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Pathogenesis of the *Ilheus virus* in golden hamsters (*Mesocricetus auratus*)

Estudo experimental sobre a patogenicidade do *Vírus Ilhéus* em hamsters dourados (*Mesocricetus auratus*)

Estudio experimental sobre la patogenicidad del *Virus Ilhéus* en hámsteres dorados (*Mesocricetus auratus*)

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ABSTRACT

The pathogenesis of the *Ilheus flavivirus* (*Flaviviridae*) was investigated in golden hamsters (*Mesocricetus auratus*) using an inoculum of 9.8 LD_{50} via intraperitoneal (IP). For ten days, two infected and one control animals were anesthetized, and blood and viscera fragments (brain, liver, heart, lung, spleen and kidneys) were collected on a daily basis for determination of viral titers in newborn mice and antigens/antibody by complement fixation and hemagglutination inhibition tests. Additionally, the pathology of animal tissues was studied by the the hematoxylin and eosin method, viral antigens were detected by immunochemistry, and all collected viscera showed histopathological changes. Large amounts of ILHV antigens were detected by immunohistochemistry in the brain, and in lower quantities in the liver, spleen and kidneys, corroborating with newborn viral titers in them. This inoculum resulted in a fatal outcome of all infected animals seven days after experimentation. Viral antigens were not found in the heart and lungs, suggesting that the viral titers obtained were caused by viremia and not by viral damage. The information in this study confirms the neurotropism and neuropathogenicity of ILHV.

Keywords: *Ilheus Virus*; *Flavivirus*; Encephalitis Arbovirus; Virulence; Models Animal.

INTRODUÇÃO

The *Ilheus Virus* (ILHV) belongs to the genus *Flavivirus* of the family *Flaviviridae*, and it is included in the Ntaya virus group[7]. However, a recent molecular study demonstrated that ILHV, together with the *Rocio Virus* (ROCV), represent a genetic line distinct from the Ntaya virus group[10]. After the first viral isolate was obtained in 1944, many other isolates from patients with fever, mosquitoes and a variety of animals, particularly wild birds and bats, were obtained in Brazil, Colombia, Panama, Argentina and Trinidad[8,11,12,19,23]. In the Brazilian Amazon, the virus has been isolated from patients with fever, sentinel monkeys, bats and various species of mosquitoes, especially *Psorophora ferox*, which is considered its main vector[14,19,20,22]. Wild birds have been implicated as probable vertebrate hosts of the virus. However, antibodies have also been found in other vertebrates, such as rodents, marsupials, edentates, bats and monkeys, from which the virus has also been isolated[4,6,8].

The disease in humans is usually reported sporadically and is associated with exposure in forested regions. Its clinical spectrum ranges from asymptomatic infections to encephalitis. Febrile illness, which is responsible for the majority of known cases, is characterized by sudden onset

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with a moderate or high fever, headache, chills, photophobia, arthralgia, myalgia and asthenia. It lasts three to five days, with complete recovery without sequelae. The small number of clinically diagnosed cases contrasts with the high prevalence of antibodies against the agent, suggesting that most infections are inapparent or oligosymptomatic. Another relevant factor is its short incubation period. During the observation of eight acute cases of ILHV infection that occurred in Brazil, Trinidad, Argentina, Colombia and Panama, changes in the central nervous system that led to suspicion of encephalitis were reported in only one patient[13].

There are few studies of ILHV pathogenicity available. However, it is known that viremia has been observed in experiments in small animals, such as mice and other rodents, as well as nonhuman primates[8,9]. Tesh and colleagues[21] and Xiao and colleagues[24, 25] used golden hamsters (*Mesocricetus auratus*) as an experimental model to study the pathogenesis of flavivirus, and those experiments demonstrated great susceptibility of the animals to the virus. Consequently, as they are an excellent alternate model in substitution for nonhuman primates, we decided to perform intraperitoneal (IP) injection experiments with ILHV in these animals.

MATERIALS AND METHODS

The ILHV BeH 7445 strain used in this study was obtained from the brain suspension of infected Swiss albino mice after 21 serial passages in these mice and kept at -70° C without a previous passage in hamsters. This viral strain was isolated in 1959 from the blood of a febrile patient, who was a resident of the District of Caraparu, located in the municipality of Santa Isabel do Pará, in Pará State, Brazil[2].

The inoculum was prepared from brains of albino mice infected with the ILHV strain in a phosphate buffered saline solution pH 7.4 containing 0.75% bovine albumin and antibiotics (100 IU/mL of penicillin and 100 μg/mL of streptomycin), and kept at temperatures below 8° C. Twenty male hamsters, 4 to 5 weeks of age, were inoculated with 0.1 mL of suspension by IP injection, and ten animals were reserved for negative controls (not inoculated). To detect the applied dose, 2- to 3-day-old mice were also inoculated with the viral solution in serial dilutions of 10^{-1} to 10^{-12}. The viral titer was calculated by the Reed and Muench method[16] and expressed as LD_{50}/0.02 mL. Every 24 h after inoculation, two infected hamsters and one control group hamster were anesthetized and killed for bleeding and organ collection (brain, liver, heart, spleen, kidney and lung). An aliquot of the serum was stored at -70 °C for studies of viremia and detection of antigens, and another at -20 °C for antibody detection. Fragments of the collected organs were divided into two parts. One part was frozen at -70 °C for the detection of viral antigens and for viral titration of the tissue, and the other was fixed in a 10% formaldehyde solution for use in histopathological test and immunohistochemistry. The collected specimens were, therefore, used for determination of viremia by titration in newborn mice, antigen detection by complement fixation (CF) test and immunohistochemistry (IHC), detection of antibodies by CF tests and hemagglutination inhibition (HI) and tissue examination (histopathology).

To detect the presence of ILHV and to analyze the viremic curve produced by this virus in the biological specimens obtained from the experimental test, virus titration was performed separately, from serum and organ fragments, with a 10% macerated suspension in PBS containing bovine albumin and antibiotics. The supernatant was used to prepare serial dilutions of 10^{-1} to 10^{-12}, which were inoculated into a group of six newborn mice, and 0.02 mL was administered via intracerebral (IC) inoculation into each animal. The titrations were also calculated by the Reed and Muench method[16].

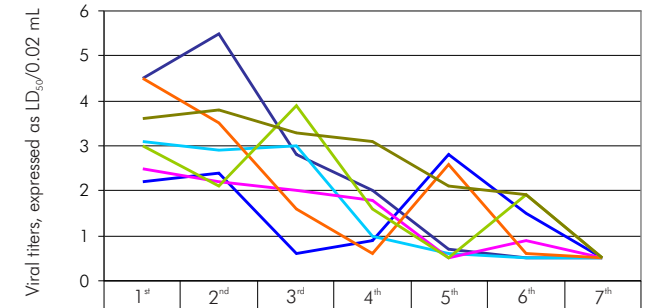
The CF test was used to detect the ILHV antigens and antibodies. The tests were performed according to the technique modified by Fulton and Dumbell[5] and adapted for microplate by Shope and Sather[17], in serial two-fold dilutions (1:8 to 1:128 for the antigen sources and 1:8 to 1:64 for the antibody sources) using two units of guinea pig complement, antigen (used as a control test) and the hyperimmune ascitic fluid of ILHV (anti-ILHV serum), *Bunyavirus Tacaiuma*, BeAN 73 viral strain (anti-VTCM serum) and sensitized sheep erythrocytes. The reading was done according to the hemolysis rates observed, and positive titers were recorded as the highest dilution rate with up to 25% of hemolysis.

For the study of HI antibodies, the technique of Clarke and Casals[3] was adapted for microplates using serum dilutions of 1:20 to 1:1280 tested against four units of the ILHV antigen negative control (VTCM).

The organ fragments that were fixed in a 10% formaldehyde solution were processed for light microscopy by the Hematoxylin-Eosina[15]. The studied organs were semi-quantified on a scale ranging from 0 to 3: 0= no lesion, 1= mild lesion, 2= moderate lesion and 3= severe lesion[25]. Sections of 5 μm were removed from all the visceral fragments of the infected and control animals that were already embedded in paraffin and were placed on Super-frost®/Plus (A. Dalgger & Company) slides. The sections were stained, according to the procedures described by Barros[1], using ILHV hyperimmune ascitic fluid.

RESULTS

The LD_{50}/0.02 mL titer of the BeH 7445 strain of ILHV inoculated through IP injection in young hamsters was 9.8. Collection of blood (to obtain serum) and organ fragments occurred within seven days because after this period all of the animals died. Although the viral titers found in the experiment were relatively low, the titers were detectable within the first 24 h, and the serum collected on the second day post-inoculation (p.i.) was the highest titer (LD_{50}/0.02 mL = 5.5). In fragments of liver, heart and spleen we observed that the peak had already occurred at 24 h p.i. In fragments of brain, kidney and lung we observed that the peak occurred on the fifth, third and second days after infection, respectively (Figure 1).



	1st	2nd	3rd	4th	5th	6th	7th
SERUM	4,5	5,5	2,8	2	0,7	0,5	0,5
BRAIN	2,2	2,4	0,6	0,9	2,8	1,5	0,5
LIVER	3,1	2,9	3	1	0,6	0,5	0,5
HEART	2,5	2,2	2	1,8	0,5	0,9	0,5
SPLEEN	4,5	3,5	1,6	0,6	2,6	0,6	0,5
KIDNEY	3	2,1	3,9	1,6	0,5	1,9	0,5
LUNG	3,6	3,8	3,3	3,1	2,1	1,9	0,5

Figure 1 – Viral titers, expressed as LD_{50}/0.02 mL, from specimens collected in the ILHV experimental study performed by IP injection

In this study we observed that in the collected serum samples the viremia was detectable within the first 24 h p.i., with a duration of four days, and it peaked on the second day p.i. In the organ fragments, the presence of ILHV was detected within the first 24 h p.i., with a duration that ranged from four to six days. It was interesting to observe the biphasic behavior of the virus titration in the brain. In fact, on the first two days after inoculation an LD_{50}/0.02 mL viremic titer slightly greater than 2 was observed. On the third and fourth days p.i., a decline to 0.6 and 0.9 LD_{50}/0.02 mL, respectively, was evident. On the fifth day p.i., the titer reached the maximum (2.8 LD_{50}/0.02 mL), decreased to 1.5 LD_{50}/0.02 mL on the sixth day p.i., and was no longer detected on the seventh day, as observed for all studied specimens (Figure 1).

The studies performed to detect antibodies showed the appearance of complement fixing antibodies from the fourth day p.i., as well as hemagglutination-inhibiting antibodies from the fifth day after experimental infection (Table 1). As stated above, despite the appearance of antibodies, all inoculated animals subsequently died.

Table 1 – Viral titers, expressed as LD_{50}/0.02 mL, from specimens collected in the ILHV experimental study performed by IP injection

Test	Day post-infection (p.i.)						
	1°	2°	3°	4°	5°	6°	7°
IH	0	0	0	0	1:40	1:40	1:40
FC	0	0	0	8/16	128/64	128/64	128/64

Note: 0 = negative.

The histopathology study (HS) revealed leptomeningitis, mild edema and congestion in the brain, which became more severe as from the fifth day p.i. Congestion of mild to moderate intensity and severe edema, more visible in the perivascular space, were detected in the cortex and white matter from the first to fifth days p.i.. Congestion and edema were accompanied by inflammation, which was determined by the presence of isolated or small groups of cells showing retracted and acidophilic cytoplasm and pycnotic nuclei. From the fifth to seventh days p.i., the edema was moderate in the pericellular space, and there was an increase in the number of areas containing acidophilic neurons or cellular necrosis, which was documented by the presence of residual nuclear pyknosis (Figure 2). IHC showed immunoreactivity from the fifth day p.i. (120 h p.i.), initially in the basal ganglia, extending to the hippocampus and cerebral cortex on the remaining days post-infection (Figure 3).

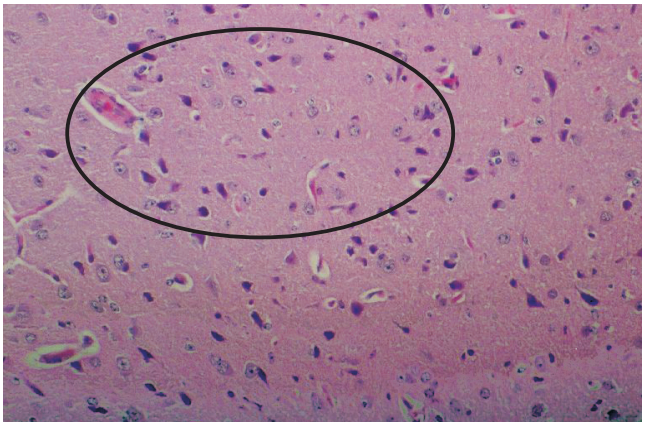


Figure 2 – Changes in brain tissue collected on the seventh day p.i. at a 20x magnification. Moderate edema, areas of cellular necrosis (presence of residual nuclear pyknosis) and a mononuclear inflammatory reaction in the brain tissue (circle) was noted

In the liver, the trabecular arrangement remained the same throughout the experiment. On the first day p.i., severe cellular swelling associated with cytoplasmic acidophilia in isolated parenchymal cells, especially in the zones two (midzone) and three (efferent zone) of the liver acinus, and the presence of rare inflammatory cells in sinusoids were detected. On the second day p.i., in addition to cellular swelling, acidophilic retraction of the cytoplasm of some cells that presented pycnotic nuclei was still detected. Furthermore, the emergence of corpuscular structures with the characteristics of the Councilman-Rocha Lima bodies with a sparse distribution (apoptotic cells) was observed. The sinusoids were enhanced and displayed hypertrophy and hyperplasia of the Kupffer cells, as well as a moderate inflammatory reaction that was mainly represented by mononuclear cells. The inflammatory reaction was mild in the portal tracts. In the third and fourth days p.i. the lesion presented moderate intensity, which was characterized by the presence of sparse focal cellular necrosis in the parenchyma, a higher number of Councilman bodies and a moderate inflammation in lobules and in the portal tracts. On the fifth day p.i., in addition to the changes noted above, focal sinusoidal congestion was detected, though hepatocyte binucleation was already present, which is a typical sign of the onset of the regenerative process. From the sixth to seventh days p.i., regression of lesions and accentuation of the liver regenerative process were noted. IHC revealed marked hepatocytes only on the second day (48 h) p.i., and viral antigens were not found on the other days of the experiment (Figure 4).

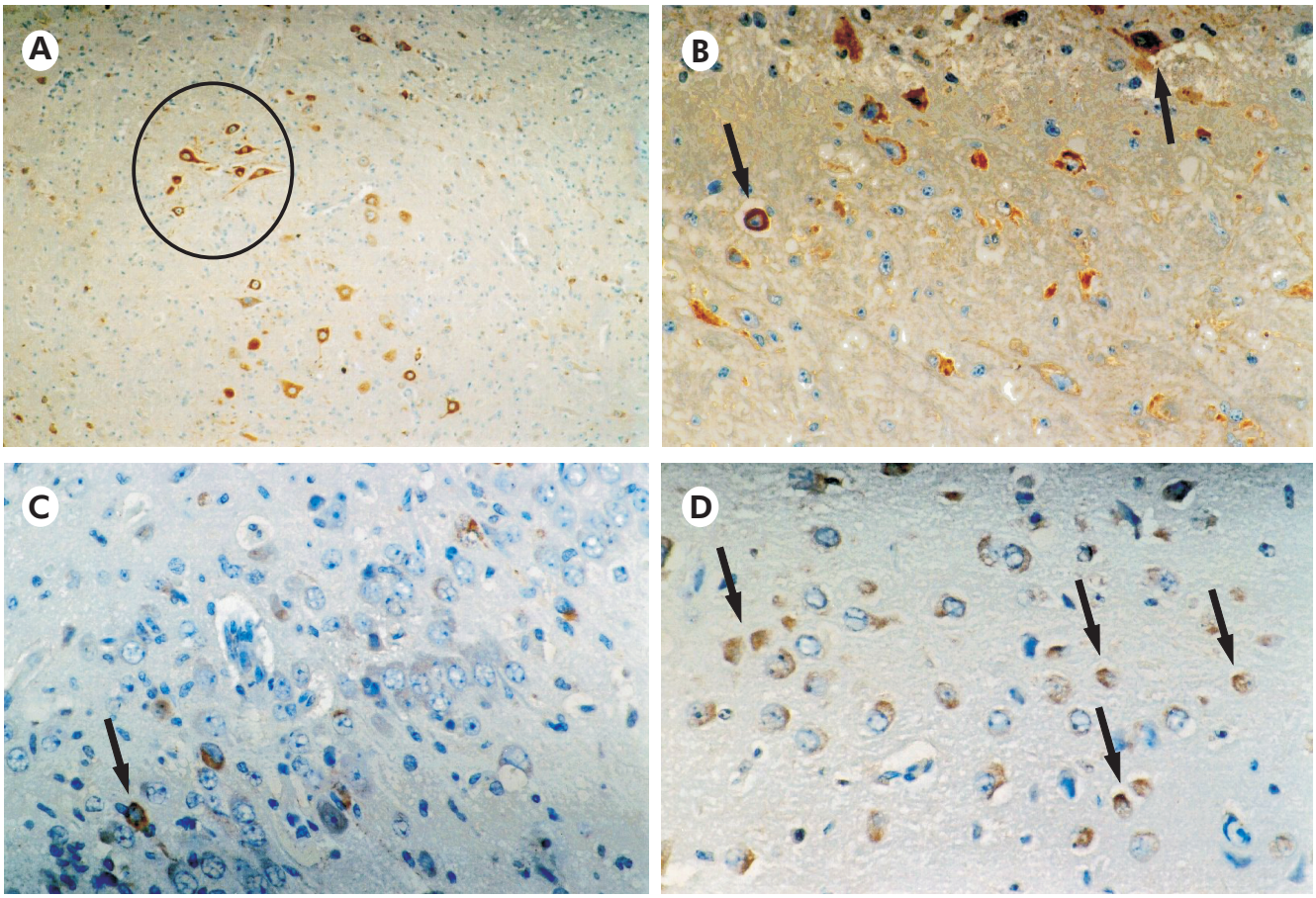


Figure 3 – Detection of ILHV antigen in the brain. **A**: neurons of the basal ganglia on the sixth day p.i. (circle), 10x magnification. **B**: neurons of the basal ganglia on the sixth day p.i. (arrows), 40x magnification. **C**: cells of the hippocampus on the sixth day p.i., 40x magnification. D: cells of the cerebral cortex on the seventh day p.i. (arrows), 40x magnification

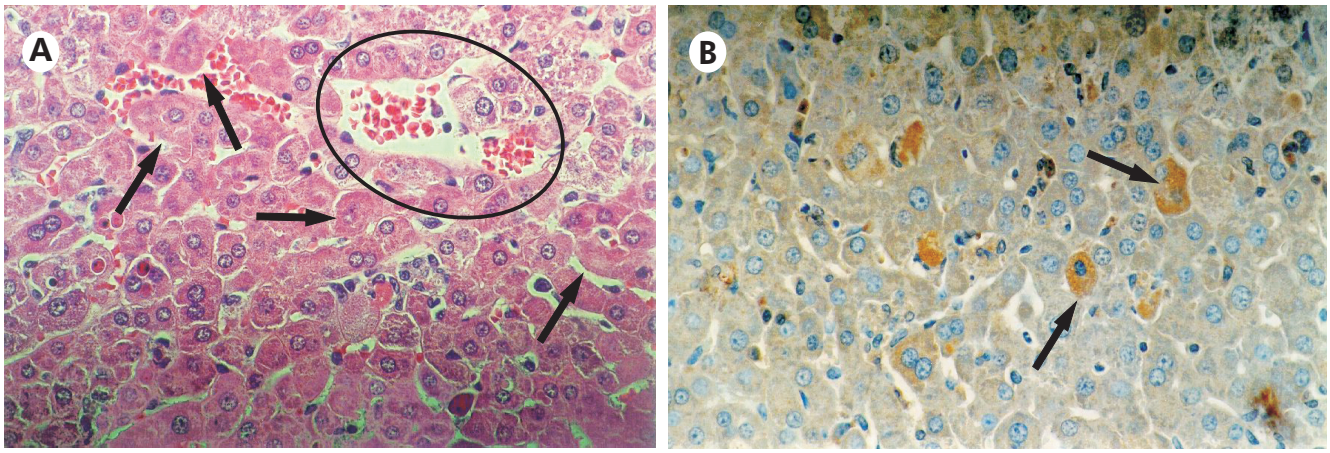


Figure 4 – Liver fragments. **A**: second day p.i., 40x magnification. Sparse mononuclear inflammatory infiltration, congestion (circle) and apoptosis (arrows) were observed. **B**: detection of antigen in hepatocytes (arrows) on the second day p.i., 10x magnification

In the spleen, on the first day p.i., we observed that the white pulp showed follicular hyperplasia and the red pulp already exhibited some degree of congestion. From the second to fifth days p.i. the presence of necroapoptosis of the lymphocytes was noted in the white and red pulps, which was accompanied by the appearance of tingible body macrophages. By the third day p.i., the lymphocytes were no longer intact and only apoptotic bodies and nuclear dust could be identified. On the fifth day p.i., the presence of centroblasts, immunoblasts and some plasma cells was observed amid the cellular remains. As the days passed there was a gradual increase of these cells. In the red pulp, moderate congestion was still detected on the second day, which became intense and was followed by the appearance of hemorrhagic areas on the third, fourth and fifth days p.i.. From the sixth to seventh days p.i., a mild persistence of cellular remains was observed, and there was a decline of the congestive process, as well as an intense proliferation of centroblasts and immunoblasts, which indicated the occurrence of the tissue regeneration phase. The presence of plasma cells was also observed. In the white pulp, immunostaining of viral antigens was detected

on the third day (72 h) p.i. As noted in the liver, the presence of viral antigens in splenic cells was not observed in the remaining days of the experiment (Figure 5).

In the kidneys only intertubular focal congestion was detected on the first day p.i. From the second to fifth days p.i. the glomeruli remained preserved; however, slight swelling appeared in the tubular cells. In addition, we noted that congestion in the intertubular stroma became intense as the experiment progressed, and this was more evident in the medullary portion. On the sixth and seventh days p.i. glomeruli and tubules were preserved. Nonetheless, mild interstitial congestion was observed, and this was far more evident in the renal cortical portion. In IHC the presence of viral antigen in the renal tubules was observed on the fourth and fifth days p.i.. On the remaining days of the experiment the presence of a viral antigen was not detected (Figure 6).

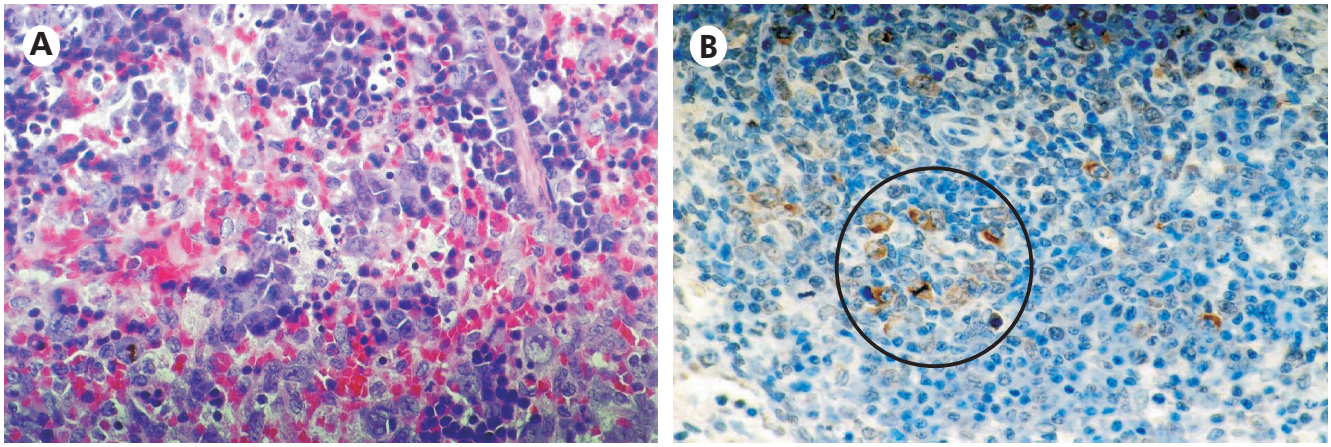


Figure 5 – Spleen fragment. **A**: HS on the fifth day p.i., 40x magnification. It shows the destruction of lymphocytes and other elements, as well as areas with hemorrhage and severe edema. **B**: detection of ILHV antigen (white pulp) on the third day p.i., 40x magnification (circle)

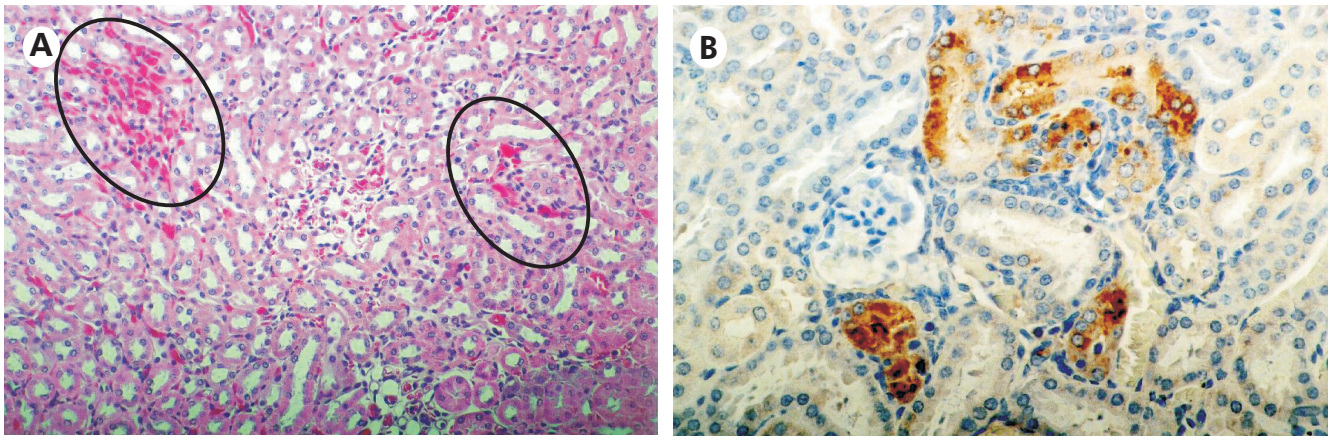


Figure 6 – Kidney fragment. **A**: fifth day p.i., 20x magnification. Severe congestion in the medullary area was observed. **B**: detection of antigen on the fifth day p.i., 40x magnification

In the heart, histological changes were detected from the second day p.i. They were characterized by mild interstitial edema and diffuse hyperemia, and this was far more evident in the myocardium. These lesions lasted until the fifth day p.i. On the sixth and seventh days p.i., morphologic changes were no longer detected in the heart. On the first two days p.i., histological changes were not detected in the lungs. From the third to fifth days p.i., areas that showed alveolar collapse and moderate congestion of alveolar walls and intra-alveolar hemorrhage of mild to moderate intensity were observed. On the sixth and seventh days p.i., only mild congestion of the alveolar walls was noted. In both heart and lung, immunostaining was not found on any day of the experiment.

DISCUSSION

Although the viral strain used in the experiment was serially passaged 21 times in newborn mice, it had never been inoculated into hamsters. Despite the possibility of mutations having occurred during successive IC passages in mice, it was interesting to note that the tested animals developed low titer infections in all organs, which lasted longer in the lungs than in serum or any other organ. Is it possible that the animals have died of pulmonary lesions? Histopathology and IHC, however, did not confirm this possibility because the lungs were the organ with the smallest changes observed in the histopathological study. Moreover, viral antigens were not detected by IHC in this organ. However, the occurrence of progressive leptomeningitis and tissue necrosis in the brain were

observed by HS. These findings, supported by the detection of viral antigen by IHC in the brain on the fifth day p.i., demonstrate that ILHV caused meningoencephalitis in these animals (Figure 3). In the collected liver, spleen and kidney fragments, the titers were low. However, ILHV antigens were detected by IHC on the second day p.i. in the liver, on the third day p.i. in the spleen and on the fourth and fifth days p.i. in the kidney. This demonstrates the tropism of ILHV in these organs and highlights the ability of ILHV to produce antigens in these organs during different phases of infection (Figures 4, 5 and 6). In the lung and heart fragments, although viral titers and slight tissue changes were observed, IHC did not detect the presence of viral antigen. This may mean that the viral titers and the detected tissue lesions are caused by the passage of the virus by these organs through bloodstream, as mentioned earlier.

When we compared the viral titers with the histological changes by HS and viral antigen detection by IHC, we observed severe cellular staining with ILHV antigens on the two days preceding the death of the animals; however, the viral titer was low in the tissues. One hypothesis to explain this finding is that the antigen staining represents immune complexes. If this is true, the low viral titers are justified. Nonetheless, this contrasts with the extent of lesions and cellular staining of antigens observed in the brain.

Considering that the brain lesion was the cause of death of the hamsters in the test, how is it possible to explain the low viral titers that we found? We understand that the intraperitoneally inoculated virus was slow to reach and establish itself in the CNS. When the virus was established and began to replicate the viral titer observed was low because there was time for the production and circulation of antibodies that attached to the viral antigens in the CNS, and this reduced the viral titer in the brain as observed in this study. It is possible, however, that other factors have interfered, culminating in the death of the animals on the seventh day p.i. One factor may be the high dose of inoculum. In fact, the inoculum of 9.8 LD_{50}/0.02 mL is extremely high, and this led to the death of all animals on the seventh day p.i. Indeed, in the work of Tesh and colleagues[21], the authors used a dose of 3.0 LD_{50}/0.02 mL of yellow fever virus (YFV). Even considering that the LD_{50}/0.02 mL titer of ILHV for hamsters was slightly superior, the dose in the IP injection was considered exaggerated.

The study on the pathogenicity of ILHV in humans, carried out by Southam and Moore[18], showed that subcutaneous inoculation of ILHV in humans results in febrile illness for several days. However, in some cases, patients developed signs of mild encephalitis during the course of the illness. This reinforced the previously documented neurotropism of ILHV, which was confirmed in this study.

CONCLUSION

Based on the results obtained in this study, we conclude that the brain was the organ that presented the greatest extent of tissue changes. The presence of the virus in this organ is observed in two stages (biphasic evolution). The first stage occurs 24 h after experimental infection, and the second stage, on the fifth day post-inoculation. All the organs studied showed tissue changes that were detectable by histopathology and viral titers in tissues. Viral antigens, observed through immunohistochemistry, were detected in a severe form in the brain, whereas in the liver, spleen and kidneys the presence of viral antigen was transient and of mild severity. Viral antigens could not be detected in the heart and lungs, which suggests that the titers (LD_{50}) found in these organs during titration in mice resulted from the presence of ILHV in the bloodstream, i.e. from viremia. Severe histopathological lesions and the presence of large amounts of antigen in brains observed by IHC, supported by the viral titers in this organ, suggest that the death of the animals occurred due to encephalitis. This demonstrates that, regardless of the inoculation path, ILHV presents severe neurotropism.

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Estudo experimental sobre a patogenicidade do *Vírus Ilhéus* em hamsters dourados (*Mesocricetus auratus*)

RESUMO

Visando investigar a patogenicidade do *Flavivirus Ilhéus* (VILH) foi inoculada, via intraperitoneal, 9,8 DL_{50} de suspensão viral em hamsters dourados jovens (*Mesocricetus auratus*) e, diariamente, soros e vísceras (cérebro, fígado, coração, baço, rins e pulmões) de animais infectados e de controles não-infectados foram obtidos sob anestesia. Durante o experimento foi determinado o título viral do VILH em soros e vísceras infectados, em camundongos recém-nascidos. Ademais, a detecção de antígeno e os níveis de anticorpos por testes de fixação do complemento e inibição da hemaglutinação foram realizados nos soros. Exame histopatológico por HE e a detecção de antígenos virais por Imunohistoquímica (IHQ) foram realizados nos tecidos dos animais. A dose inoculada ocasionou a morte dos animais por encefalite no sétimo dia pós-inoculação. Todos os órgãos estudados apresentaram alterações teciduais detectáveis por histopatologia. Volumosa presença de antígeno viral foi detectada por IHQ no cérebro, e, em menor quantidade, no fígado, baço e rins; porém, nestes órgãos, a presença de antígeno viral foi transitória e de leve intensidade, o que corroborou com os títulos virais obtidos nesses órgãos. Não foram encontrados antígenos virais em coração e pulmões, sugerindo que os títulos (DL_{50}) observados nesses órgãos, durante a titulação em camundongos, decorreram da presença do VILH na corrente sanguínea (viremia). Os achados deste estudo reforçam o importante e conhecido neurotropismo do VILH.

Palavras-chave: *Vírus Ilhéus*; *Flavivirus*; Encefalite por Arbovírus; Virulência; Modelos Animais.

Estudio experimental sobre la patogenicidad del *Virus Ilhéus* en hámsteres dorados (*Mesocricetus auratus*)

RESUMEN

Con el fin de investigar la patogenicidad del *Flavivirus Ilheus* (VILH), se inoculó por vía intraperitoneal (VIP) 9,8 DL_{50} de suspensión viral en hámsteres dorados jóvenes (*Mesocricetus auratus*), y se obtuvieron diariamente bajo anestesia muestras de sueros y órganos (cerebro, hígado, corazón, bazo, riñones y pulmones) de animales infectados y controles no infectados. Durante el experimento se determinó el título viral del VILH en el suero y órganos infectados, en ratones recién nacidos. Además, se realizó en los sueros la detección de antígeno y los niveles de anticuerpos, a través de pruebas de fijación del complemento y de inhibición de la hemoaglutinación. El examen histopatológico por HE y la detección de antígenos virales por inmunohistoquímica (IHQ), se llevó a cabo en los tejidos de los animales. La dosis inoculada ocasionó la muerte de los animales por encefalitis en el séptimo día tras la inoculación. Todos los órganos estudiados mostraron cambios en los tejidos detectables por histopatología. Se detectó por IHQ una presencia masiva del antígeno del virus en el cerebro y en menor medida en el hígado, bazo y riñones, aunque en estos órganos la presencia de antígeno viral fue transitoria y leve, lo que corroboró con los títulos virales obtenidos en estos órganos. No fueron encontrados antígenos virales en el corazón y en los pulmones, lo que sugiere que los títulos (DL_{50}) observados en estos órganos, durante la titulación viral en los ratones, son el resultado de la presencia de VILH en el torrente sanguíneo (viremia). Los resultados de este estudio refuerzan el importante y conocido neurotropismo del VILH.

Palabras clave: *Virus Ilhéus*; *Flavivirus*; Encefalitis Arbovirus; Virulencia; Modelos Animales.

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