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Evidence of *Babesia microti* penetration of hepatocytes based on *in vitro* and *in vivo* studies

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Abstract

Introduction and Objective. Babesiosis is a tick-borne disease of animals and humans caused by the intraerythrocytic protozoa Babesia genus. The objective of this study was to demonstrate that *B. microti* can invade not only blood cells but also cells of other parenchymal organs, and to examine the effects of this parasitemia. An additional objective was to ascertain whether there were differences in the response of *B. microti* to hepatocytes *in vitro* and *in vivo* conditions.

Materials and Method. Wistar rats and the reference hepatocyte cell line Clone 9 isolated from rat livers were used. The rats and cell cultures were infected with an inoculum of *B. microti*. The investigation of cells *in vitro* and tissues *in vivo* was conducted using a light microscope, transmission electron microscope, atomic force microscope, and molecular methods.

Results. The research findings revealed substantial structural damage to cells, including hydropic degeneration and mitochondrial swelling in *B. microti*-infected hepatocytes under culture conditions. Similar damage to hepatocytes and thrombosis formation in liver blood vessels were observed. These changes indicated severe liver dysfunction and inflammation of the liver.

Conclusions. *B. microti* in intermediate hosts can infect erythrocytes, lymphocytes, and other cells, such as hepatocytes. *B. microti* affects liver cells directly in *in vitro* conditions and causes significant liver dysfunction in infected animals. The observed alterations in the blood vessels, including the adhesion of erythrocytes and thrombocytes to the vessel endothelium, provided compelling evidence that the infection of the intermediate host, *B. microti*, results in haemodynamic disturbances that significantly impact liver dysfunction.

Key words

liver, histopathology, *Babesia microti*, ultrastructure, parasitemia

INTRODUCTION

Babesia microti belongs to *Apicomplexa*, a polyxenic intraerythrocytic parasite characterized by metagenetic development with full development occuring within two hosts [1]. The definitive hosts are ticks, while the intermediate hosts are vertebrates, including humans, who might be infected by ticks *via* the transplacental route [2, 3] or through transfusion of blood and blood products [4]. An increase in tick population and lack of screening for infections by *Babesia* spp. in the human population causes it to be an epidemiological problem [5, 6]. Based on a study in the United States, the seroprevalence of babesiosis in humans was between 2% – 10% of the general population. Furthermore, it was found that in the blood donor population, between 1% – 4.9% of individuals were infected [7]. The condition features non-specific symptoms and a high proportion of sparsely symptomatic or asymptomatic cases [8, 9]. The most common acute babesiosis complications include damage to the kidneys, liver, and spleen [10–12]. According to literature data, *B. microti*, unlike *P. falciparum*, penetrates directly into erythrocytes during its developmental cycle [13, 14]. *B. microti* penetrates erythrocytes inducing their disintegration, causing a syndrome of multiple symptoms, such as anaemia, thrombosis, renal and hepatic dysfunction, and during prolonged parasitaemia – splenic rupture [10, 15]. The liver is well supplied with blood which increases the likelihood of contact with the intra-erythrocyte parasite.

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OBJECTIVE

Because many questions remain unanswered regarding the pathophysiological effects of infection with this parasite [16], the aim of the study was to show that during *B. microti* infection, the parasites can penetrate not only blood cells but also cells of other organs, such as the liver. The study also shows the possibility of thrombus formation under the influence of *B microti* infection, and to observe the effects of invasion on the structure of hepatocytes and other elements of the liver, as well as the circulation of blood in this organ.

MATERIALS AND METHODS

One of the important aims of the study was to compare the effects of *Babesia microti* on hepatocytes under *in vitro* and *in vivo* conditions. To this end, a series of studies were conducted using a range of methods, including histological, ultrastructural, and molecular approaches, in both configurations.

***In vivo* studies.** 20 young, sexually mature male Wistar rats were selected for the *in vivo* study in order to exclude the influence of hormonal variation's, and minimize the number of experimental animals. The selection of male rats eliminated the potential influence of the estrous cycle, thereby reducing variability and facilitating the reproducibility of results.

The animals were kept under the standard conditions required by the Bioethics Committee: 20–22 °C, about 50–60% humidity, and equal light cycles of 12h light and 12h darkness. The animals had *ad libitum* access to water and food.

In the first stage of the study, 2 rats were intraperitoneally infected with the reference strain of *Babesia microti* (Franca) Reichenow ATCC 30221 (Manassas, VA, USA). At the same time and under the same conditions, physiological saline was injected into 2 other rats. Parasitaemia control was performed 3 times in all rats at weekly intervals. It was assessed by observing blood smears and calculating the percentage of infected erythrocytes, 500 in each smear. After 3 weeks of the experiment, when parasitaemia reached 20% of erythrocytes, blood was collected from the infected rats. Following this, 0.5ml of infected blood was injected into each of 7 rats in the study group. Seven rats from the control group were injected with blood without *B. microti*, which was collected from rats previously administered with physiological saline. The rats were then sacrificed – anesthetized by inhalation of isoflurane, under American Veterinary Medical Association Guidelines for the Euthanasia of Animals, and tissues collected for further tests. The blood was fixed with methyl alcohol. Liver samples collected for histological studies were preserved in Bouin's solution, and samples intended for ultrastructural analyses were preserved using Karnovsky's buffered solution.

***In vitro* studies.** Cell culture was carried out on the Clone 9 cell line (Sigma-Aldrich, St. Louis, MO, USA). According to the manufacturer's instructions, rat liver cells were cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 5% foetal bovine serum, and 1% essential amino acids. Cultures were grown in sterile 12-well Nunclon plates (Nunc, Wiesbaden, Germany). After reaching 80% confluence, 0.5 ml of blood from rats with confirmed presence of *B. microti* was added to the 5 wells with cell culture. Control groups were cultured in a medium with blood from healthy rats (5 wells) and without blood added (2 wells). The cultures were maintained at 37 °C for 48h in an atmosphere of 5% CO_2. After this, the cultures were washed with DMEM and fixed in 2.5% PFA. Cells were cultured on slides in Falcon Culture Slides for molecular analysis. *In situ* analyses were performed using the FISH method. The culture was carried out under identical conditions.

To confirm the reliability of the *in vitro* tests and the presence of *B. microti* in the monolayer of cells, 0.5 ml of the cell suspension was taken from the culture and injected intraperitoneally into healthy rats (2 individuals). After 7 days, the presence of parasitic merozoites in the blood of infected rats was checked.

Parasitaemia control. May-Grünwald-Giemsa staining was performed to confirm the presence of *B. microti* in rats' blood smears. The procedure was performed following the manufacturer's instructions. The presence of protozoan DNA was also confirmed in blood smears, Clone 9 cells, and rats' livers using fluorescent *in situ* hybridization (FISH). Molecular detection of *B. microti* in rat liver was performed in unfixed cross-sections obtained using the cryostat. Histology FISH Accessory Kit (DAKO, Carpinteria, CA, USA) was used to detect *B. microti* genetic material. The probe sequence was as follows: 5′-fluoresceins-GCCACGCGAAAACGCGCCTCGA-fluorescein-3′ (Metabion, Planegg, Germany). The probe complemented the *B microti* 18S rRNA gene fragment [17]. The preparations were analyzed using an Olympus BX60 epifluorescence microscope in which the excitation radiation source was a 150W xenon lamp. Excitation wavelength – 475–490nm**,** emission wavelength – 510–520nm with peak fluorescence.

Histological and ultrastructural studies. The hepatocytes from the *in vitro* culture of Clone 9 were embedded in epoxy resin, as were the liver samples from rats. The material obtained from the *in vitro* culture was dehydrated in ethanol-acetone series and embedded in epoxy resin directly on the culture plates. Semi-thin sections were stained with 1% methylene blue solution. The ultrastructural observations were performed by TEM FEI Tecnai G2 BioSpirit, at an accelerating voltage of 120kV. For observations, a NanoWizard®3 BioScience atomic force microscope (AFM) by JPK Instruments AG (Berlin, Germany) was used, in combination with a Zeiss Axio Observer (Jena, Germany) inverted optical microscope. The AFM was located in a specially designed laboratory with acoustic and vibration isolation to ensure absolute stability. The optical microscope with DIC contrast and a maximum of 400 magnification was used to aid the positioning of the AFM tip to the desired face and location on the sample surface. All experiments were performed in air using the intermittent contact mode (AC mode) with the Z range set to 15µm and a scan rate of 0.5–1Hz. Imaging was carried out using the Tapping Mode NSC15/AL BS etched silicon probes with a resonance frequency of 265–410kHz (MikroMasch, Portland, USA). During measuring, the drive amplitude and set point were adjusted to minimize tip artifacts. The JPK SPM Software was used for AFM operation and later imaging processing. The fixed liver section for histological examination were stained using the AZAN trichrome method. The individual stages of the experiment are presented shown in Figure 1.

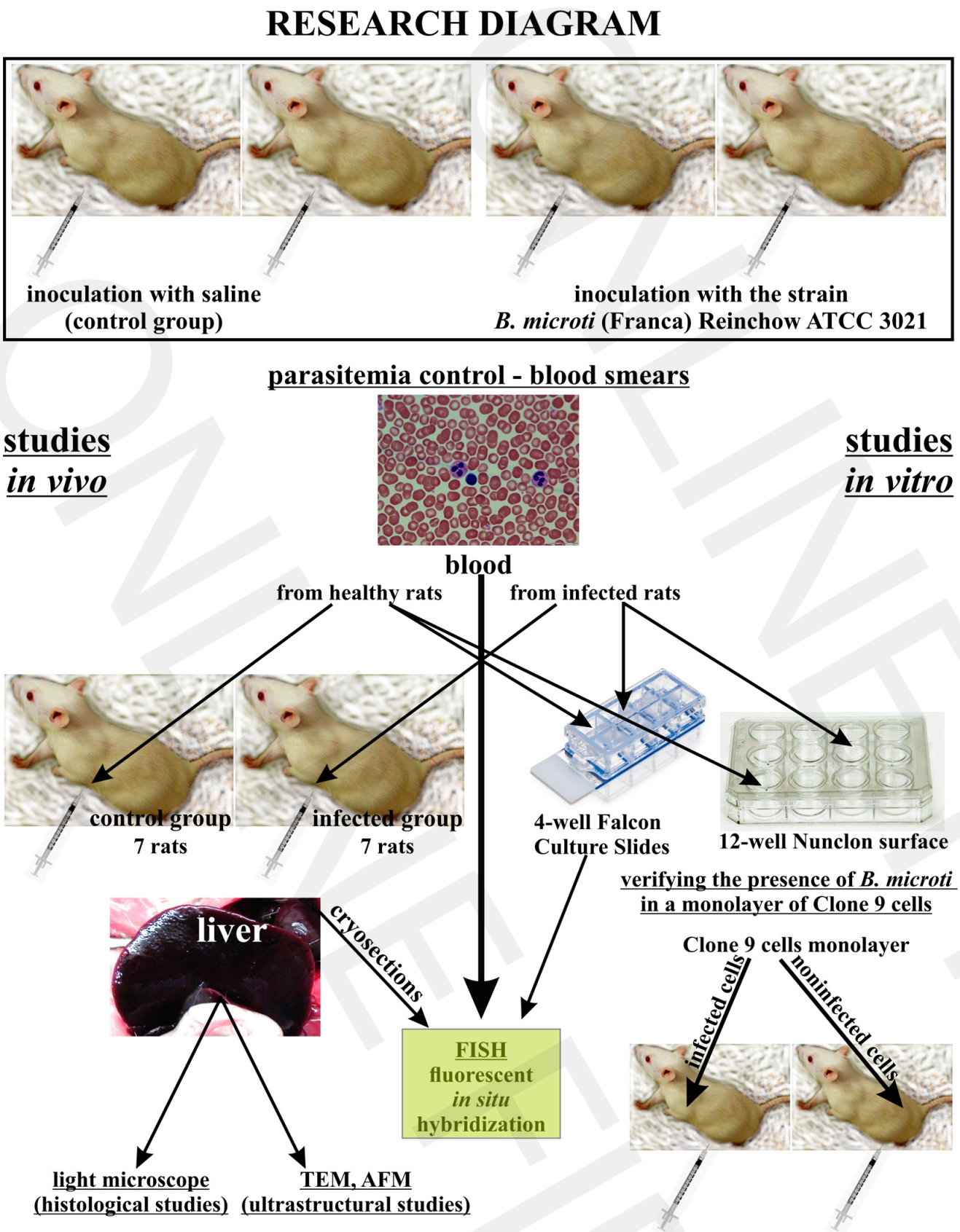

Figure 1. Scheme of an experiment

RESULTS

Parasitaemia control using MGG and FISH methods. Rats' blood at the highest stage of parasitaemia was used as the inoculum to infect hepatocyte cultures during *in vitro* studies. The highest stages of parasitaemia were obtained using both routine methods, May-Grünwald-Giemsa (MGG) staining of smears and detection of *B. microti* genetic material by fluorescent *in situ* hybridization (FISH). All the techniques used confirmed the presence of protozoa in the infected cells and tissues. Using the FISH method, green fluorescence signals were found in the blood of infected animals. Moreover, no fluorescent signals were detected in blood smears of control rats, on control plates with Clone 9 cells, and liver sections of groups of control rats (Fig. 2A-F).

Light microscope observations of Clone 9 cells cultured in medium with the addition of rat blood infected with *B. microti*. On the cross-semithin sections through the monolayer of hepatocytes cultured with the addition of blood infected with *B. microti*, many shadows of erythrocytes and membrane residues from the destroyed morphotic elements of blood, were observed. In some erythrocytes, the *B. microti* had a characteristic ring-like form located over the apical surface of the hepatocytes monolayer (Fig. 3A). Similar images were also observed in the control group. No piroplasms occurred in the control inoculum. Regressive changes were noted in many observed sections across the monolayer, manifested by a free plasma mass with loss of plasmalemma integrity and absence of normal cells. Numerous vacuoles occurred within the cytoplasm

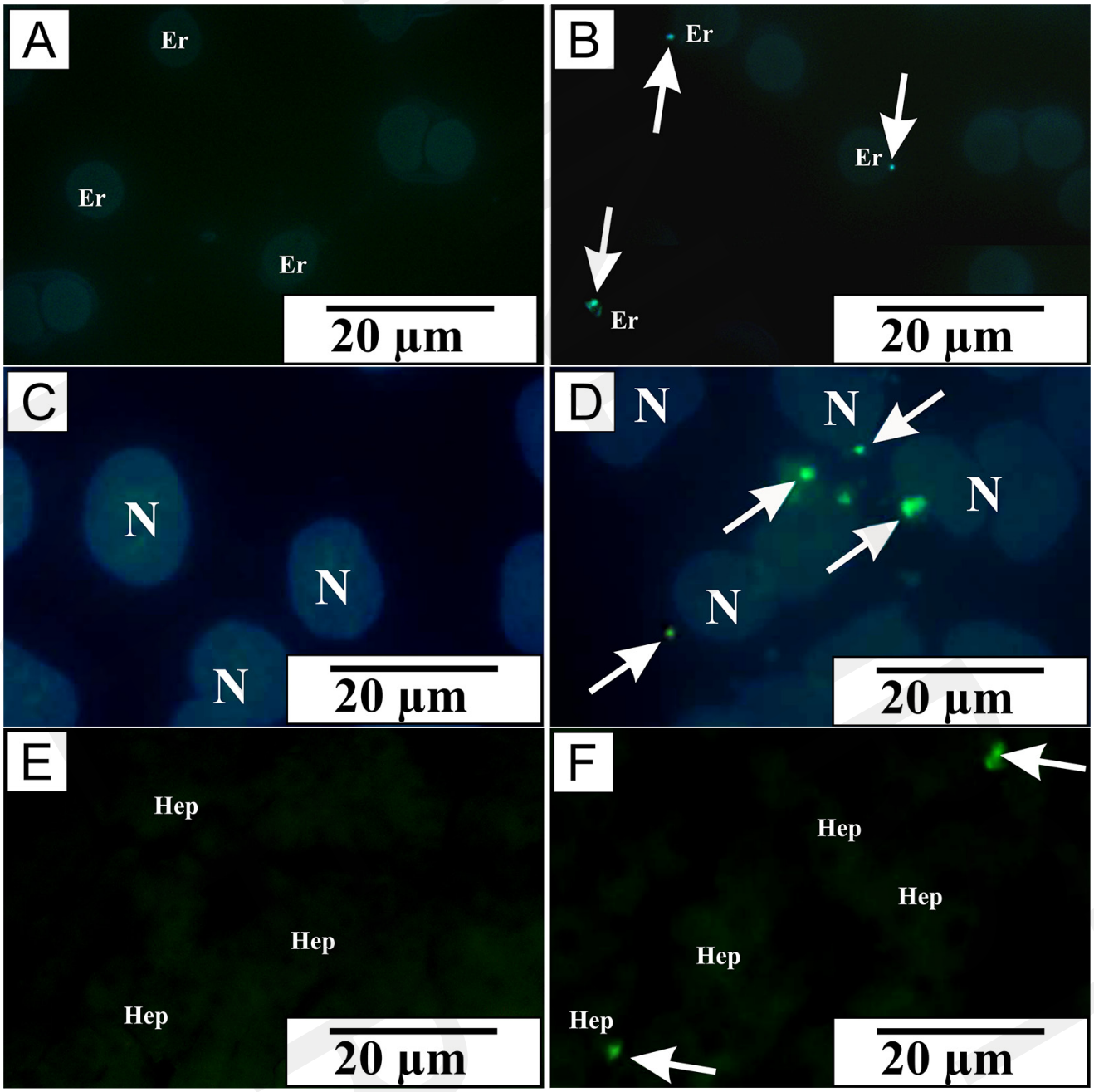

Figure 2A-F. Microphotography of Clone 9 cell monolayer. A: erythrocytes in blood smear of rats from the control group; B: blood smear of rats infected with B. microti; C: liver cells of Clone 9 in control culture; D: liver cells of Clone 9 line cultured with blood collected from infected rats; E: liver collected from control rats; F: liver collected from rats infected with B. microti. FISH

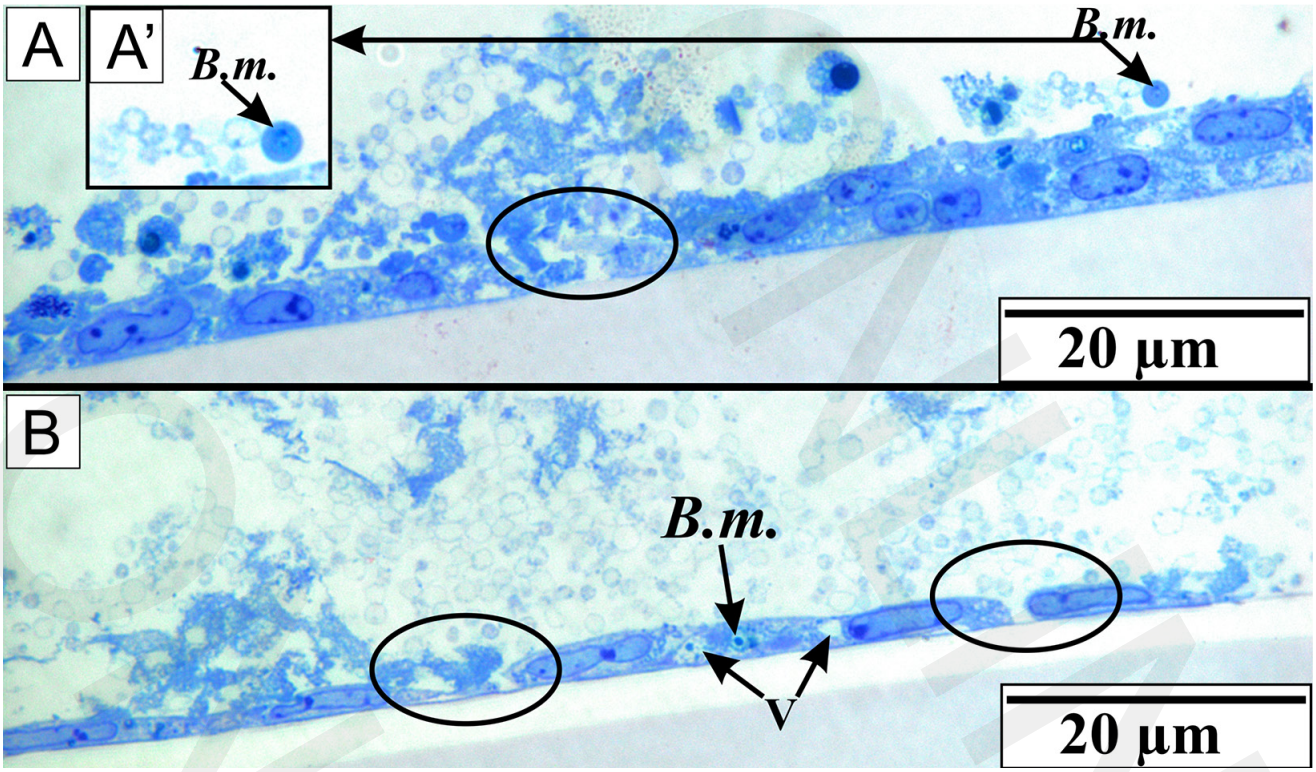

Figure 3A-B. Semithin cross-sections of Clone 9 cells cultured in medium with the addition of rat blood infected with B. microti. Regressive changes of monolayer – oval shapes; B. microti merozoites within the cytoplasm of erythrocyte of inoculum – characteristic ring form (A, A'); B. microti merozoite within the cytoplasm of hepatocyte (B); B.m. – B microti merozoites; V – vacuoles. Slides stained with methylene blue

of the other hepatocytes. Inclusions of a size and structure suggestive of *B. microti* merozoites, were observed in a few hepatocytes (Fig. 3A, 3B).

Observations of the Clone 9 liver cells using TEM. Ultrastructural analyses of Clone 9 line monolayers from the control group showed continuity of cells in each field of view. The cells varied in height, some were flattened, and their cytoplasm showed poor differentiation. The cells adhered tightly to each other, and intense growth was responsible for developing layers of cells on each other in some areas. Other cells were higher and contained typical organelles within the cytoplasm, such as mitochondria, and rough endoplasmic reticulum (Fig. 4A, 4B). Most mitochondria were orthodox in structure, with a few short microvilli on the free surface of the cells. Filaments of the cytoskeleton were visible, especially under the plasmalemma. Tight junctions were visible in some sections (Fig. 4A).

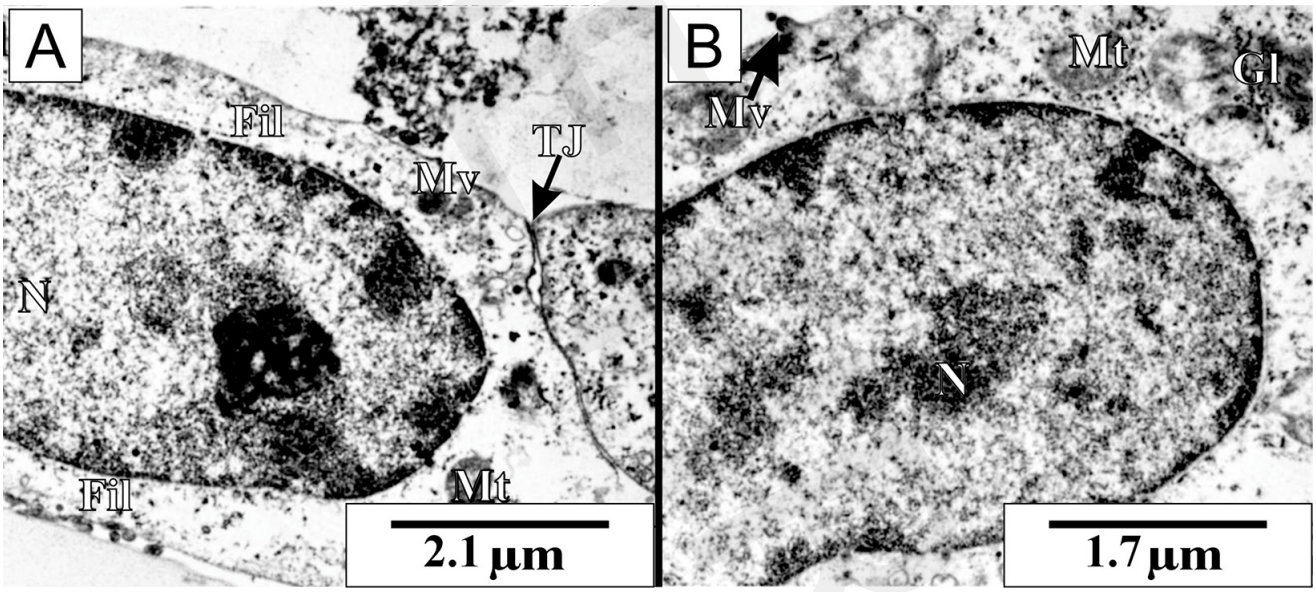

Figure 4 A-B. Ultrastructure of hepatic cells of Clone 9 with no contact with B. microti. Fil – filaments of the cytoskeleton; Gl – glycogen; Mt – mitochondria; N – nucleus; Tj – tight junction. TEM

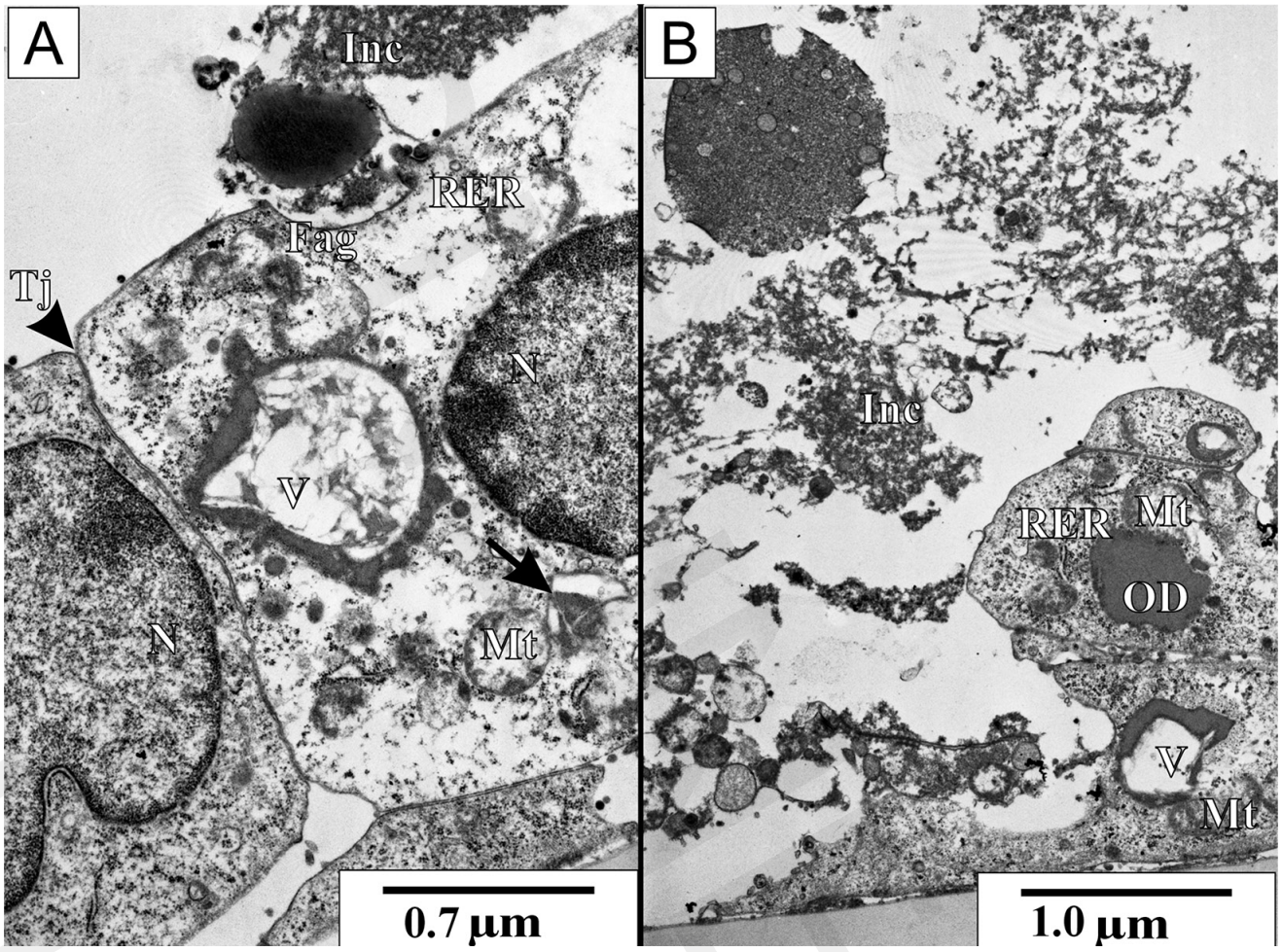

Figure 5A-B. Ultrastructure of Clone 9 cell line cultured in a medium with the addition of blood of rats infected with B. microti. Arrow – indicates pear-shaped structure (piroplasma); Fag – phagosome; Mt – mitochondria, N – nucleus; OD – osmophilic deposits; RER – rough endoplasmic reticulum; V – vacuole. TEM

Ultrastructural analyses of line Clone 9 cells cultured in a medium with the addition of blood of rats infected with *B. microti*. After exposition to the blood of infected rats varying degrees of degeneration of the hepatocytes were observed. A characteristic feature of the analyzed cells was the presence of strongly osmophilic deposits in their cytoplasm. Their shape was irregular and the internal structure was heterogeneous. The plasmalemma on the apical side of the hepatocytes was disrupted in numerous places (Fig. 5A, 5B). Within the cytoplasm of hepatocytes, hydropic degeneration was observed many times. Fragments of hepatocytes' cytoplasm and cell organelles were visible in the spaces surrounding the damaged cells. Mitochondria varied in size and shape, with features of swelling, atrophy of mitochondrial crests, and matrix with reduced osmophilicity were observed. In individual cases, inclusions resembling pear-shaped structures were seen in the cytoplasm of hepatocytes. Tight junctions were present near the apical side of hepatocytes (Fig. 5A). These connections, however, were not visible on severely damaged cells.

Histological examination of liver samples from rats infected with *B. microti*. On day 21 of parasitaemia, on the cross-section through the liver of infected rats, pear-shaped cells of *B. microti* were noted within and outside vessels. The presence of pyroplasms was mainly observed near the central vein and sinusoid vessels. In the spaces between vessels and hepatocytes an increased number of macrophages, as well as several groups of lymphocytes, were observed (Fig. 6A-C). A high accumulation of erythrocytes within the blood vessels was also seen, especially in the area of the central vein and sinusoids. On the sections through the liver, numerous mitotic figures within hepatocytes were observed (Fig. 6C). Also visible were many hepatocytes with vacuoles which had an amorphous content (Fig. 6D).

Ultrastructure rat's liver samples infected with *B. microti*. Ultrastructural analyses of the rat liver infected with *B. microti* narrowing perisinusoidal and portal spaces were poorly visible (Fig. 7A, 7B). The hepatocyte cytoplasm contained mitochondria, rough endoplasmic reticulum tubules, α-glycogen particles, a few lipid droplets, and residual bodies (Fig. 7A-B). Numerous vesicles filled with a moderately osmophilic matrix containing a paracrystalline core were visible (Fig. 7C,D). There were normal, fenestrated capillaries (Fig. 7A, 7B) and some blood vessels with poorly demarcated borders in some areas. Extravascular erythrocytes were visible. The round or oval spaces of capillaries filled with flocculent material accompanying erythrocytes were visible (Fig. 7A,B).

Moreover, within the lumen of blood vessels, aggregations of thrombocytes with groups of connected erythrocytes were observed. The cross-sections through the liver vessels observed in the atomic force mass microscope (AFM) and

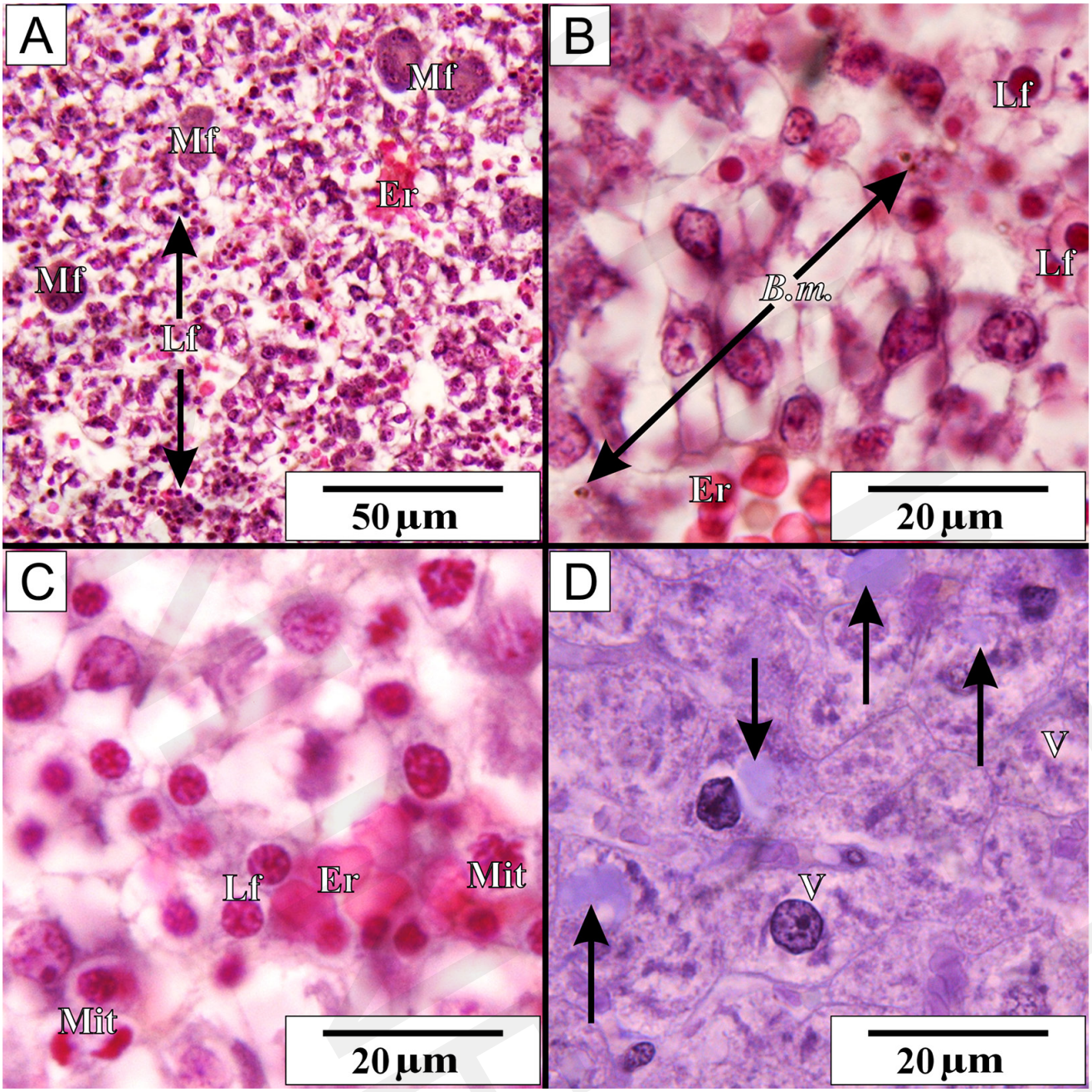

Figure 6A-D. Liver samples of rats infected with *B. microti*. B.m. – *B. microti* piroplasms; Er – erythrocytes; Lf – lymphocyte; Mf – macrophages. Slides stained with AZAN trichrome method. Arrows – amorphous matrix (mucopolysaccharides deposits) in the cytoplasm of the hepatocyte; erythrocytes; Lf – lymphocyte; Mit – mitotic figures; Mf – macrophage. Slides stained with AZAN trichrome method

transmission electron microscope (TEM), erythrocytes adhering to the endothelial surface and fibrin deposits tightly adhering to the blood vessel wall were visible. These observations were consistent with histopathological observations in which mural thrombosis was often noted in cases of *B. microti* infection. Pyroplasms of *B. microti* were observed in the cytoplasm of erythrocytes (Fig. 8 A, 8B).

DISCUSSION

Babesia microti is a parasite the full development of which occurs within 2 hosts. The definitive hosts are ticks, while the intermediate hosts are vertebrates. Many clinical cases prove that humans can be intermediate hosts of *B. microti*, with the symptoms of the infections varying. One of the symptoms may be liver dysfunction [16, 18].

Babesia has common features with *Theileria* spp. and *Plasmodium falciparum*, although this remains unclear [19], and share such similarities as the ability to invade more than just erythrocytes, multiple invasions of individual erythrocytes, and variation in schizont morphology.

This results of the current study indicate differences in the contact of *B. microti* piroplasm with rat hepatocytes *in vitro* and with rat liver *in vivo*. It can be assumed that the piroplasms can cross the blood vessels – liver tissues barrier. Similar results were obtained in earlier studies [10]. However, there is a need to verify the ability of *B. microti* to penetrate not only erythrocytes but also hepatocytes and cells of other organs of wild animals under natural

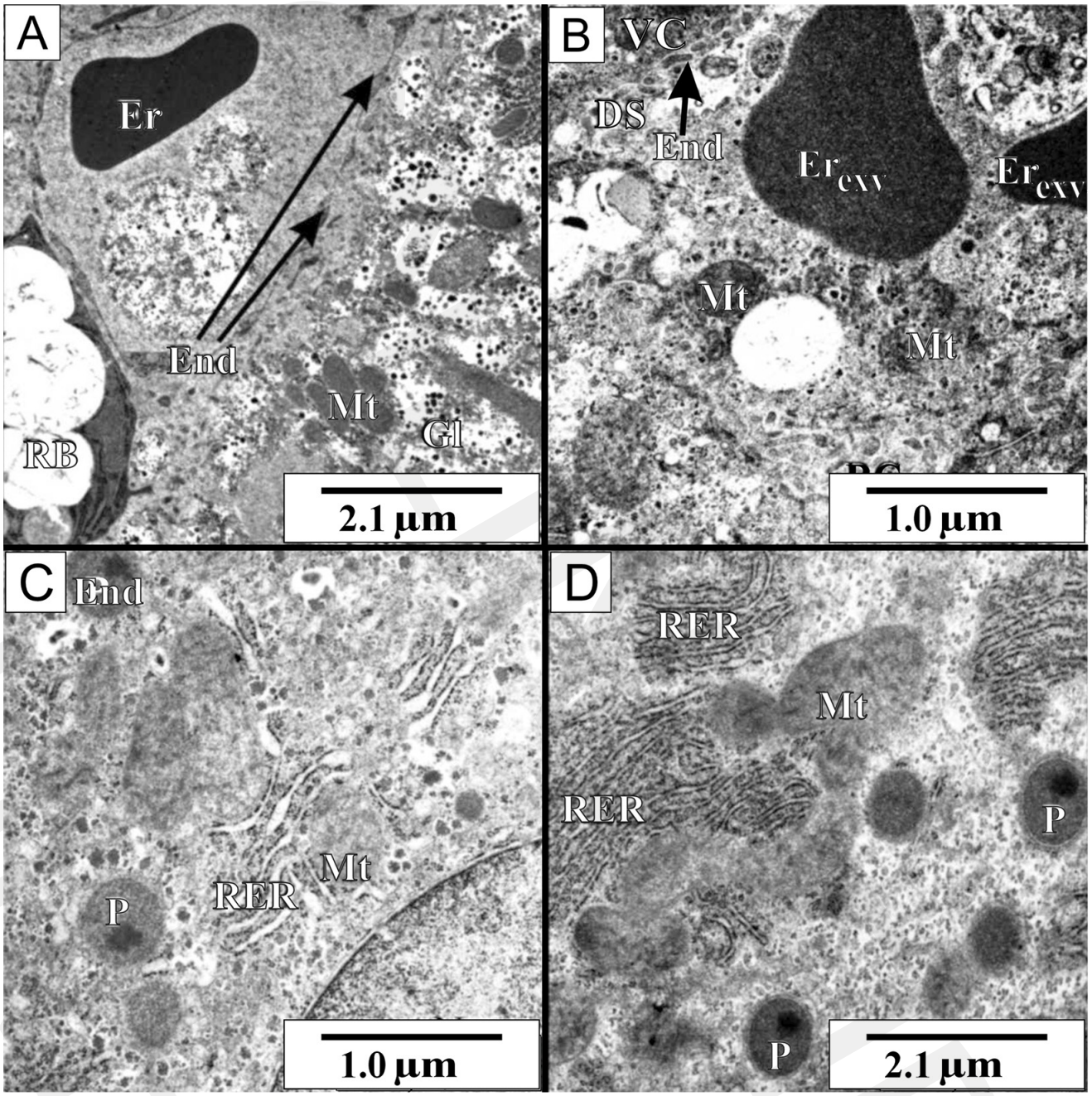

Figure 7 A-D . Ultrastructure of liver samples from rats infected with *B. microti*. BC – bile ducts; DS – Disse space; End – endothelial cells; Er – erythrocytes; Gl – glycogene (α - granules); Mt – mitochondria; RB – residual bodies, RER – rough endoplasmic reticulum, Gl – glycogene (α-granules); Mt – mitochondria; P – peroxysomes; RER – rough endoplasmic reticulum. TEM

conditions. Comparative studies of the Clone 9 cultured cells line in a medium with the addition of blood from healthy animals and animals infected with *B. microti,* indicated the destructive effect of blood from infected rats on the structure and ultrastructure of the cells studied. The results of ultrastructural studies showed swollen mitochondria, hydropic degeneration, the presence of osmophilic granules with the appearance of typical lysosomes, and localized disruption of the continuity of the cell membrane. All the noticed changes led to the destruction of the cells, resulting in discontinuity in the culture monolayer. These lesions were found in semi-thin preparations of the studied cell culture. The destructive effect of the inoculum containing *B. microti* on the tested cells should be considered. This action probably involves the effect of metabolic or enzymatic factors released from the inoculum into the culture medium, acting on the cell membranes of cultured hepatocytes and their receptors. This interpretation seems to be correct because the results of previous proteomic studies performed in infected animals have shown that an increase in the level of parasitemia in dogs and mice causes changes in the activity of many proteins [18, 20].

Based on the results obtained in the current study, it cannot be determined that the described changes in hepatocytes are the result of the migration of piroplasm into the cytoplasm of hepatocytes, or are the result of interruption of plasmalemma or binding of *B. microti* to the membrane and engulfment of piroplasm by endocytosis. However, the structural results

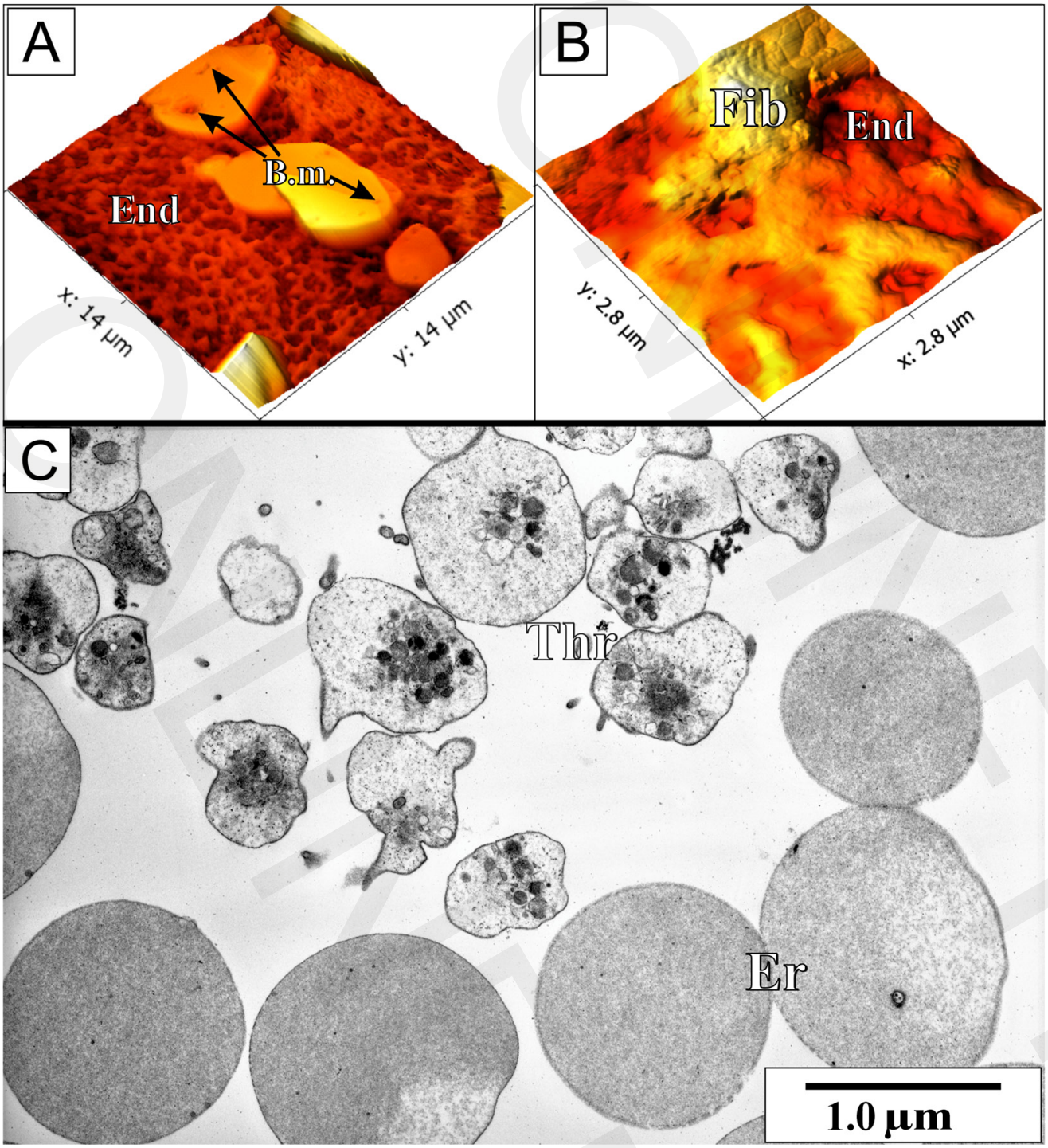

Figure 8 A-B. Ultrastructure of blood vessel lumen of liver samples from rats infected with B. microti observed with AFM method. B.m. – B. microti piroplasms inside erythrocytes; End – endothelial cell surface; Fib – fibrin deposits. **8C**. Ultrastructure of liver's blood vessel lumen from rat infected with B. microti. Er – erythrocytes; Thr – thrombocytes. TEM

obtained in the study indicate the presence of *B. microti* in the liver parenchyma of the infected rats used. Confirmation of the existence of *B. microti* in the liver parenchyma of the rats and in the culture of Clone 9 cells is provided by the results obtained by the FISH method. Structural and ultrastructural studies showed noticeable differences in the livers of *B. microti*-infected and uninfected rats, with the liver of infected rats showing narrowing of the lymphatic and secretory spaces within the lobules. These changes may cause complex liver dysfunction provoked by contact with *B. microti*. Confirmation of liver dysfunction in rats infected with *B. microti* is provided by ultra-structural changes in hepatocytes, such as a visible increase in the number of peroxisomes, and the presence of vacuoles and vesicles containing an amorphous matrix.

The results of the current study indicate the existence of hydropic degeneration and hyaline degeneration, hyalinizing changes, and glassy degeneration-type changes in hepatocytes. The results corroborate the data obtained from various organs of animals infected with *B. microti* [3,

14, 21]. It should be noted that in the liver of rats infected with *B. microti*, many macrophages and lymphocytes were found between hepatocytes. The results indicate intense diapedesis and immune response of the rats in the early stage of the disease. In addition, the increased number of mitotic figures in the studied material indicates an enhanced regenerative response of the liver [22, 23]. An essential result of the study is the presence of changes in the blood vessels of the liver of rats infected with *B. microti*.

The formation of agglomerates of erythrocytes in blood vessels and the adhesion of erythrocytes to endothelial cells, as well as the presence of aggregates of thrombocytes at a very early stage of the disease, indicate the development of sequestration and mural thrombosis. Sequestration and mural thrombosis in the studied rats were demonstrated using atomic force microscopy (AFM) methods. The data obtained are consistent with the results of clinical studies [24].

The presented results indicate similar changes in cells cultured *in vitro* and tissues collected from rats infected with *B. microti*. The observations show that, to a certain extent, *B. microti* exerts pathogenic effects directly on the cells and tissues of the liver. The secondary effects of animal infection may potentiate or inhibit parasiatemia, depending on the organism's immune condition. Nevertheless, the effects of *B. microti* on cells *in vivo* and *in vitro* are similar in many respects.

CONCLUSIONS

In summary, contrary to common belief, *B. microti* in intermediate hosts can infect not only erythrocytes and lymphocytes but also other cells such as hepatocytes. This was verified both *in vitro* and *in vivo* – the comparison of these 2 models made it possible to verify the susceptibility of liver cells to infection in the presence of the immune system and an isolated cell line. Invasion of *B. microti* into liver cells causes degenerative changes. *In vivo* studies revealed hyaline degeneration, deposition of mucopolysaccharide deposits, and vacuolation of hepatocytes.

It is very important to demonstrate changes in the small blood vessels of the liver. There was significant congestion in the central veins and the formation of clots, especially in the vessels of the marginal zone. Due to the serious clinical consequences, *B microti* infection should be diagnosed quickly and treated effectively. Clinicians' knowledge about babesiosis should be expanded.

The study provides some observations that require further research using different methods. A more extensive comparison of studies of babesiosis in animals with clinical data on human babesiosis is also required.

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