ELECTRON MICROSCOPY OF THE *PLASMODIUM FALCIPARUM* TROPHOZOITES AND THE TISSUES THESE HAVE INFECTED IN SEVERE TROPICAL MALARIA

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The paper provides the results of the comprehensive electron microscopic examination of the venous blood and internal organ tissue samples obtained when studying the imported case of tropical malaria. The study was aimed to assess the fine structure of the erythrocytic stages of *Plasmodium falciparum* and alterations of the affected tissues in severe tropical malaria. The venous blood, cerebral cortical tissue and myocardial samples were examined by light microscopy and electron (scanning and transmission) microscopy. Numerous *Plasmodium falciparum* trophozoites were found in blood. Multiple Maurer's clefts were found in the cytoplasm of the infected erythrocytes. Abnormal intercellular contacts between the infected and unaffected erythrocytes were revealed, which resulted in their adhesion and rosette formation (erythrocyte rosetting/e-rosetting). When studying cortical tissue and myocardial samples, fixation of the affected erythrocytes on the endothelium (erythrocyte adhesion) was noted in the capillary lumen. Rosetting and erythrocyte adhesion lead to capillary thrombosis, disruption of microcirculation and sequestration of tissues in vital organs (parasite sequestration). The identified morphological features of the pathogens causing tropical malaria and the affected tissues determine the parasites' capability of changing properties of the infected erythrocytes' cell membranes, which leads to formation of abnormal intercellular contacts and constitutes one of the main mechanisms underlying the *Plasmodium falciparum* virulence.

Keywords: tropical malaria, *Plasmodium falciparum*, virulence, E-rosetting, erythrocyte adhesion, parasite sequestration, PfEMP1, electron microscopy

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Compliance with ethical standards: the study was approved by the Ethics Committee of the Kirov Military Medical Academy (protocol No. 285 dated 21 November 2023) and conducted in accordance with the principleы of the Declaration of Helsinki (1964) and its subsequent updates.

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ЭЛЕКТРОННАЯ МИКРОСКОПИЯ ТРОФОЗОИТОВ *PLASMODIUM FALCIPARUM* И ИНФИЦИРОВАННЫХ ИМИ ТКАНЕЙ ПРИ ТЯЖЕЛОЙ ФОРМЕ ТРОПИЧЕСКОЙ МАЛЯРИИ

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Представлены результаты комплексного электронно-микроскопического исследования образцов венозной крови и тканей внутренних органов, полученных при изучении летального случая завозной тропической малярии. Целью работы было изучить ультраструктуру эритроцитарных стадий развития *Plasmodium falciparum* и изменений пораженных ими тканей при тяжелой форме тропической малярии. Образцы венозной крови, тканей коры головного мозга и миокарда исследовали с помощью световой, а также электронной (сканирующей и трансмиссионной) микроскопии. В крови были выявлены многочисленные трофозоиты *Plasmodium falciparum*. В цитоплазме инфицированных эритроцитов обнаружены множественные расщелины Маурера. Между инфицированными и непораженными эритроцитами выявлены патологические межклеточные контакты, что приводит к их слипанию и формированию розеток (эритроцитарный розеттинг). При исследовании тканей коры головного мозга и миокарда в просвете капилляров отмечена фиксация пораженных эритроцитов на эндотелии (эритроцитарная адгезия). Розеттинг и адгезия эритроцитов приводят к тромбированию капилляров, нарушению микроциркуляции и возникновению секвестров в тканях жизненно важных органов (паразитарная секвестрация). Выявленные морфологические особенности возбудителей тропической малярии и поряженных ими тканей определяют способность паразитов менять свойства клеточных мембран инфицированных эритроцитов, что приводит к формированию патологических межклеточных контактов и служит одним из основных механизмов вирулентности *Plasmodium falciparum*.

Ключевые слова: тропическая малярия, *Plasmodium falciparum*, вирулентность, эритроцитарный розеттинг, эритроцитарная адгезия, паразитарная секвестрация, PfEMP1, электронная микроскопия

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Tropical malaria (TM) is a serious problem for tropical and subtropical regions, despite the international community's efforts aimed to decrease the incidence and stop transmission of pathogen causing this infection, *Plasmodium falciparum* (Welch, 1897), by its vector, by female *Anopheles* mosquitoes (Meigen, 1818). The diseases insidiousness often results from the gradual onset followed by the rapid malignant progression, suddenly entering the phase of irreversible fatal complications, even in cases of using antimalarial drugs. Malarial coma, algid, acute renal failure, toxic shock syndrome (TSS), and hemoglobinuric fever can lead to death of non-immune individuals, who have got infected with TM when visiting endemic regions [1].

The severe TM pathogenesis is based on the rosetting process (formation of conglomerates consisting of the infected and uninfected erythrocytes) and erythrocyte adhesion to the capillary endothelium [2]. This leads to small vessel thrombosis and sequestration of the vital organs, especially the brain. The molecular genetic mechanisms underlying the TM pathogenesis are associated with the release of multiple proteins (MAHRP1,2, REX3, HSP40, KAHRP, etc.) by the pathogen. *Pf*EMP1 (erythrocyte membrane protein being the main factor of *P. falciparum* virulence) found on the surface of the infected cell is the most important exported protein family [3, 4]. Transport of parasitic proteins is ensured by the Maurer's clefts defined as the parasitophorous vacuolar membrane protrusions that represent highly mobile structures [5, 6]. During the early phases of parasite development these move quickly, taking part in the PfEMP1 transport to the erythrocyte surface. The main virulence factor of *Plasmodium* has the increased affinity to the major cell receptors, such as ICAM-1 (intercellular adhesion molecule-1), CD36, CR, etc. [7, 8]. The PfEMP1 specific binding to the cell receptors leads to formation of abnormal contacts between the infected erythrocytes and the healthy cells, which results in such phenomena, as rosetting, adhesion, and sequestration [9].

Light microscopy is still the main method of examining parasites in blood and other tissues of the susceptible body. However, this method does not provide the possibility of examining fine structure of microorganisms and cellular mechanisms underlying pathogenesis of malignant malaria. High resolution of the electron microscope significantly expands the possibility of exploring the interplay between the malaria parasite and the cells of blood and other body's tissues. Sample preparation complexity, labor intensity, significant time investment, and high demands on the quality of test samples significantly limit the use of electron microscopy for examination of clinical material [10]. The contributing factors also include rapid development of the *P. falciparum* erythrocytic forms, accumulation of affected erythrocytes in the capillary bed, low parasitemia, and the rapid loss of morphological traits by the parasites and erythrocytes these have infected [11]. In this regard, the samples obtained by artificial cultivation of standard laboratory *Plasmodium* strains that often have low virulence or are non-pathogenic for humans are mostly used for examination by electron microscopy [12]. The data on the ultrastructural morphological alterations in the organism of patients with malaria are sporadic. Thus, we present the results of the comprehensive study of the material obtained from the patient, who died from complications of TM, by electron microscopy.

The study was aimed to assess fine structure of the *P. falciparum* erythrocytic stages of and estimate ultramorphological alterations of the affected tissues when examining the clinical material obtained from the patient with TM.

METHODS

The materials used in the study were represented by the antemortem venous blood samples collected for diagnostic purposes and the material obtained during subsequent postmortem examination of the brain, heart, and kidneys of the patient, who died from complications of TM.

Light microscopy

Blood specimens were prepared in accordance with the generally accepted method and Romanowsky–Giemsa stained [13].

The autopsy material was fixed in the 10% neutral buffered formalin. Then the samples were dehydrated using the increasing alcohol concentrations and embedded in paraffin (Biovitrum LLC; Russia). The tissue slices with the thickness of 5 μm cut from paraffin blocks were stained with hematoxylin and eosin (Biovitrum LLC; Russia). The AxioImager A2 light microscope (Carl Zeiss; Germany) was used for examination.

Scanning electron microscopy

The venous blood samples were fixed in the 2.5% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.2–7.4, for 24 h. Then formed elements of blood were washed from the fixing solution by adding PSB three times, centrifugation, and supernatant removal. The volume of formed elements suspension was brought to 5% of the sample volume, the material obtained was resuspended and applied to the glass slides. After drying the specimens were soaked in the 2% osmium tetroxide (osmium oxide (VIII)) for 30 min, then sequentially dehydrated with the increasing ethanol concentrations (30°, 50°, 70°, 80°, 96°) and air-dried. To ensure the scanning effect, the material was treated with the gold/palladium alloy (Au/Pd (60 : 40). The 5 nm layer sputtering was accomplished using the Q150T ES system (Quorum; Germany). The specimens prepared were examined in the scanning mode using the Merlin electron microscope (Carl Zeiss; Germany) equipped by the SE2 secondary electron detector.

The internal organ tissue sections with the thickness of 5 μm were transferred to the glass slides, deparaffinized by treating with xylene three times (Ecos-1; Russia) throughout 3 days, then soaked in the 2% osmium tetroxide (Serva; Germany). The scanning layer spluttering and examination of the specimens prepared were accomplished using the above method.

Transmission electron microscopy

The venous blood and internal organ tissue samples were prepared by standard methods. The samples prepared were embedded in blocks, the plasticized Araldite resin (EMS; USA) was used as the embedding medium. The 100 nm slices cut from blocks were subjected for double contrasting with lead citrate and 1% aqueous uranyl acetate solution (Serva; Germany). The specimens prepared were examined in the transmission mode with the Merlin microscope (Carl Zeiss; Germany) using the STEM detector. The images of specimens obtained were analyzed using the ImageJ tool for analysis and processing of images (NIH; USA).

RESULTS

Female patient I., 44-years, resident of Saint Petersburg, was infected with the causative agent of TM during the short-term visit to the highly endemic region. She received no

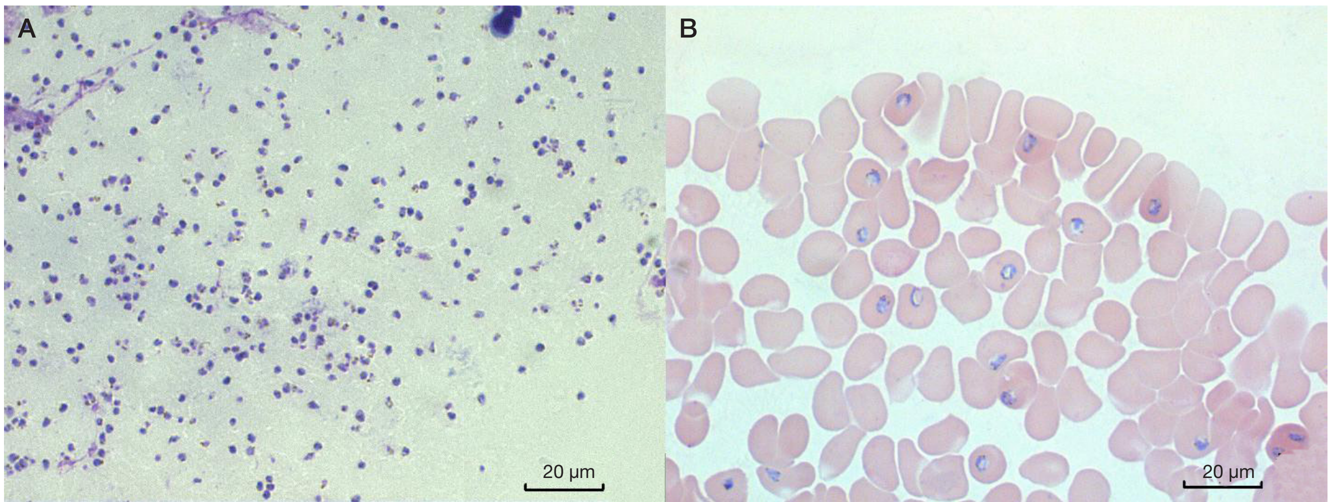

Fig. 1. *P. falciparum* young (ring) trophozoites in the thick blood smear (**A**); thin blood smear (**B**). Romanowsky–Giemsa stain, light microscopy (×1000)

chemotherapy for prevention of malaria. A week after returning from the trip the patient felt unwell, her body temperature increased. Her condition became progressively worse. The patient was admitted to the infectious diseases hospital on day 5 of the disease. Blood testing for parasites was performed in the emergency room due to clinical manifestations and epidemiological history, *P. falciparum* was revealed. Malaria treatment was started immediately. However, the patient died suddenly 13 h later. The immediate causes of death were toxic shock syndrome and edema (swelling of the brain), i.e. well known complications of malignant TM.

Examination of thick and thin blood smears revealed numerous *P. falciparum* young (ring phase) trophozoites in each field of view. Parasitemia exceeded 50,000 cells per 1 μL of blood (Fig. 1).

The images obtained when performing scanning electron microscopy of peripheral blood specimens show a large number of the deformed erythrocytes that had lost their typical biconcave shape. The affected erythrocytes have a bumpy surface repeating the outlines of the parasites developing in the cells. The infected erythrocyte membrane loses elasticity and becomes uneven due to incorporation of parasitic proteins in the membrane. Cell junctions are formed between the affected erythrocytes. This is associated with specific interaction between proteins of the main *P. falciparum* virulence factor incorporated in the membranes of affected erythrocytes and the cell receptors of adjacent cells. The same mechanism underlies the rosetting phenomenon associated with formation of conglomerates consisting of affected and healthy blood cells. It is assumed that parasites avoid exposure to the cellular immunity factors by surrounding themselves with intact cells (Fig. 2).

According to the literature data, the infected erythrocytes' shape becomes rigid, and the proteins responsible for rigidity are directly related to virulence, which further demonstrates that secretome affects the infection severity [14]. Deformation of erythrocytes results from the emergence of large protrusions over trophozoites. This is where the cytoplasmic membrane areas carrying the *Pf*EMP1 parasite adhesion proteins are located [15].

The fine structure of the parasitic cells and erythrocytes these had affected in blood specimens was assessed by transmission electron microscopy (Fig. 3).

DISCUSSION

The observational random slices of venous blood specimens showed that there were *P. falciparum* trophozoites surrounded by the parasitophorous vacuolar membrane in the erythrocytes. The parasites' nuclei have an amorphous structure, chromatin is not condensed, the nuclear membrane contour is fuzzy, which represent the signs of incipient schizogony accompanied by the asynchronous sequential replication cycles [16]. Specific mechanisms of the *P. falciparum* multiple fission are poorly

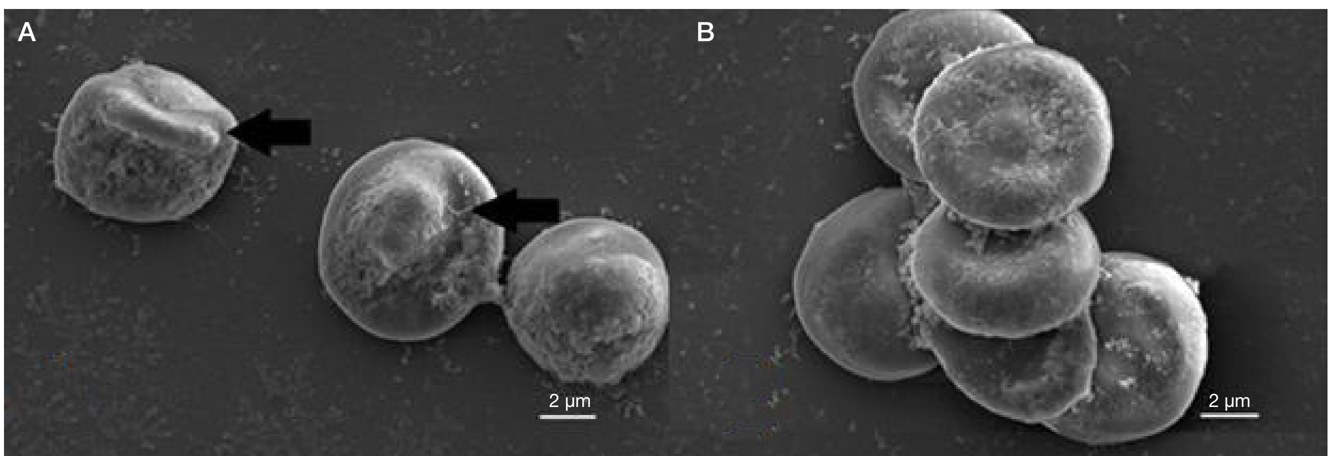

Fig. 2. Scanning electron microscopy of blood specimens. **A**. Deformation of the erythrocyte cytoplasmic membrane over the trophozoite (*arrow*). **B**. Rosetting — formation of conglomerate consisting of infected and uninfected erythrocytes

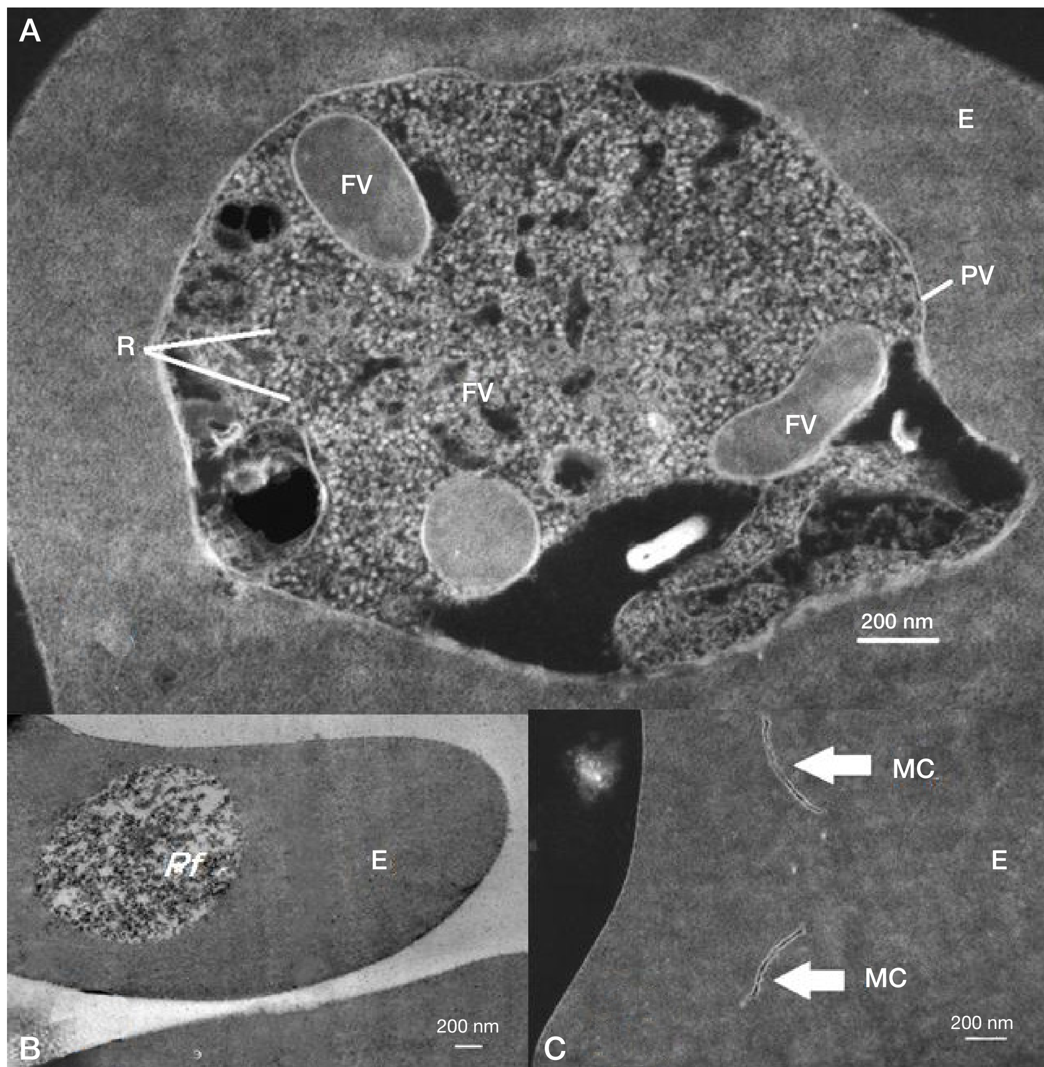

Fig. 3. Transmission electron microscopy: inverted (**A**, **C**), direct (**B**). Random slices of *P. falciparum* trophozoites located in the erythrocytes. E — erythrocyte; *Pf* — *P. falciparum*; FV — food vacuole; PV — parasitophorous vacuole; R — ribosomes; MC — Maurer's clefts in the infected erythrocyte cytoplasm

understood. It has been found that the processes occurring in the *Plasmodium* cells significantly differ from reproduction of other eukaryotes [17]. The food vacuoles are filled with the electron-dense substance similar to hemoglobin. It is well known that hemozoin, the product of the metabolism of hemoglobin ingested by *P. falciparum*, is accumulated in the parasites' food vacuoles (modified lysosomes) [18]. Many unbound ribosomes are found in the trophozoite cytoplasm, which suggests active synthesis of specific proteins essential for its membrane and exomembrane systems by the parasite. The infected erythrocyte's cytoplasm has a loose fine-grained structure; the membrane loses clear contours. Tubulovesicular structures with the electron-dense walls and electronically transparent content, the Maurer's clefts, were clearly visible inside erythrocytes. The Maurer's clefts that emerge at the early stages of parasites' development consist of the processes and whorls extending from the parasitophorous vacuolar membrane; these mature forming the functionally independent structures attached to erythrocyte cytoplasmic membrane [19].

Examination of the myocardium and cortical tissues has revealed the signs of the capillary lumen obliteration with the rosettes of infected erythrocytes attached to the endothelium. Erythrocytes of the rosettes still look as individual cells, their walls are clearly visible (Fig. 4).

Examination of the brain tissue and myocardial slices by scanning electron microscopy has revealed multiple facts of erythrocyte adhesion on the surface of the capillary endothelium. The infected and uninfected erythrocytes are deformed, they form rosettes, there are cells of spherical shape among them. This is probably associated with the changes in the erythrocyte cytoplasmic membrane structure resulting from incorporation of parasitic proteins in the membrane. It is well known that spherocytes can be considered as prehemolytic stage erythrocytes [20]. Apparently, the erythrocyte membrane permeability is impaired, when the membrane structure is changed, however, it remains unclear at which stage of the *P. falciparum* life cycle this occurs (Fig. 5).

In the erythrocytes organized in rosettes on the surface of endothelial cells, the lack of the fibers of fibrin being normally the key contributor to the blood clot formation attracts attention. The lack of fibrin masses observed in the erythrocyte adhesion sites suggests the differences in the mechanism underlying blood clot formation in TM and blood coagulation. It has been proven that conglomeration of erythrocytes into rosettes and their adhesion on the capillary endothelium results from abnormal interaction between parasitic proteins and cell receptors of erythrocytes and endothelial cells [21]. The key role of abnormal cell–cell interaction in the pathogenesis of malignant TM is confirmed by identification of tight junctions between

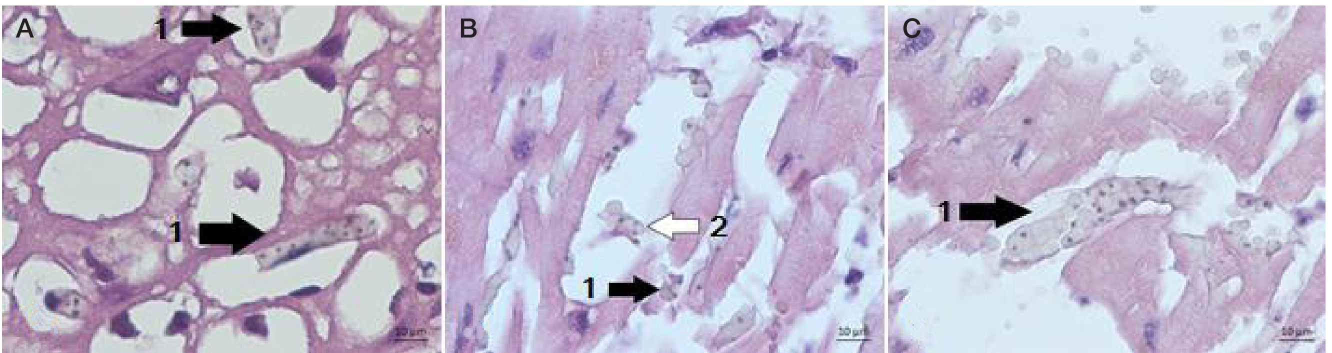

Fig. 4. Light microscopy of the autopsy material (hematoxylin and eosin stain, 1000× magnification): cerebral cortex (**A**); myocardium (**B**, **C**). 1 — rosetting in the small capillary lumen; 2 — adhesion of the rosettes of erythrocytes infected with *P. falciparum* to the capillary wall

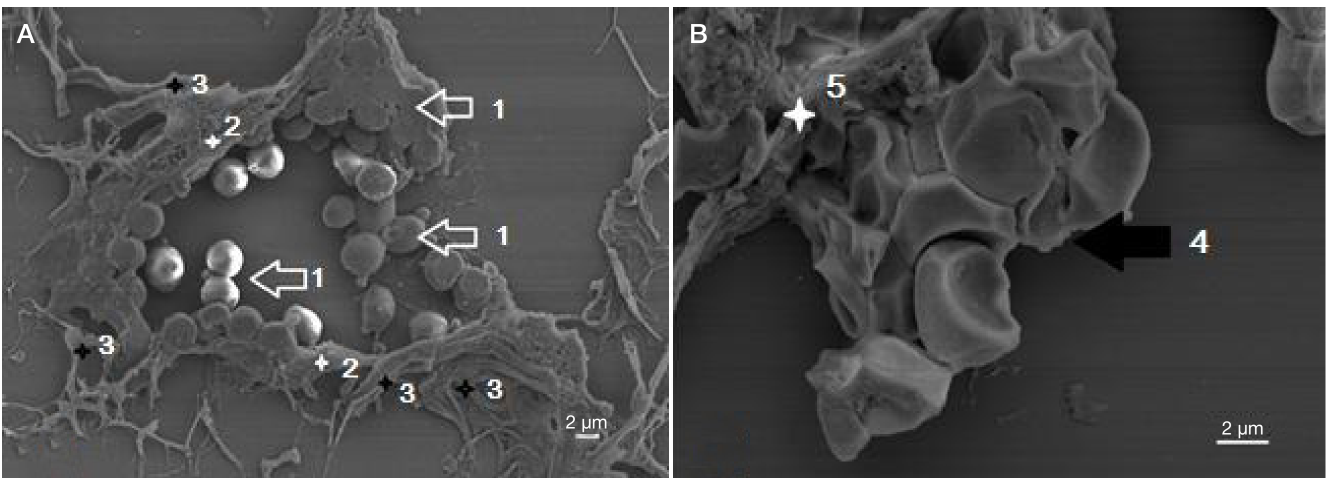

Fig. 5. Scanning electron microscopy of the slices of myocardium (**A**) and cerebral cortex (**B**). 1 — adhesion of spherical erythrocytes to the membranes of endothelial cells; 2 — endothelial cells; 3 — fibers of loose fibrous connective tissue along the periphery of the capillary; 4 — resetting in the capillary lumen; 5 — capillary endothelium

membranes of the erythrocytes forming rosettes in the cerebral capillaries. Of special importance are convoluted channels of the Maurer's clefts located in the area of the parasite adhesion to the membrane of the affected erythrocyte (Fig. 6).

CONCLUSIONS

The paper provides the results of morphological examination of the venous blood erythrocytes, myocardium, and brain tissues in severe tropical malaria. The analysis of the results of assessing ultrastructural changes of *P. falciparum* and erythrocytes during erythrocytic schizogony in *Plasmodium* confirms that there are complex molecular genetic and cellular mechanisms underlying the parasite's adverse effects on the host cells. Such interaction results in the changes of the infected erythrocyte cytoplasmic membrane surface architecture, formation of erythrocyte conglomerates, adhesion of those on the capillary endothelium in the myocardium and cerebral cortex. The changes observed led to microcirculatory

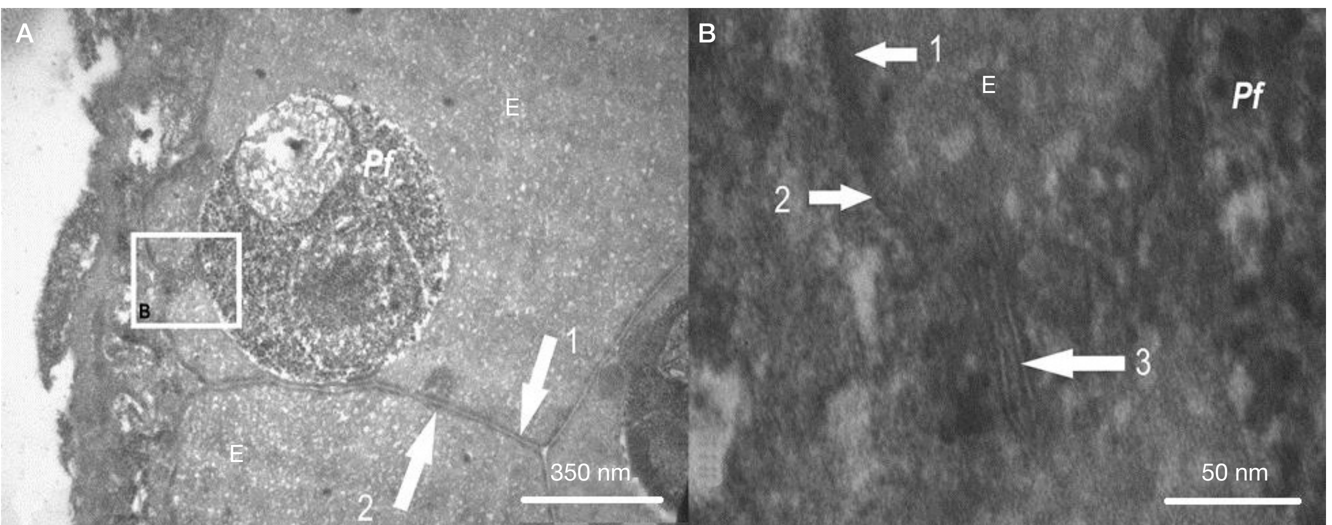

Fig. 6. Transmission electron miscroscopy of brain tissues. **A**. Slice of mature *P. falciparum* (*Pf*) trophozoite inside the erythrocyte (E) fixed on the capillary endothelium surface. **B**. Abnormal endomembrane system of the infected erythrocyte. 1 — cell membrane of the infected erythrocyte; 2 — cell membrane of the adjacent erythrocyte; 3 — channels of the Maurer's cleft located between the parasitophorous vacuolar membrane and the cell membrane of the affected erythrocyte

disturbances in the tissues of vital organs. The ultrastructural changes revealed confirm the parasite's capability of changing properties of cell membranes of the infected erythrocytes, which leads to formation of abnormal cell–cell contacts and serves as one of the main mechanisms of *P. falciparum* virulence determining the malignant course of tropical malaria.

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