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Neurohistological and Immunohistochemistry of Rabies- Experimental Study in Rats

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Abstract:

The histopathology of rabies studied with conventional methods reveals only subtle changes, for this reason, it has been suggested that the disease is more based on dysfunction of biochemical origin. This work was carried out with the purpose of studying the effect of rabies virus infection on neuronal morphology in the cerebral cortex of mice based on the Golgi method and establishing a relationship between dendritic pathology and the expression of the microtubule- associated protein MAP-2, a marker of dendrites in the adult brain. As a complement, an ultrastructural study of the dendrites was carried out to look for possible effects of the infection on their organelles. The results revealed the existence of notable alterations in the structure of the pyramidal neurons: decrease in the size of the soma, decrease in the thickness of the dendrites, more evident in the apical dendrite. Likewise, the number of dendrite ramifications and the total length of the dendrites were lower. This information was quantified with the help of the NeuronJ program. Additionally, there was a notable loss in the density of dendritic spines. On the other hand, by means of immunohistochemistry an increase in the expression of the MAP-2 protein was found, until making the neuronal body of the pyramidal cells visible, an unexpected result. With the electron microscope, loss of microtubules was observed and electron-dense inclusions (myelin figures) were found that suggest damage to organelles, especially mitochondria. These results are an important contribution to the knowledge of the pathogenesis of rabies. most evident in the apical dendrite. Likewise, the number of dendrite ramifications and the total length of the dendrites were lower. This information was quantified with the help of the NeuronJ program. Additionally, there was a notable loss in the density of dendritic spines. On the other hand, by means of immunohistochemistry an increase in the expression of the MAP-2 protein was found, until making the neuronal body of the pyramidal cells visible, an unexpected result. With the electron microscope, loss of microtubules was observed and electron-dense inclusions (myelin figures) were found that suggest damage to organelles, especially mitochondria. These results are an important contribution to the knowledge of the pathogenesis of rabies. most evident in the apical dendrite. Likewise, the number of dendrite ramifications and the total length of the dendrites were lower. This information was quantified with the help of the NeuronJ program. Additionally, there was a notable loss in the density of dendritic spines. On the other hand, by means of immunohistochemistry an increase in the expression of the MAP-2 protein was found, until making the neuronal body of the pyramidal cells visible, an unexpected result. With the electron microscope, loss of microtubules was observed and electron-dense inclusions (myelin figures) were found that suggest damage to organelles, especially mitochondria. These results are an important contribution to the knowledge of the pathogenesis of rabies. This information was quantified with the help of the NeuronJ program. Additionally, there was a notable loss in the density of dendritic spines. On the other hand, by means of immunohistochemistry an increase in the expression of the MAP-2 protein was found, until making the neuronal body of the pyramidal cells visible, an unexpected result. With the electron microscope, loss of microtubules was observed and electron-dense inclusions (myelin figures) were found that suggest damage to organ

Keywords: *Rabies, pyramidal neurons, dendritic pathology, MAP-2, neuronal ultrastructure, Golgi technique.*

1. INTRODUCTION

Rabies is a disease caused by a neurotropic virus, generally transmitted through the bite of infected animals. According to the World Health Organization (WHO) it is currently estimated that some 60,000 people a year die as a result of this deadly disease (1). In Colombia and most Latin American countries, vaccination campaigns have significantly reduced the impact of rabies of canine origin, but the risk posed by wild animals that are vectors of the rabies virus persists, especially bats (not only blood-sucking ones). and some carnivores such as foxes, skunks, and raccoons (1,2). About rabies there are historical records dating back to 2300 years before the Christian era. In ancient Mesopotamian legal documents, dog owners were required to answer for deaths caused by the bites of their pets. In 1885, without yet knowing the causative agent of the disease, Louis Pasteur successfully supplied the first rabies vaccine; thus the study of rabies entered the modern era. After decades of discussion, electron microscopy made it possible to confirm the viral origin of the disease in the mid-20th century (3). The clinical signs of rabies and its fatal nature, once its first symptoms appear, make this a disease that causes panic in the patient and terror in the population. Paradoxically, the macroscopic and histological neuroanatomical examination of the affected nervous tissue only reveals subtle changes (4). To a large extent, postmortem confirmation of the diagnosis of rabies requires the demonstration of Negri bodies in nervous tissue. These correspond to small intracytoplasmic eosinophilic inclusions observed in some neurons that were first described by Adelchi Negri in 1903 and are considered the pathognomonic feature of rabies (4,5). Immunohistochemistry contributed to facilitating its localization and increasing the reliability of the diagnosis (6). Examination of nervous tissue affected by rabies reveals no appreciable histopathologic changes; Neither lysis nor neuronal death was observed. For this reason, different researchers have defended the hypothesis according to which the effects of the infection are more of a biochemical and metabolic type associated with neuronal dysfunction but preserving the integrity of the nerve cells (7-11). Even the phenomenon of cell death by apoptosis, very frequent in other infectious diseases, is not an important component of the disease. togenesis of rabies (8,11,12). However, in preliminary studies by our work group we have found alterations in neuronal morphology, described qualitatively, mainly in the cell body and dendritic arborization, using the Golgi technique, the method that best describes the external structure of the components of a neuron (13-

15). In accordance with this background, the purpose of this work was to deepen the knowledge of the effect of rabies virus infection on neuronal structure based on the Golgi method studied with quantitative analysis tools. This was complemented with the immunohistochemical study of the expression of the microtubule-associated protein (MAP-2), an essential molecule for the stability of the neuronal cytoskeleton, mainly of the dendrites (16,17). Additionally, brain tissue was processed for electron microscopy in order to investigate possible changes in the fine structure of the dendrites and their organelles that could be associated with dendritic pathology.

2. PATIENTS AND METHODS

Viral inoculation and initial handling of animals.

Two types of rabies virus were used for this study: wild virus (street type) isolated from the brain of an infected dog and laboratory adapted virus (fixed type) of the CVS (Challenge Virus Standard) strain. These were supplied in aliquots (in 1:10 dilution) by the Virology Laboratory of the National Institute of Health (INS). From these aliquots, the titration of each one of the viruses was carried out by inoculating suckling mice intracerebrally according to the Reed and Muench method (18). For the development of the experimental tests, 28-day-old female mice (young adults) of the ICR strain (Institute of Cancer Research) were used. A group of 20 animals was inoculated with 'fixed' virus and another group of 20 animals was inoculated with previously titrated 'street' virus. Each group, needles very thin and short (No. 27 gauge). For the experiment with fixed virus, each mouse was inoculated with 0.03 ml of an aliquot with 10 dilution.-1 equivalent to 109Fixed virus LD50. For the study with street virus, each animal was inoculated with 0.03 ml of an aliquot with 10 dilution-1equivalent to 106LD50. The differences in the LD50 correspond to the titration previously carried out for each of the types of virus. The animals were kept in the high-security room of the INS vivarium under constant temperature (20±2ºC) and humidity (60±5%) conditions, as well as food and water availability at will. This project and its animal handling protocol were approved by the INS Ethics Committee. The ethical and legal standards required for research with laboratory animals in Colombia were followed (Law 84 of 1989 and Resolution No. 8430 of 1993 of the Ministry of Health). The animals were kept in the high-security room of the INS vivarium under constant temperature (20±2ºC) and humidity (60±5%) conditions, as well as food and water availability at will. This project and

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Collection and initial preparation of brain tissue.

When the mice infected with the rabies virus reached signs of advanced disease (at 5-6 days post-inoculation for fixed virus and at 10-12 days for street virus) manifested in weight loss, decreased body temperature, bristly hair and few displacement movements, they were sacrificed to obtain the brains. In accordance with our experience and that of other authors, the effects of the infection become more evident only in advanced stages of the disease (7,19). The protocol for the sacrifice and obtaining the sample from each animal was as follows: Intraperitoneal injection of 1 milliliter of an aqueous solution of chloral hydrate prepared at 30% (dose of 300mg/Kg). In 3 minutes the animals were already deeply anesthetized. A 27-gauge needle, one centimeter long, was inserted through the left ventricle, attached to the terminal end of a hose connected to a peristaltic pump. By this route, each animal was given 50 ml of phosphate buffered saline (PBS) or 0.9% saline solution. A fixative solution was then allowed to run according to the study technique to be followed with each specimen: 4% paraformaldehyde (PFA) for immunohistochemistry and Golgi-Colonnier or a mixture of 1% PFA and 3% glutaraldehyde (GA) for electron microscopy. Animals destined for the Golgi- Cox technique were only perfused with saline. The brains were then removed, immersed in the same fixative solutions used in the perfusions, and transferred to the laboratory to continue with the processing protocol for each technique. Samples from animals infected with the two types of virus, and their respective controls, were divided into four groups of five each to be processed separately using the four techniques used in the study: Golgi-Colonnier, Golgi-Cox, immunohistochemistry, and electron microscopy. . Before starting each of these procedures, the presence of the viral infection was verified in all the brains of animals inoculated with rabies, especially in the cerebral cortex, by means of an immunohistochemical test (as described below) with an antibody and an elaborate protocol. by group (6). The study area for all experiments was the frontal motor cortex in the plane coronal and dorsal to the corpus callosum. Valverde's mouse brain text-atlas (20) was used as a guide.

Golgi technique.

The Golgi-Colonnier method was followed for the study of the morphology of cortical pyramidal neurons according to the previously standardized protocol (13). Slices of brains obtained in a coronal plane, 0.5 cm thick, fixed in 4% PFA, were successively transferred to a mixture of 2% potassium dichromate (DP) and 5% GA for 5 days, then to a 3.5% DP solution for 24 hours followed by a 0.75% silver nitrate (NP) solution for 24 hours. After cleaning the precipitates formed on the surface of the slices, the DP and NP steps were repeated and the samples were immersed in a series of solutions of increasing concentration of glycerol (20%, 40%, 60%, 80%, 100%). These slices were mounted on a vibratome to obtain 150-micrometer- thick sections, which were dehydrated in solutions of increasing concentration of ethanol, passed through xylol, and mounted in Canada balsam. The histological preparations were observed in a microscope equipped with a digital camera and computer programs that allowed the capture of images of the impregnated neurons, reconstructions, and quantitative analysis of the neuronal morphology using the Neuron J software. As a complement, schematic drawings of some neurons were obtained in a microscope with camera lucida (drawing arm). The Golgi-Cox method was used to also obtain Panoramic images of the cerebral cortex with neurons and neural processes imbued. The protocol followed was that of Gibb and Kolb (21), briefly: the brains extracted from the animals perfused with saline solution were submerged in a solution composed of 5% potassium bichromate, 5% mercuric chloride, and saturated potassium chromate. 5% (Cox solution prepared five days in advance) in which they were left for 14 days. Then they were transferred to a 30% sucrose solution for 5 to 10 days. Coronal sections 200 micrometers thick were obtained on a vibratome and collected in 6% sucrose. The sections were then briefly washed in distilled water, treated with ammonium hydroxide for 30 minutes, washed again and transferred to Kodak photographic fixer solution in the dark for 30 minutes. Finally, the sections were dehydrated in ethanol and xylene and mounted in Canada balsam. Digital panoramic images of the cerebral cortex were taken under the microscope, which were processed for densitometric study with the ImageJ program to evaluate the density of ramifications of neuronal processes.

Immunohistochemistry

The specimens fixed in 4% PFA were processed for MAP-2 immunohistochemistry following a basic protocol already standardized in previous work by the group for the study of other antigens (6,19,22-26). The procedure is summarized as follows: the brains were cut on a vibratome to obtain slices 50 micrometers thick in the coronal plane and in the rostrocaudal direction of the frontal cortex. The sections were collected in small Petri dish-type glass containers (2.5 cm in diameter x 1 cm in height), one for each specimen. Initially, they were washed in PBS, overnight with constant stirring and at room temperature (approx. 20°C). All other steps were done under the same conditions. After the initial washing, the sections were successively treated with ammonium chloride for 30 min (to remove traces of aldehyde fixation) and hydrogen peroxide for 30 min (to block endogenous peroxidase) with PBS washes after each step. The sections were then incubated for 30 minutes in a solution prepared in PBS to which bovine albumin, horse serum and newt were added (to block non-specific sites). Immediately and without washing, the blocking solution was removed and the sections were they were incubated overnight in the solution containing the primary antibody (monoclonal anti- MAP-2 Santa Cruz, 1:1000 dilution). This treatment was chosen after testing three different brands of antibodies and dilutions ranging from 1:100 to 1:2500. The following day the sections were washed in PBS and incubated for two hours in the secondary antibody (Sigma anti-mouse, 1:400 dilution). After washing in PBS, the sections were treated, for two hours, with a solution of avidin- biotin-peroxidase (ABC Kit) in PBS prepared according to the manufacturer's instructions (Vector). Finally, after a final wash in PBS, development was carried out with the DAB-Nickel chromogen (Vector Kit). The sections were mounted on slices previously treated with gelatin for later observation.

electron microscopy

The brains of animals fixed with 1% PFA and 3% GA were cut with a vibratome to obtain coronal slices 200 micrometers thick, at the level of the frontal cortex above the corpus callosum. These were processed using small Petri dishes to change the reagent solutions in a similar way to how the samples for immunohistochemistry were handled but at a temperature of 4°C. After washing them with phosphate buffer solution (TP), they were post-fixed with 1% osmium tetroxide for 1 hour and again washed with TP. The sections were then dehydrated using a series of solutions of increasing concentrations of ethanol (50, 70, 80, 95, 100%) for 15 minutes each, followed by treatment with propylene oxide

(OP) in two changes. 15 minutes each. Fragments of sections containing the complete cortex were embedded in EA resin (Polysciences) in molds flat to maintain their orientation in the coronal plane and were polymerized at 70°C for 24 hours. Ultrathin sections (60 nanometers thick) were obtained from this material on an LKB ultramicrotome; these were collected on 400 mesh copper grids, sequentially treated with uranyl acetate and lead citrate to increase contrast, and observed under an electron microscope. Photographs were taken on Tmax ASA 100 black and white film, the negatives were developed and scanned.

3. RESULTS

dendritic pathology

The Golgi-Colonnier technique allowed the observation of complete neurons, most of them pyramidal cells that exhibited their characteristic morphology. This consists, first of all, of a long apical dendrite emerging from the neuronal body (soma) in the direction of the surface of the cerebral cortex. In addition, basal dendrites originate from the soma and many secondary and tertiary lateral branches from the apical dendrites. Most of the dendrites are covered by spines. In samples from control animals, an average of three complete pyramidal cells were impregnated in 1 mm2 of cortical area that corresponds to frontal motor cortex. In animals inoculated with any of the types of rabies virus, alterations in neuronal morphology and particularly in the pattern of the dendritic tree were evident. These are summarized as follows: decrease in the size of the soma, decrease in the thickness of the dendrites, more evident in the apical dendrite. Likewise, the number of dendrite ramifications and the total length of the dendrites were lower. This information was quantified with the help of the NeuronJ program (**Table 1**). The Golgi-Cox technique impregnated a greater number of neurons and dendrites than the Golgi-Colonnier technique. The morphological characteristics observed both in the neurons of control samples and in those corresponding to samples of animals inoculated with rabies were very similar with the two techniques, but the greater density of cells and neuronal processes impregnated with the Golgi-Cox allowed a densitometric analysis of loss of dendritic arborization in infected samples. For this, panoramic images were taken (**Figure 1 a and b**) in which the density of processes was quantified with the ImageJ program (**Table 2**). With each of the two Golgi methods used, they observed other abnormal features such as marked loss of dendritric spines and the formation of nodules or bulges along the dendrites

and on some axons. With the Golgi- Colonnier method, mainly pyramidal neurons were impregnated, while the Golgi-Cox method also revealed the presence of interneurons, especially basket cells. Some of them in samples from infected animals developed an abnormal proliferation of short and densely packed cell processes around the soma (**Figure 1**).

Expression of the microtubule-associated protein MAP-2.

Immunohistochemical images to reveal the presence of the MAP-2 protein showed marked microtubule labeling in the apical dendrites of pyramidal neurons. In samples from infected animals, immunoreactivity (immunostaining) was higher. The effect of infection with the rabies virus was so evident that, in addition to increasing the expression of the protein in the dendrites, the immunoreactivity also became visible in the somas of the pyramidal neurons of layer five of the cerebral cortex (**Figure 2**). The increase in MAP-2 is analyzed by optical densitometry with the help of the ImageJ program and it was statistically significant (**Table 3**).

dendritic ultrastructure

The electron microscopy study focused on observing ultrastructural details of the dendrites of pyramidal neurons. In the controls, the dendrites showed normal ultrastructural characteristics such as the presence of microtubule bundles oriented parallel to the plasma membrane and abundant mitochondria as well as a regular contour at the limits of each dendrite. The most significant feature found in the infected tissue was the formation of electron-dense (dark) structures within the dendrites (**Figure 3**). These exhibited an appearance similar to what are known as myelinated figures and took different forms; some of them were eventually found in the axons. Dendrites containing these abnormal inclusions tended to be more thickened and irregular in outline, and loss of microtubules and disorganization of those that were preserved was evident within them. Another relevant detail was the low number of mitochondria observed. Additionally, in some dendrites they formed vacuoles that interrupted the continuity of the protoplasmic content within the dendrite.

Table 1. Quantification (*NeuronJ*) of the length of dendrites and size of the neuronal bodies (somes) of pyramidal neurons from control mice and those infected with the rabies virus. Data obtained with animals inoculated with 'street' virus are presented. Qualitatively the observations made in samples infected with 'fixed' virus were similar.

dendrites	Controls	Infected
	length in pixels	length in pixels
apical 1st	649 ± 36	402 ± 33
apical 2nd	532 ± 39	338 ± 38
Apical 3rd	286 ± 37	97 ± 31
Baseline 1ª	654 ± 38	320 ± 42
Baseline 2ª	512 ± 32	156 ± 36
Baseline 3rd	278 ± 48	94 ± 45
Somas (n=50)	average area in pixels	average area in pixels
	20579	9457

* Statistically significant. P. value dendrite length = 0.030*. P. value soma area = 0.025*

Table 2.Optical densitometry data obtained with the programImage Jin panoramic images of coronal sections of control and infected mice processed with the Golgi-Cox technique. The loss of optical density in the gray scale (0-255) of the infected is evidenced by a greater number of transmitted light and reflects a decrease in neuronal processes in the neuropil.

samples	Average scale of transmitted light (0-255) controls	Average light scale transmitted (0-255) Viruses 'fixed'	Average light scale transmitted (0-255) 'street' virus
1	124,729 ± 57	186,182 ± 46	163,093 ± 61
2	133,511 ± 52	187,051 ± 46	167,851 ± 58
3	120,240 ± 46	181,625 ± 52	154,287 ± 48
4	131,150 ± 43	169,554 ± 57	158,327 ± 54
5	129,348 ± 45	177,457 ± 39	140,833 ± 45
P. value		0.0079*	0.0079*

Table 3.Densitometric measurement with the programImageJof MAP-2 immunoreactivity in the cerebral cortex of mice. On the left are data from animals inoculated with 'fixed' rabies virus and on the right with 'street' virus. The values correspond to the number of pixels of black tone. The higher values in samples from infected mice represent the increase in protein expression.

Black tone frequency values (0-255)				Black tone frequency values (0-255)			
Sample	Infected	Controls	p value	Sample	Infected	Controls	p value
1	67481	73604	0.057*	1	35213	46297	0.0143*
2	60955	75065		2	44241	48993	
3	51339	65794		3	41772	49960	

4. DISCUSSION

In the histopathological descriptions of the nervous tissue affected by the rabies virus, it is traditionally stated that the observed changes are almost imperceptible and that only the presence of the Negri body confirms the diagnosis of the disease. For this reason many authors have adhered to the hypothesis according to which the characteristic dramatic symptoms of rabies are due more to effects of the infection on the metabolism of neurotransmitters and other compounds, that is, that the damage is more biochemical and functional than structural (4, 7-11). But when using the Golgi technique, profound alterations in the structure of the neurons induced by this viral infection became evident, as we qualitatively demonstrated in previous studies (13,14). Previously, and despite the importance of rabies in public health, only one reference was found in which the effect of the virus on dendrites was very briefly reported using the Golgi method. This was carried out by the scientist who discovered the technique at the end of the 19th century, who inoculated rabbits with intracerebral fixed virus and made brief descriptions of the histopathology with various techniques, including his method (27), when he had not yet acquired the relevance and development that Santiago Ramón y Cajal later gave it and that earned them the Nobel Prize in Medicine (15). The Golgi technique continues to be recognized as the procedure that makes it possible to better demarcate complete neuronal morphology and therefore the "gold-standard" for studying dendritic pathology (15,20,21,28-31). In this work we confirm our preliminary findings, but now through two different protocols (Golgi- Colonnier and Golgi-Cox) and the use of computer tools that facilitated a quantitative and we could say more scientific analysis of the importance of dendritic pathology in rabies. Therefore, it can be affirmed without any doubt that rabies

does induce structural neuronal damage. This includes the marked loss of dendritic spines, which is consistent with the recent finding that dendritic spine loss in hippocampal pyramidal neurons is associated with the depolymerization of F-actin, a very important protein of the dendritic spine cytoskeleton (32). This has profound implications for everything related to the integration of neural circuitry. that this protein establishes bridges between microtubules and confers stability to the neuronal cytoskeleton (16,17,33,34). However, cases of neuropathologies with dendritic alterations and increased MAP-2 have also been reported (34-37). What we can infer is that both the increase and decrease of this protein would give rise to imbalances in the cytoskeleton structure capable of altering dendritic morphology and stability. In a previous study, loss of MAP-2 was reported in cultures of neurons infected with a strain of fixed rabies virus (38) and more recently, the effect of infection with street virus on MAP-2 immunofluorescence was studied. pyramidal neurons of the hippocampus. The internal structure of the dendrites observed with the electron microscope confirms the effect of structural damage caused by the virus. The presence of relatively large and abundant myelinated figure- type structures, together with the loss of microtubules and mitochondria in the dendrites, had apparently not been previously reported, despite the fact that numerous ultrastructural studies of virus-infected brain tissue have been performed. of rage. The most likely explanation lies in the orientation given to the brain tissue when processing the samples in accordance with our goal of observing the dendritic arborization of cortical pyramidal cells in a coronal plane of the brain slice allowing a sagittal view of the soma, the apical dendrite and its ramifications. We believe that the myelin figures observed here may correspond to mitochondria due to their location and size and because they coincide with the loss of normal mitochondria, which are abundant in controls. In addition, mitochondrial dysfunction has recently been reported by biochemical methods in neurons due to rabies (39). Myelin figures are structures that can appear in tissues as a result of alterations in membranous organelles such as mitochondria and the endoplasmic reticulum (40,41). In some natural or experimentally induced neuropathologies, myelin figures associated with degeneration of mitochondria into dendrites and axons have been found (42-45). In conclusion, with this work we have fully demonstrated that the rabies virus does induce structural neuronal damage, contrary to the hypothesis defended by other authors based on conventional histopathology. Therefore, it cannot be affirmed that the dramatic clinical manifestations of

rabies correspond only to biochemical disorders. In other studies, brief descriptions have been made with different techniques that make partial reference to the effect of rabies on the dendritic structure (32,38,46) while in our work morphological aspects were integrated (using the technique that best describes the neuronal structure).), with the study of the expression of the cytoskeleton protein that supports the dendritic structure and the details of the fine structure observed with the electron microscope The quantitatively analyzed dendritic pathology in rabies, as well as the unexpected increase in the expression of the cytoskeletal protein MAP-2 and the ultrastructural findings revealed here are an important contribution to the knowledge of the pathogenesis of rabies. In addition, we work with an animal model and confirm our results with street virus, the wild virus that circulates in nature, inoculated by the intramuscular route, which best approximates the natural contagion conditions of the disease. Most research in rabies employs fixed virus (laboratory-adapted virus), primary neuron cultures, and when inoculation of live animals is carried out, it is generally done by the intracerebral route.

Ethical Issues: All ethical issues were approved by the authors from the Iraqi Ministry of Health. Verbal and signed informed consents were obtained from all patients who included in the study during their first visit.

Conflict of interest: None

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