PRACA ORYGINALNA
ORIGINAL ARTICLE

EXPERIMENTAL STUDY OF THE LIVER ULTRASTRUCTURE IN THE PRESENCE OF INFECTION WITH HERPES SIMPLEX VIRUS 1

EKSPERYMENTALNE BADANIE ULTRASTRUKTURY WĄTROBY W TRAKCIE INFEKCJI WIRUSEM HSV1

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ABSTRACT

Introduction: Understanding of HSV-1liver infection pathogenesis is of great scientific, social and economic significance, since this is one of the main latent infections in population. However reactivation of this infection remains understudied.

The aim: This experimental research aimed at studying the ultrastructure changes occurring in the liver in the presence of HSV-1infection.

Materials and methods: Experiments were conducted on 12 BALB/c line mice weighing 18-20 g. They were divided into 2 groups: experimental, and control. Experimental animals were infected with the attenuated HSV-1. On day 40 the animals were withdrawn from the experiment by decapitation. Liver fragments were excised and studied ultramicroscopically.

Results: Liver disorders were represented by the focal damage of hepatic lobuli cells. Ultrastructure changes were found both in the microvascular endothelium and hepatocytes. The vascular disorders included swelling of endotheliocytes, their demise and desquamation into the lumen, disruption of the basal lamina integrity and diapedesis of blood cells into the subendothelial space. Finding virions in the endotheliocytes allowed to explain the possible pathway of the infection into the interstitium and hepatocytes via systemic circulation from the primary source of infection. Electron microscopy has not revealed any virions in hepatocytes, with only the following changes: significant cytosole density of the osmiophylic granules, lisosomes and lamellar bodies found. These were considered to be the consequence of the infectious process. Findings of the experimental study enable understanding of the causal relationship between the acute infection and liver damage.

Conclusions: Ultrastructure changes in the liver of mice infected with HSV-1 were focal, and more rarely diffuse in nature. Non-specific cytopathological changes (swelling of the cytoplasm and reduction of the endoplasmatic reticulum, and mitochondria) were found both in the endotheliocytes of the sinusoid capillaries and hepatocytes. Endotheliocytes of the sinusoid liver capillaries in mice infected with HSV-1 lose their barrier function, which leads to direct and indirect damage of hepatocytes and development of dystrophic changes in the liver.

KEY WORDS: HSV-1, liver, endothelium, experiment, electron microscopy

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INTRODUCTION

Herpes simplex virus 1 (HSV-1) is the most prevalent infection in the population [1]. HSV-1 exists in its latent form in the nervous system, however immune suppression may cause reactivation of the infection [2]. Although the specific features and consequences of the acute HSV-1infection in the brain are more or less understandable and described in clinical studies [3, 4], the damage of other organs, which are not the primary source of the latent HSV-1infection, liver in particular, remain poorly studied.

Assessment of the clinical studies [5] shows, that liver damage caused by HSV-1is detected rarely and late. The authors associate acute liver insufficiency with HSV-1only in 1-2% of cases [6, 7]. As a rule, herpetic hepatitis is diagnosed based on the autopsy results, since in the majority of cases the disease is asymptomatic. The structural underpinnings of the infection development and viral reproduction in the liver are underinvestigated. Changes in liver structure have been adequately described only based on the autopsy findings. Pathology reports only state the hepatic lobular necrosis [8]. However these changes are also found in other liver disorders. As a rule morphological changes are not specific, and confirmation of the herpetic damage requires the use of additional immunohistochemical and molecular techniques (PCR, ELISA). At the same time these methods do not enable finding of the very site of viral reproduction. Electron microscopy provides more advantages in this, allowing detection of viral particles and consequences of their reproduction in various types of cells in the liver. Analysis of the literature data, which provide a

limited description of the specific features of herpetic liver and other organs [9] damage development and methods of detecting viral infection [10, 11] leads to the conclusion that the processes of HSV-1infection development in the liver are poorly studied.

THE AIM

The aim of this research was to study the ultrastructure changes in the liver in the presence of HSV-1infection.

MATERIALS AND METHODS

Experiments were conducted on 12 BALB/c line mice weighing 18-20 g. They were divided into 2 groups: experimental (9 mice), and control (3 mice). Experimental animals were infected with the attenuated HSV-1(the museum strain of the virus specifically adapted to studies on laboratory mice) in the amount of 4.0 lg $LD_{50.}$ HSV-1was cultured on the cell lines at the Gromashevsky Institute of Epidemiology and Infectious Diseases (Kyiv, Ukraine). Initially the virus was received from the museum of viruses of the Institute of Virology named after D.I Ivanovskii, RAMS.

The intracerebral infection of mice can cause high fatality, which limits the capability and usefulness of such experimental model, that is why the mode of infection was modified. For this purpose the mice were infected at the junction of the zygomatic and the squamous bones of the skull, which is located retroorbitally and allows injection without damaging the animals' brain. 0,01-0,02 mL of the solution corresponds to 4,0 lg LD50. This allows to reduce the mortality of animals up to 20-35% within the first 3-6 days. The virus is likely to penetrate via the vascular system. Those animals who have successfully survived after the infection have become the HSV-1carriers, with the presence of the virus being confirmed *in vitro* on the Vero culture model. On day 40 the animals were withdrawn from the experiment by decapitation.

The animals were kept at a 12-hour light-dark cycle, at 22° C, fed with standard granulated cereal with free access to food and water.

To study the ultrastructure changes the animals' liver fragments were excised and fixed in 2,5% solution of glutaraldehyde on the phosphate buffer with postfixation in 1% solution of osmium tetroxide (on PBS, pH=7,4). Dehydration was conducted in alcohols with ascending concentration (70%, 80%, 90%, 100%) and acetone. The specimens were impregnated and embedded in the Epon-Araldite mixture. For targeted orientation the semithin sections were stained with Toluidine blue, followed with the preparation of ultrathin sections on Reihart (Austria) ultratome. Contrasting was done with 2% solution of Uranil acetate and Lead citrate. The ultrathin sections were studied and photographed under the electron microscope Tescan Mira 3 LMU (Chech Republic) with 10-80 thousand times magnification.

Ethical consideration: All laboratory (virology and experimental) procedures on animals were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health Guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). The research was approved by the Bioethical committee for human subjects or animal research at Bogomolets National Medical University, Minutes №12, December 30, 2015. Animal handling strictly accorded with the guidelines approved by these entities.

RESULTS

Specific features of HSV-1replication, virions formation and their release from the cells have been so far described mostly on cell cultures (*in vitro*). It is related to the fact that the culture cell lines method allows to obtain in fact the unlimited amount of virus in the proliferating cells [12]. However, it is obvious, that several experimental findings do not adequately represent the viral infection *in vivo*. To ensure the more realistic understanding of the damage of liver ultrastructure associated with infection caused by HSV-I, we have conducted electron microscopy of the liver samples obtained from adult animals (*in vivo)*.

Distorted liver ultrastructure was found in all the specimens (Fig. 2-6) from experimental group. Whereas the control demonstrated no signs of damaged cell ultrastructure: hepatic lobules were formed by plates of hepatocytes, which were separated by sinusoids and bile canaliculi. Hepatocyte microvilli were present in perisinusoidal spaces. Also found were the central veins, having in their structure the intact endothelium.

The evaluation of the ultrastructural changes in the infected animals' liver has shown the focal nature of the damage, significantly rarer the diffuse one.

Fig. 2-6 demonstrate changes found in the studied specimens, from the intact cells to dystrophy, hepatocytes and endothelium destruction. In assessing the changes special attention was paid to the hepatic vessels in order to understand the histophysiological mechanism of the damage inflicted on the organ by viral infection. Changes at the level of vascular system of the hepatic lobuli included the endothelial disruption, demise and destruction of endotheliocytes, and desquamation of cells and cell detritus into the microvascular lumen (Fig. 2, 3). In small and large vessels (the lobular veins and arteries) no signs of stasis have been found, however there was RBC stasis in hemocapillaries. Vascular damage was not limited to the endothelial disruption, with the destruction of the venous and capillary basal lamina found as well. In the subendothelial space singular neutrophils and RBCs were found which can be explained by the diapedesis of certain formed elements through the areas of focal disruption of the hemocapillaries integrity. These findings indicate the loss of endothelial barrier function in the hepatic microvessels, which can explain the penetration of infection to the organ via circulatory system.

Changes in hepatocytes were quite variable. Cells without signs of ultrastructural damage and cells with different levels of organelles reduction and cytoplasm swelling were

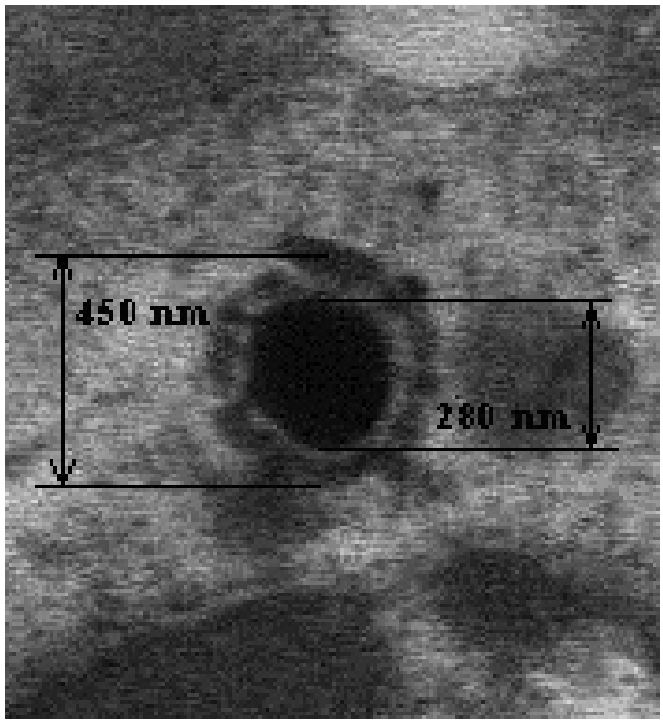

Fig. 1. Transmission electron microscopy of the murine liver with HSV-1: virion in the endotheliocyte with morphometric data (× 38700);

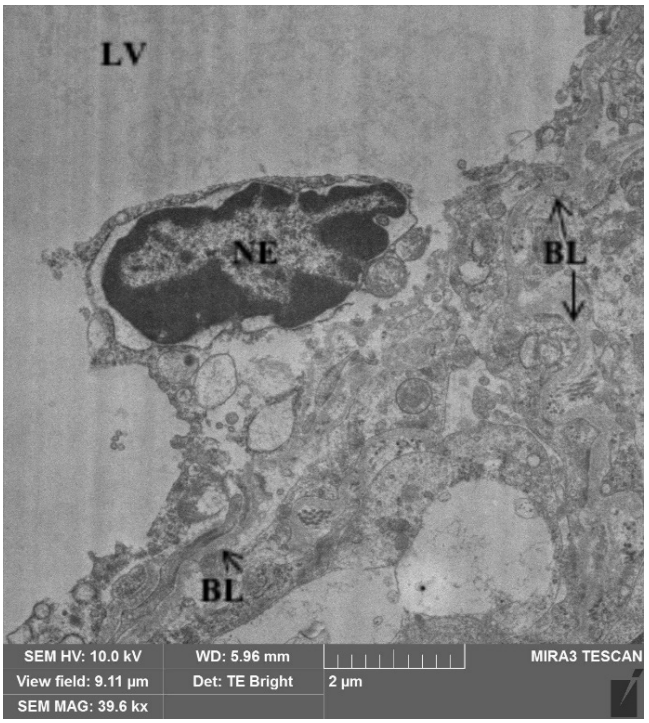

Fig. 2. Desquamation of the endotheliocyte in the lumen of the central vein: LV – lumen of the central vein; NE – nucleus of endotheliocyte; BM – basal lamina;

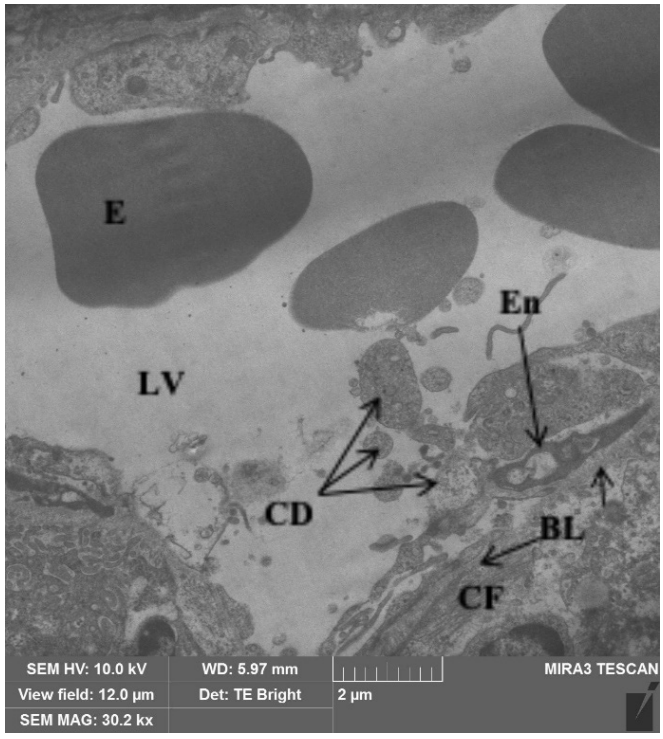

Fig. 3. Desquamation of cell detritus in the lumen of the central vein: : LV – lumen of the central vein; BL – basal lamina; E – erythrocyte; CD – cell detrite; CF – collagen fibers; En – endotheliocyte;

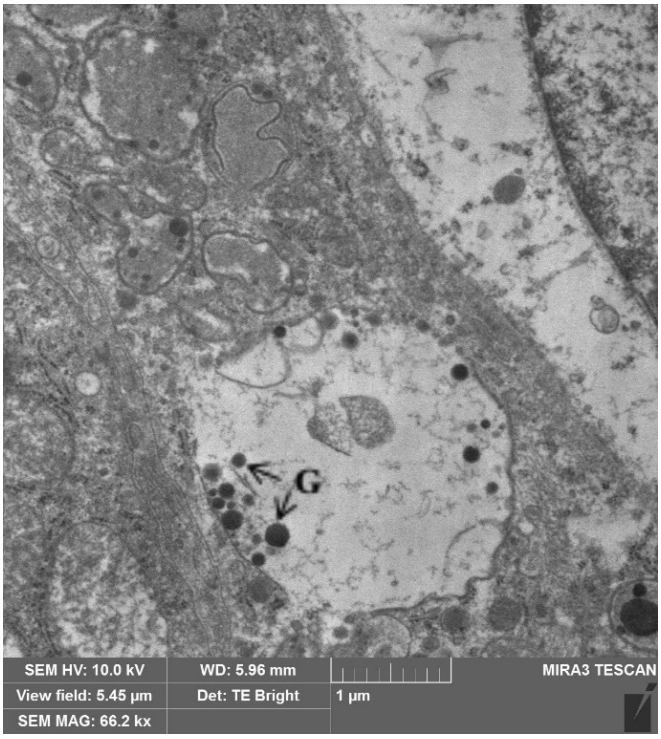

Fig. 4. Dystrophic changes in the hepatocytes (swelling of the cytoplasm, reduction of organelles): G – granules of glycogen;

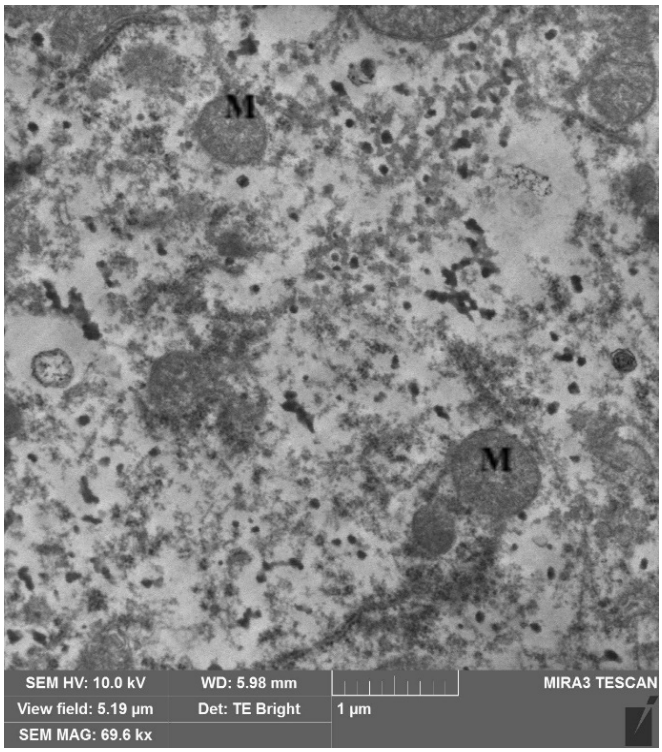

Fig. 5. Reduction of organelles in the cytoplasm of hepatocyte and appearance of osmiophilic granules: M – mitochondria;

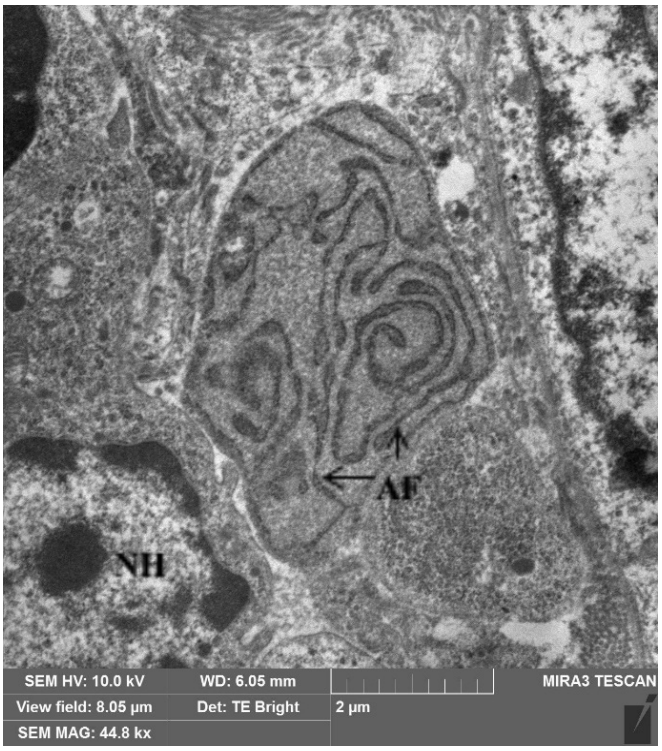

Fig. 6. Autophagy (concentric cisterns of autophagosomes): NH – nucleus of hepatocyte; AF – autophagosomes.

found (Fig. 4, 5). Cells with swollen cytoplasm contained a large number of osmiophilic granules, lyzosomes and lamellar bodies. No virions were found in any one of the specimens, and the described ultrastructural changes may be considered the consequence of the infectious process. Osmiophilic granules seem to be the residues of the unformed protein elements of the viral capsid. Accumulations of glycogen granules and lipid inclusions were found in hepatocytes more seldom. At certain parts of the hepatic lobuli signs of autophagy were noted (Fig. 6). At the same time the hepatocytes with intact structure, cells with the signs of functional rearrangements, hypertrophy of the endoplasmatic reticulum in particular were found in the hepatic lobuli.

DISCUSSION

HSV-1virions were found only in the endotheliocytes, and only in 1 out of 9 mice. According to the morphometric assessment the diameter of the electron dense "core" of the virion equals to up to 280 nm, with additional electrondense envelope found around the viral particle (the diameter was 450 nm, the outer contour included) (Fig. 1). The data obtained by other researchers during autopsy give smaller dimensions of virions (100-200 nm) [13]. The difference between these data additionally confirm that specific features of HSV-1virion formation *in situ* have been inadequately studied, and the study of structural changes in the affected organs was limited to the description of the morphological changes only.

The earlier experiments have revealed morphological changes in the HSV-1infected animals liver. Light microscopy has demonstrated dystrophic changes in the liver, which included swelling and hypertrophy of hepatocytes, reduced cell density, infiltration of mononuclear phagocytes (macrophages) with signs of fibrosis around the interlobular arteries [14, 15]. At the same time changes in microcirculatory system, structural disintegration of the hemocapillaries walls and the central veins, local hemorrhages were found. These data are consistent with the biopsy findings of patients with herpetic hepatitis [16, 17]. Morphological disorders in the liver of the infected animals described earlier [14, 15] are often present in other liver pathologies [17, 18, 19] and brain damage [19, 20, 21, 22], which prevents complete understanding of the relation between the found changes with local reproduction of HSV-1, even when HSV-1 has been confirmed *in vitro* [23]. Molecular methods significantly broaden the perception of the changes in the organ and often are required to confirm the presence of the virus. Unfortunately the *in vitro* methods (PCR and ELISA) being the principle ones in diagnosing and confirming the infectious causative agent [24], do not allow to establish the specifics of the infectious process development at the cellular level. That is why to establish the association between the dystrophic liver changes and the presence of the infectious process we have used electron microscopy.

Finding HSV-1 virions by electron microscopy was found to be a quite challenging task. There are several

explanations of it. Firstly, classical electronograms of the virions are as a rule obtained in *in vitro* studies of the cultured cells. Secondly, as noted in [25], the liver of mice infected with HSV-1 eliminates the virus quickly and that is why only consequences of the damage can be identified at the ultrastructural level, and often they are represented by small necrotic foci. Early signs of necrotic changes are found as well along with the morphological basis of the direct cytopathic effects of HSV-I. They manifest themselves by necrosis of hepatocytes: nuclear deformation and fragmentation, chromatin margination, accumulation of internuclear viral particles, consisting of electron dense nucleus and surrounded by capsid. Sometimes small areas of steatosis can be found [26].

All changes in hepatocytes found in this study, such as cytoplasm swelling, organelle reduction, and inclusions were described in [27] in other infections, in hepatitis C virus in particular. That is why such changes cannot be considered specific. Moreover, changes in hepatocytes, which cannot be plausibly interpreted as the consequences of infectious damage, such as swelling of cytoplasm are likely to be secondary. These changes may be caused by water-electrolyte dysbalance while the lamellar bodies in the cytoplasm of hepatocytes may have resulted from the impaired bile production. Besides this, the presence of lipid inclusions, which are normally often found in hepatocytes, is not the consequence of damage but rather a sign of the preserved functional state of the cell. Small number of virions found in liver specimens, absence of signs of the viral formation machinery, complexity of identifying the capsidless virus by electron microscopy do not enable better understanding of the specificity of infectious process in the liver. New data about the ultrastructural signs of HSV-1 reproduction in the microvascular endotheliocytes of the liver obtained in the experiment give grounds for better understanding of the causal relation between the acute infection and liver damage. Supplementary methods of HSV-1 detection (immunohistochemistry, PCR, virus titration) had demonstrated correlation between the phase of the infectious process development and the degree of damage, which were valuable for the description of the pathological process and assessment of the prognosis and differential diagnosis [26]. However, molecular methods of HSV-1detection, PCR in particular, are not always available, and their utilization is time-consuming. Moreover, the authors emphasize the potential association between the liver damage and sharp increase in the liver transaminases activity (100-1000 fold increase of AST and ALT level), with the bilirubin level remaining relatively normal or even low [28, 29, 30]. In such cases it is not possible to quickly find the cause of these disorders. That is why liver biopsy (both histological and electron microscopic study) are considered to be compulsory in diagnostic. However, it is obvious that such studies are conducted quite seldom, which is demonstrated by the scarcity of evidence, and findings of electron microscopy of the detected HSV-1 virions. Our current results have added new data regarding HSV-1 infection and its development in the liver.

Study of the infected cells in the consecutive intervals following the infection [29] has shown that the first viral forms to emerge are capsids containing low density cores, with electron dense nuclei emerging only afterwards. At the same time non-typical viral particles, capsid-like material without the dense nucleus may occur, while the naked particles are rare. Virion with the defective capsid is unstable outside the cell. Packaging of viral particles may take place in the nuclear matrix in the immediate proximity to the nuclear surface and in the cytoplasm. Some particles had several envelopes. It is not unlikely that some envelopes are the endoplasmatic reticulum derivatives, since the virions were found within the EPR canaliculi

Electron microscopy findings are of special interest illustrating as they do the process of viral release from the cell. Unfortunately, these materials were obtained on cell cultures, and we have not found any microphotographs clearly showing this phenomenon in hepatocytes in the literature. In our current research we have not found such cells either. However, the fact that virions are found in endotheliocytes is a new evidence of HSV-1 liver infection which additionally confirms that HSV-1 is polytropic. This explains the mechanism of infection and development of liver pathology given that in the experiment brain was the primary source of the virus. Penetration of the virus into blood during infection cannot be excluded, since the presence of HSV-1 in blood plasma was also confirmed *in vitro* on the Vero cell culture and by PCR. The virus was also found in the murine blood plasma as the HSV-1 titer was increasing after the simulation of intracerebral hematoma [24]. Thus, the new data provided by electron microscopy explain the mechanism of liver infection, and significantly broaden the perception of viral reproduction in the liver. These results are of great importance for the study of histophysiological underpinnings of the infectious liver damage as well as of the mechanisms responsible for combatting infection in this organ.

CONCLUSIONS

1. Ultrastructure changes in the liver of mice infected with HSV-1 were focal, and more rarely diffuse in nature. Changes in blood vessels were represented by the distortion and dystrophy of the endothelial layer (death of endotheliocytes, destruction of the basement membrane, penetration of the dead cells derivatives into the microvessels lumen), being more prominent in the capillaries.
2. Non-specific cytopathological changes (swelling of the cytoplasm and reduction of the endoplasmatic reticulum, and mitochondria) were found both in the endotheliocytes of the sinusoid capillaries and hepatocytes. The cytoplasm of hepatocytes revealed specific changes which consisted in accumulation of a significant number of osmophylic granules and lamellar bodies. Accumula-

tion of lysosomes and signs of autophagy in hepatocytes were found.

3. Endotheliocytes of the sinusoid liver capillaries in mice infected with HSV-1 lose their barrier function, which leads to direct and indirect damage of hepatocytes and development of dystrophic changes in the liver.

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Authors' contributions:
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