USA). During post-mortem examinations, liver samples were collected for histological and ultrastructural analysis. Tissues were fixed in the respective fixatives.

The samples for histological analysis were fixed in Bouin's solution at room temperature. At the next stage, tissues were rinsed in 80% ethyl alcohol, and then embedded in paraffin blocks according to standard histological procedure. Paraffin sections (6-μm thick) were stained by the Masson trichrome method. The dried preparations were embedded in a synthetic resin DPX Mounting Medium (POCh, Gliwice, Poland), and analyzed using an Olympus BX60 microscope equipped with XC50 digital camera and Olympus cellSens Standard software.

The liver samples for transmission electron microscopy (TEM) were fixed in Karnovski's solution at 4°C. After rinsing in 0.1 M of phosphate buffer (pH 7.4), the tissues were fixed again in 1% buffered osmium tetroxide solution (Sigma-Aldrich, St. Louis, MO, USA) for 2 hours. At the next stage samples were rinsed in phosphate buffer and dehydrated in an ethyl alcohol and acetone series according to standard histological procedure. Dehydrated tissues were embedded in epoxy resin – Poly/Bed® 812 Embeeding Media/DMP-30 Kit (Polyscience, Inc., Warrington, PA, USA). Resin polymerization was conducted at 60°C for 72 hours. The semi-thin sections (0.5-μm thick) were stained with methylene blue (AppliedChem, Darmstadt, Germany) and observed by means of light microscopy. The ultrathin sections (80-nm thick) were contrasted with uranyl acetate (Polyscience, Inc., Warrington, PA, USA) and citrate lead (Sigma-Aldrich, St. Louis, MO, USA). The ultrastructural observations were performed with a transmission electron microscope Hitachi H500 at an accelerating voltage of 75 kV.

Fluorescence *in situ* hybridization (FISH)

To detect *B. microti* DNA using the FISH technique, tissues were fixed in a neutral buffered formalin solution, dehydrated and embedded in paraffin. The blocks with samples were cut using a microtome, and then tissue sections were rinsed in xylene in order to remove paraffin. The peripheral blood smears for the FISH technique were fixed in 2.5% paraformaldehyde solution. Detection of *B. microti* genetic material in tissue sections and blood smears was performed using a commercially available kit, i.e. Histology FISH Accessory Kit (DAKO, Carpinteria, CA, USA). The preparation procedures of tissues and blood were carried out according to the manufacturer's instructions. In this study, a fluorescein-labeled probe complementary to the fragment of *B. microti* 18S rDNA gene was used. The probe sequence was as follows: 5'-fluorescein-GCCACGCGAAAACGCGCCTCGA-fluorescein-3' (SHAH & HARRIS 2010). Oligonucleotide synthesis was performed in the Institute of Biochemistry and Biophysics in Warsaw. The thermal profile of FISH was as follows: denaturation at 84°C for 5 min, and hybridization at 42°C for 30 min. After hybridization, rinsing was conducted according to the manufacturer's instructions. The analysis of preparations was performed with an Olympus BX60 epifluorescent microscope with a 150W xenon lamp using a blue filter for fluorescein and non-fluorescent immersion oil.

Results

The perpetuation of parasitemia in rat blood was confirmed during the first stage of the experiment. Microscopic observations of the peripheral blood smears stained by the MGG method demonstrated the presence of cyst-like basophilic intraerythrocytic inclusions in many areas. In addition, parasitemia was confirmed by thick smears of hemolyzed blood stained with MGG. After staining, the peripheral blood was mixed with distilled water in volume ratio 1:1. This step caused erythrocyte hemolysis and release of *Babesia* merozoites, hence protozoa concentration was observed in small areas of slides. This method is commonly used in veterinary diagnosis of babesiosis (Fig. 1A). Furthermore, the presence of *B. microti* DNA was demonstrated in blood cells with the FISH method (Fig. 1B). Spot signals of fluorescence were also observed in the samples of rat liver (Fig. 1C).

The macroscopic observation of livers collected from rats with chronic invasion of *B. microti* was performed during post-mortem examination. None of the organs was enlarged when compared with controls. More detailed histopathological analyses showed signs of intensive inflammatory processes in rats with babesiosis.

During a morphological analysis, numerous lymphocytic infiltrations were observed with the use of small magnification and light microscopy (Fig. 2A). The increase in lymphocyte count compared with the control liver (Fig. 2A') suggests inflammatory background of liver damage in the course of babesiosis. Ultrastructural observations confirmed these results. Other elements of the hepatic mononuclear phagocyte system (MPS) also showed increased activity. The concentration of MPS was visible in the area of blood vessels.

In the course of chronic babesiosis numerous hepatocyte injuries were also observed (Fig. 2B and 2C) compared with the control liver (Fig. 2B' and 2C'). The cells were enlarged, and there was vacuolization in the cytoplasm.

Observations with the use of TEM confirmed the presence of hydropic changes which constitute an initial stage of necrosis. Many cells in disintegration phase were observed in the area of blood vessels. The perivascular, ultrastructural analyses

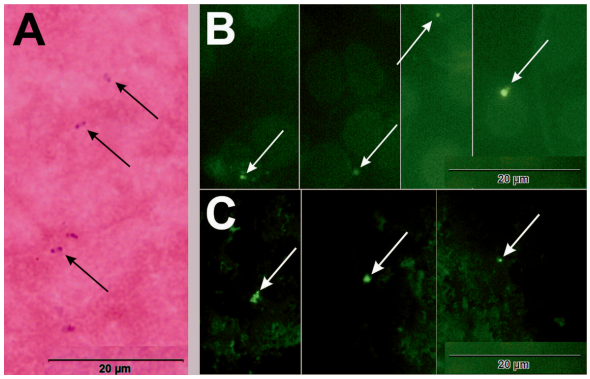

Fig. 1. A – The confirmation of presence of *B. microti* merozoites in rat erythrocytes during chronic invasion. Microphotograph of thick smears of hemolyzed blood stained with MGG. Black arrows indicate cyst-like basophilic, intraerythrocytic inclusions of protozoan merozoites. B – FISH spots in the rat erythrocytes identified genetic material of *B. microti* (white arrows). C – FISH spots in the hepatic tissue (white arrows).

showed cellular debris near vessels. A concentration of peroxisomes (microbodies surrounded by a single lipid bilayer membrane) was visible in the perivascular hepatocytes which were of different size and shape. This may indicate intense production of new microbodies (Fig. 3A).

In the areas of degenerative changes of hepatocytes, abnormalities in the perisinusoidal space (space of Disse) were also observed. In many areas, the space of Disse was discontinuous and irregular, in extreme cases it was completely compressed (Fig. 3B). In bile ducts, ultrastructure showed no pathological changes compared with the controls.

Moreover, the electronogram analysis demonstrated features of hepatic fibrosis manifested by the accumulation of collagen in spaces between hepatocytes (Fig. 3C). Over time this process can result in cirrhosis of the liver in the course of chronic parasitemia.

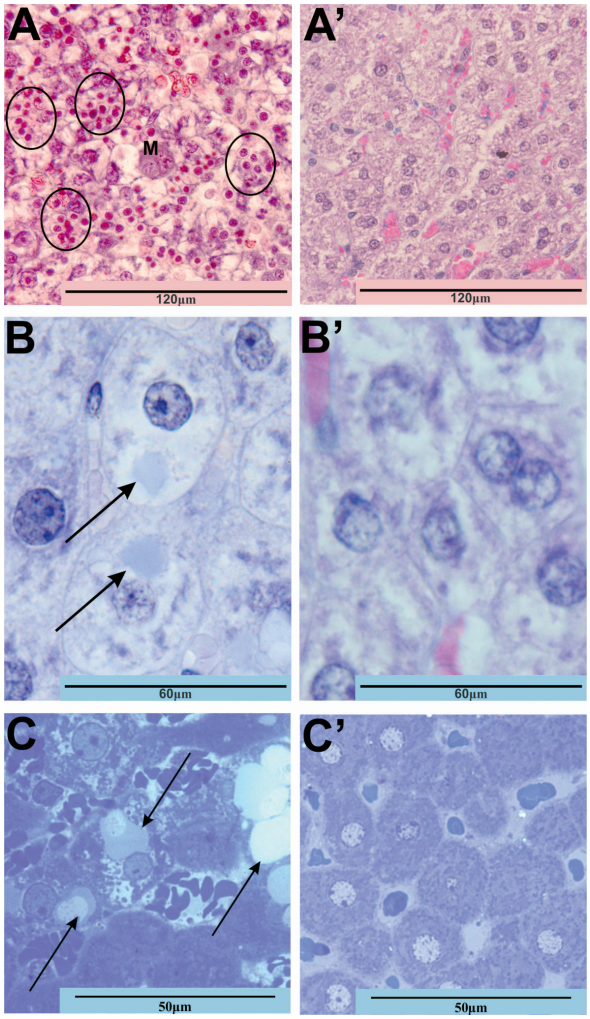

Fig. 2. A – Lymphocyte concentration (loops) and macrophages (M) in rat liver in the perivascular space during chronic invasion of *B. microti*. A'. The normal structure of rat liver from healthy control analyzed using small microscopic magnification. Elements of hepatic MPS were rare. B – Section with hydropic changes in the hepatic tissue during chronic invasion of *B. microti* (arrows). B' – A normal morphology of healthy hepatocytes analyzed using oil immersion microscopy. Histological sections in Figures A, A', B and B' were stained by the Masson trichrome method. C – Changes in the rat liver structure induced by a chronic *B. microti* invasion. Arrows indicate hydropic changes in the perivascular area. C'. Semi-thin sections show normal histological structure of rat liver. Histological semi-thin sections in Figures C and C' were stained with methylene blue.

Discussion

From a public health perspective, babesiosis seems to be increasingly important. Ticks, as vectors of parasites, exist in places of human activity, not only in forests but also in public gardens and recreational areas. In addition, more and more individuals are carriers of *Babesia* spp. with an asymptomatic course of disease. The subclinical phase of babesiosis may persist for months or years. Many patients who are invaded by the parasite never experience clinical symptoms (VANNIER & KRAUSE 2009). In addition, there is a possibility of transmitting parasites during transfusion of blood collected from an asymptomatic donor. Many casuistic reports confirm this fact (MINTZ *et al.* 1991; LEIBY 2006; LEIBY 2011; NGO & CIVEN 2009; HAAPASALO *et al.* 2010).

In this study, we presented observations concerning a comparison of ultrastructural and morphological changes in liver between *B. microti* infected rats and a healthy control group. The liver seems to play an important role in the course of babesiosis and other diseases in which parasites invade blood cells (BEN MUSA & DAWOUD 2004; ABRAMOWICZ 2007; DKHIL *et al.* 2010; DELIĆ *et al.* 2010; DKHIL *et al.* 2013). In hepatic tissue, asexual

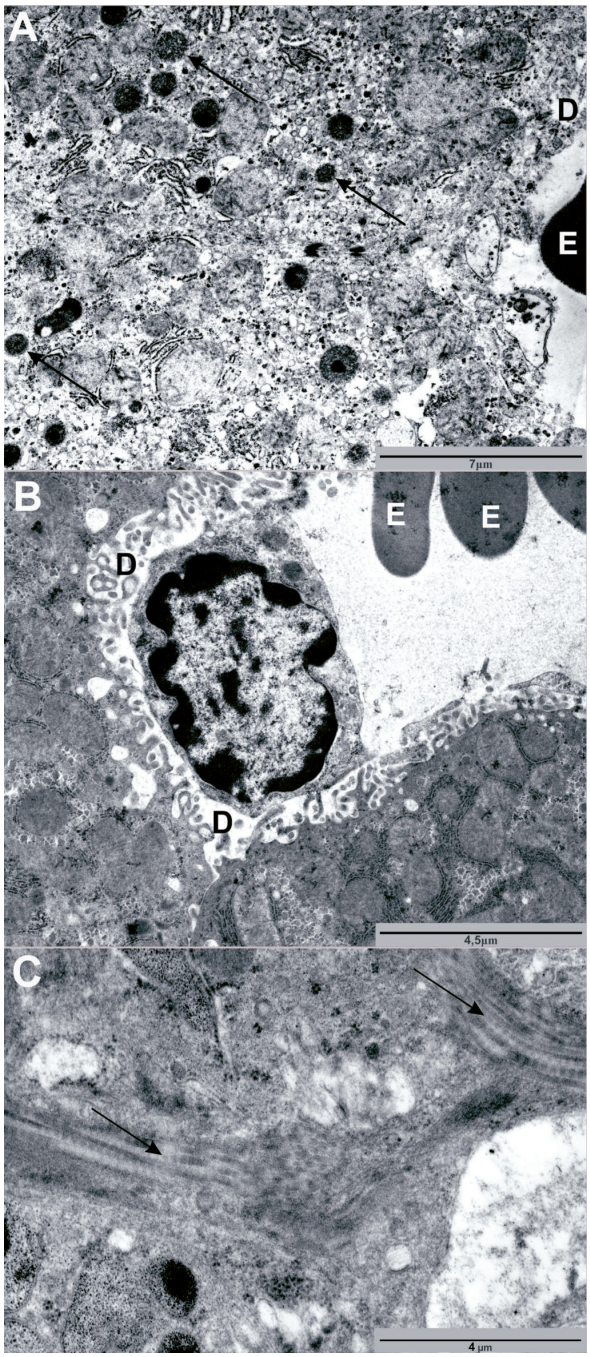

Fig. 3. A – The ultrastructure of rat hepatic tissue. Perivascular hepatocytes were disintegrating and the space of Disse was discontinuous and irregular. Arrows indicate peroxisomes. B – In extreme cases the space of Disse was completely compressed. C – Collagen fibers between hepatocytes were observed in many fields (black arrows). The architectural organization of functional units of the liver was disrupted. Abbreviations: D – space of Disse, E – erythrocyte.

stages of babesial parasites are detected (DKHIL *et al.* 2010). The immune response to invaded erythrocytes and the fight against protozoan pre-erythrocytic stages occur in the liver (DKHIL *et al.* 2013). The spleen plays an important role in the immune response to *Babesia* spp. invasion. An increased number of parasites caused by disintegration of erythrocytes in the red pulp and direct proximity of the white pulp suggest that the immune response is similar to the response observed in the liver. We can assume that immunological processes within the spleen are more violent due to the structure and function of this organ. Therefore, severe pathological changes in red and white pulp can cause splenomegaly and even spontaneous rupture of the spleen (DKHIL *et al.* 2014).

From the biopharmaceutical perspective, every change in enzyme system activity responsible for metabolizing drugs due to the invasion of the parasite may have an impact on the efficacy of pharmacotherapy, and the generation of additional adverse effects (SHIMAMOTO *et al.* 2012).

Our structural observations showed pathological changes characterized by necrotic and inflammation lesions, especially in the areas of blood vessels. Ultrastructural analyses also confirmed abnormalities in the perisinusoidal space. DKHIL *et al.* (2010) published similar results for the Mongolian gerbil (*Meriones unguiculatus*) infected with *B. divergens*. The authors presented hepatocytes with hydropic changes in the cytoplasm which correspond to our observations. The number of immune cells (lymphocytes, plasma cells, histiocytes) were also increased in the perivascular and parenchymal areas of hepatic tissue. The liver mononuclear phagocyte system was characterized by increased activity which is also consistent with our results. Molecular studies of tissue collected from mice infected with *B. microti* confirmed an increase in gene expression encoding pro-inflammatory cytokines (SHIMAMOTO *et al.* 2012).

We detected a concentration of peroxisomes in hepatocytes localized near vessels. An increased number of microbodies suggests an intensive fight with oxidative stress. Biochemical and histological analyses by DKHIL *et al.* (2013) indicate that during the course of babesiosis, symptoms of oxidative stress occurred in Mongolian gerbil livers. The parasite invasion causes an increase in free radicals which are responsible for cell membrane failure, protein dysfunction and DNA damage. Pro-inflammatory gene expression increase is the result of oxidative stress. Overproduction of reactive oxygen species (ROS) is observed under the influence of other infectious factors (not only *Babesia* spp. but also *Hepatozoon* spp. and *Theileria* spp.). ROS is responsible for liver tissue damage. Babesiosis causes a decrease in antioxidant system activity, reflected by reduced glutathione level and catalase activity, and total antioxidant capacity of the liver. Therefore hepatic tissue is more sensitive to damaging factors (HOMER *et al.* 2000; DKHIL *et al.* 2013; EL-FAR *et al.* 2014; HAMID *et al.* 2014). It seems that the immune re-

sponse to *Babesia* spp. invasion in spleen is similar to the response observed in the liver at the histological level. DKHIL *et al.* (2014) presented histological results of spleen obtained from Mongolian gerbils with babesiosis caused by *B. divergens*. The study described vacuolization of splenic cells. Disappearance of the boundary between red pulp and white pulp of the spleen was observed in the morphology. High splenic index indicates an increased number of immune cells (B-, T-lymphocytes and macrophages), presence of necrotic/apoptotic cells and pigments. Structure disorder can cause splenic function impairment, splenomegaly and even spleen rupture (KUWAYAMA & BRIONES 2008, DKHIL *et al.* 2014). We observed similar changes in the number of immune cells.

Results published by SHIMAMOTO *et al*. (2012) showed the influence of babesial invasion on activity of hepatic cytochrome P450. These authors observed a decrease in the expression of genes encoding CYP3A11, protein and activity of cytochrome P450 isoform in mice infected with *B. microti*. Babesiosis may cause a toxicity increase in antiparasitic drugs.

Many authors indicated changes in hepatic enzymes in the blood serum. In the course of babesiosis in humans, dogs, and gerbils an increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities is observed (HATCHER *et al.* 2001; BARIĆ RAFAJ *et al.* 2007; DKHIL *et al.* 2010). *B. divergens* invasion in gerbils also causes an increase in lactate dehydrogenase activity, and a decrease in unconjugated bilirubin and albumin levels in the blood (DKHIL *et al.* 2010; DKHIL *et al.* 2013). However, BARIĆ RAFAJ *et al.* (2007) observed an increase in blood bilirubin level in dogs with babesiosis caused by *B. canis*.

In conclusion, the morphological and ultrastructural analyses of rat liver with chronic babesiosis caused by *B. microti* showed significant pathological changes in the perivascular areas, which may be the cause of hepatic dysfunction. This study confirmed that the liver plays an important role in the development and course of the disease, and the immune response to a parasite invasion.

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