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RESEARCH ARTICLE

Cerebrolysin Improves Motor Abilities and Reverses the Long-Term Memory Acquisition Deficit in Rats with Hypoxic-Ischemic Encephalopathy

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ABSTRACT

Background: Hypoxic-ischemic encephalopathy (HIE) is a neurological condition that leads to motor disabilities and even death in neonates. Unfortunately, few therapeutic alternatives can contribute to brain recovery after HIE damage. Cerebrolysin is a neuropeptide mixture that exerts neuroprotective and neurotrophic effects on injured brain tissue after systemic administration.

Aims: This study evaluated the short- and long-term beneficial effects of Cerebrolysin administration in a rat model of HIE.

Methods: Neonatal 7-day-old rats were subjected to hypoxia-ischemia injury and then intraperitoneally administered Cerebrolysin (10 mL/kg of body weight) once a day for 7 days, from postnatal days 8 to 14. Growth development, blood-brain barrier permeability, and neurobehavioral tests were performed.

Results: Cerebrolysin administration after hypoxic-ischemic insult minimized brain damage, edema and increased cellular viability. Furthermore, this neuroprotective effect improves some motor abnormalities and, during adulthood, reverses the long-term memory acquisition deficit caused by HIE.

Conclusion: Repeated Cerebrolysin administration can safely and effectively reduce HIE motor disabilities and reverse long-term memory acquisition deficits in neonatal rats.

Keywords: Neuroprotection, novel object recognition, learning and memory, neonatal brain injury, Cerebrolysin.

1. Introduction

The hypoxic-ischemic (HI) events in the newborn significantly damage the central nervous system (CNS). The immature brain has high baseline oxygen requirements and high sensitivity to oxidative damage due to its high lipid composition and low levels of antioxidant molecules^{1,2}. In neonates, hypoxic-ischemic encephalopathy (HIE) increases mortality and morbidity^{3,4}. Also, it produces long-term sequels such as cerebral palsy, movement disorders, and cognitive deficits accompanied by loss of brain volume, mainly in the ipsilateral lesion area. These deficits can account for attention deficit hyperactivity disorder characteristics, such as decreased attention and increased impulsivity and compulsivity^{5,6}. Thus, HIE remains a severe clinical problem worldwide despite efforts to provide therapies to mitigate and even reverse brain injury^{7,8}. Therapeutic strategies aim to enhance neurorestoration, block neuroinflammatory response, exert neuroprotection and stimulate neurogenesis during the HIE development. However, no pharmacological treatment that induces all of these effects has yet not been developed. Cerebrolysin is a peptide mixture that mimics the action of neurotrophic factors as shown by clinical trials and experimental assays in neurodegenerative diseases, cerebral ischemia, and traumatic brain injuries. Cerebrolysin overcomes other therapeutic alternatives because of its ability to activate neuroprotection and repair mechanisms, including increased cell proliferation, neural survival, and neurogenesis⁷⁻¹¹. Cerebrolysin has been tested as a therapeutic agent in adult patients suffering from brain stroke, showing beneficial effects on function and global outcomes in the early rehabilitation phase¹². However, to date, very little is known about its benefits after HI injury in immature brains. A report has shown that Cerebrolysin administration in infants with severe brain damage from perinatal injury improves communication, symbolic language, and social interaction¹³. The present study investigated the Cerebrolysin effects on growth development and neurobehavior after intraperitoneal administration in a neonatal HIE rat model.

2. Materials and Methods

2.1. ANIMALS

Unsexed, postnatal day (PD) 7 Sprague-Dawley rats with their biological mother were housed in relative humidity of $60 \pm 5\%$ and room temperature of $22 \pm 2^\circ\text{C}$ in a light/dark cycle (12

hours each). After weaning at PD30, food and water were provided ad libitum. The Institutional Committee approved all experimental procedures for the Care and Use of Laboratory Animals of CINVESTAV according to the NOM-062-ZOO-1999 Official Mexican Standard. A total of 149 rat pups were included in the study and were randomized into 4 groups: 1) Control: Intact animals, 2) Control+Cerebrolysin: Animals administrated with Cerebrolysin from PD8 to PD14, 3) HIE: Animals subjected to the HIE model, and 4) HIE+Cerebrolysin: Animals subjected to the HIE model treated with Cerebrolysin since PD8 to PD14.

2.2. HYPOXIC-ISCHEMIC ENCEPHALOPATHY MODEL AND DRUGS ADMINISTRATION

Rat pups (10-20 g) were subjected to a modified HIE model previously described by Rice-Vanucci¹⁴. First, pups were anesthetized with an intraperitoneal injection of 10 mg of Ketamine/2 mg of Xylazine mixture. Then, after performing a midline neck incision and tissue dissection, the left common carotid artery (CCA) was isolated and occluded with a vascular micro clamp for 30 minutes. Finally, the wound was closed by using a nylon monofilament 6(0) suture. After surgery, the animals were returned to cages for 2 h and then placed in a hypoxic chamber (92% O₂ and 8% N₂) for 100 minutes. The temperature was maintained at 37 °C. Animals recovered in cages for 30 min and then were housed with the dam.

A dose of 10 mL/kg of Cerebrolysin (EVER Neuro Pharma GmbH, Austria) was administered intraperitoneally to neonatal rats at 24 h after HI injury (PD8), and the treatment was continued daily until PD14, for a total of 7 administrations (Figure 1).

2.3. GROWTH DEVELOPMENT

2.3.1. Body weight gain

The animals' body weight was recorded at PD7, PD14, PD21, PD28, PD35 and PD60. Data were expressed as body weight gain (BWG) based on the weight at PD7.

2.3.2. Eye-opening

Rat pups were examined daily since PD8 at the same hour. Eye-opening age was registered when two palpebral fissures were opened.

2.4 NEUROBEHAVIORAL TESTS

Five different tests were used to evaluate the motor activity at PD10, and cognitive deficits were evaluated during the adult stage (PD59-PD60).

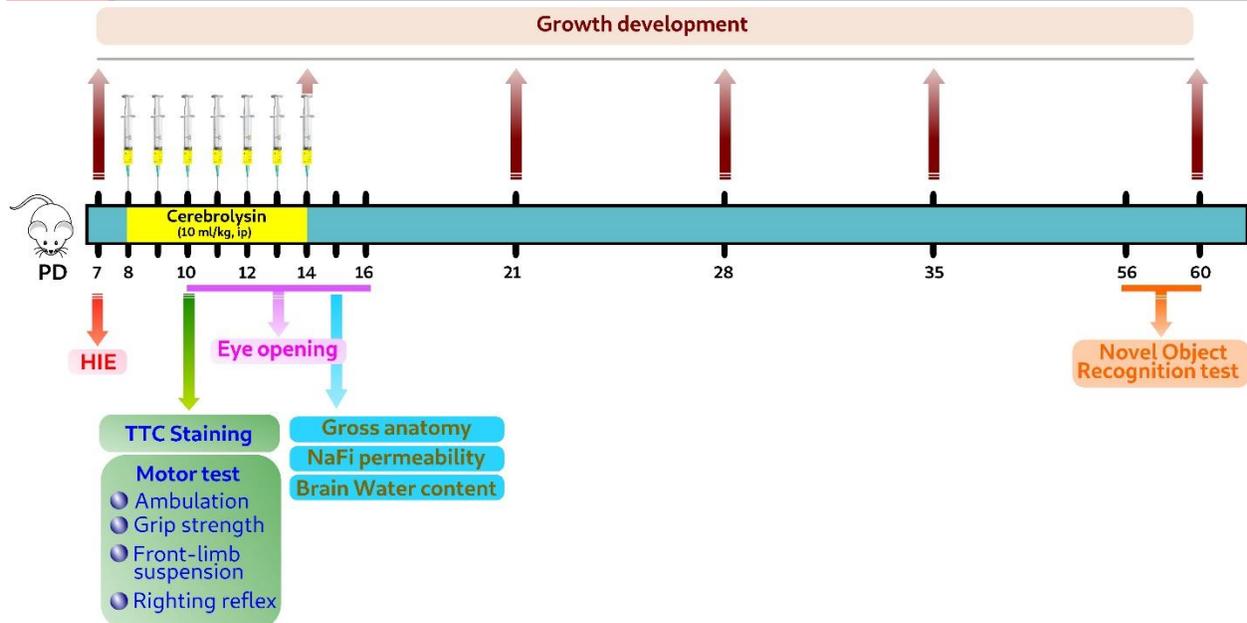


Figure 1. Experimental design showing the HIE induction in 7 days old rats, the Cerebrolysin treatment scheme, and the evaluation times of growth, cell viability through TTC staining, eye-opening, gross anatomy, brain water content and NaFl permeability, motor tests and the novel object recognition test. Hypoxic-ischemic encephalopathy (HIE), intraperitoneal (ip), postnatal days (PD), NaFl (sodium fluorescein).

2.4.1 Motor tests

2.4.1.1 Ambulation test

The animals were placed on an open acrylic box, and movements were video-recorded for 3 minutes. A score was assigned with the following parameters: 0 = no movement, 1 = crawling with slow and asymmetrical movements, 2 = crawling with slow but symmetrical movements, and 3 = fast crawling/walking, according to Feather-Schussler and Ferguson¹⁵.

2.4.1.2 Hindlimb foot angle

The rats were placed on an open acrylic box, and the movements were video-recorded for 3 minutes. The hind-limb angle from the longest middle toe line to the heel end was measured during the walk. Three angles per animal were measured to obtain the average¹⁵.

2.4.1.3 Grip strength

The suspension capacity of 4 limbs was evaluated on a metal mesh tilted 180° and positioned at 25 cm above a table. The rat was suspended on the mesh, and the falling time was recorded during the 60 s trials. Hanging impulse was calculated as follows: weight(g) x latency time (s)¹⁵.

2.4.1.4 Front-limb suspension

The strength of the forelimbs was evaluated by suspending the rat on a metal bar. The body was firmly held as it approached the bar to allow the rat to hold it with both forelimbs. Finally, the rat was released, and the time it remained on the bar was

recorded before falling. Three trials were performed¹⁵.

2.4.1.5 Righting reflex

Pup ability to change from dorsal position to ventral position was evaluated¹⁵. The pup put on a dorsal position was released. Then, the time to return to the ventral position was recorded in a maximum of 1-min trial. Three trials per rat were performed, and the following score was assigned: 0 = No response, 1 = Greater than 1s, and 2 = Less than 1s.

2.4.2 Novel object recognition test

Evaluations were performed in 2 phases, according to Reger et al.¹⁶. Habituation phase: Rat freely explored an empty arena (70 x 70 x 50 cm) for a 10 min-session. A total of 3 sessions were performed (1 session/day) at PD56-PD58. Familiarization phase: 2 identical objects (A + A) were placed in the arena in a straight line at a distance of 11 x 11 cm from one of the box walls. Free exploration for 3 min was allowed. Trial phase: 1 object used previously and 1 novel object (test 1 = A + B and test 2 = A + C) were located in the same conditions mentioned. Test trials were performed at 1 and 24 hours post-familiarization, corresponding to PD59 and PD60, respectively. The behavior was video-recorded, and the novel object recognition memory was expressed as discrimination index = $(N-F/N+F)$ where N is time spent exploring the novel object and F is time spent exploring the familiar object.

2.5 BLOOD-BRAIN BARRIER PERMEABILITY

2.5.1 Brain water content

The brain was removed at PD14 and a medial sagittal cut was performed to obtain the left and right hemispheres. Each hemisphere was placed on pre-weighed aluminum foil to determine the wet weight. Tissue samples were dried in an incubator at 100°C for 24 hours and weighed to determine the dry weight. The water content was calculated with the following formula: % H₂O = (1 - Wet weight / dry weight) x 100.

2.5.2 Sodium fluorescein permeability

Rats at PD14 were administered intraperitoneally with 5% sodium fluorescein (NaFl) in phosphate buffered saline (PBS), at the dose of 10 µL/g of body weight. After 2 hours, the animals were anesthetized and intracardial perfusion with PBS was performed to wash out excess sodium fluorescein in blood. The brain was removed and the cerebral cortex of each hemisphere was obtained. Tissue samples were homogenized with 500 µl of 80% trichloroacetic acid to precipitate proteins. It was centrifuged x 10 min at 13600g. 150 µl of supernatant and 50 µl of 5M NaOH were added in triplicate and absorbance at 490 nm was measured using a spectrophotometer. Data were expressed as a percentage of permeability to NaFl based on the permeability of the control group.

2.6 BRAIN TISSUE ANALYSIS

Brain tissues were obtained on days 3 and 7 after the HI event. 5 animals of each group were injected with pentobarbital (50 mg/kg, intramuscular), and then their brains were rapidly removed to analyze their general anatomical structure. The 2,3,5-triphenyl tetrazolium chloride (TTC) staining was performed on fresh coronal brain sections (2 mm of thickness) and dissected using a rat brain slicer matrix. The TTC (1% in saline solution) staining was performed for 30 min incubation at 37 °C. Slices were photographed using a digital camera.

2.7 STATISTICS

Results of growth development were expressed as mean ± SEM. Data were analyzed with one-way ANOVA. Differences between groups were detected with Tuckey's post-hoc test. The age of eye-opening was evaluated using Fisher's test.

Neurobehavioral and blood-brain barrier (BBB) permeability tests data were expressed as median ± SD. Their statistical analysis was performed using the Kruskal-Wallis test and Holms-adjustment. Differences between groups were detected with Dunn's post-hoc test. In all cases, statistically significant differences were established with $p \leq 0.05$. Statistical analysis was processed on GraphPad Prism version 5.0 (San Diego, California, USA).

3. Results

Of the 89 pups subjected to CCA occlusion, just 30 subjects survived out to 30-min CCA occlusion followed by a 100 min. hypoxia paradigm. Brain tissues from Control and Control+Cerebrolysin groups were positive for TTC staining in all observed areas. Also, animals with HIE administered with Cerebrolysin showed positive staining. In contrast, no staining was observed in the cortex, hippocampus, corpus callosum, and caudate-putamen of the ipsilateral hemisphere to the HIE group and a few contralateral cortex regions (Figure 2A). Besides, the HIE group showed structural alterations consisting of edema and tissue liquefaction in the ipsilateral lesioned area 7 days post-HI injury. Interestingly, Cerebrolysin also partially prevented the HI-induced brain tissue damage because the anatomical structure of the brains was similar to the Control and Control+Cerebrolysin groups (Figure 2B).

3.1 CHANGES IN BODY WEIGHT AND EYE-OPENING DURING GROWTH

The hypoxic-ischemic impact on rat growth was evaluated through the body weights from neonatal PD7 to adulthood at PD60 and expressed as BWG on PD7. BWG showed no significant changes among experimental groups from PD7 to PD35. However, in adulthood (PD60), the untreated HIE (247 ± 7.5 g) and treated with Cerebrolysin (225 ± 17.1 g) groups showed a significantly lower BWG compared with the Control (297 ± 10.4 g) and Control+Cerebrolysin (265 ± 5.9 g) groups (Figure 2C). Eye-opening no significant differences among the groups were found; generally, this event occurred on PD14 in 50% of all animals and, the rest of the pups completed the process on PD16 (Figure 2D).

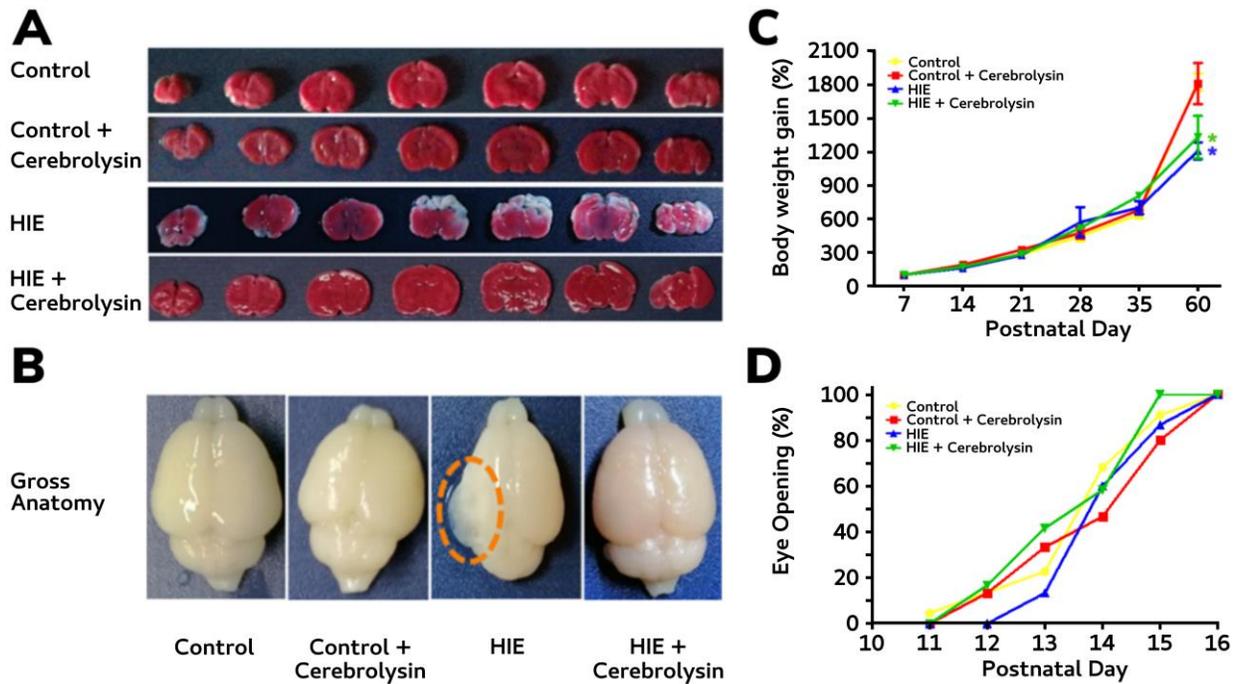


Figure 2. Therapeutic effect of Cerebrolysin on neonatal HIE. A) Brain tissue damage, all brain slices were stained with TTC at 3 days post-HI insult. B) Brain macroscopic structure 7 days post-HI damage. Orange dotted lines indicate liquefaction and edema areas. C) Body weight gain (BWG). Data are expressed as mean \pm SEM. * = $p < 0.001$ vs Control and Control+Cerebrolysin groups. D) Eye-opening patterns during postnatal development are expressed as percentages.

3.2 CHANGES IN THE BRAIN WATER CONTENT AND SODIUM FLUORESCHEIN PERMEABILITY

Brain edema was evaluated using brain water content. In the ipsilateral hemisphere, water content was $83.79 \pm 0.2 \%$ and $83.54 \pm 0.5 \%$ in the Control and Control+Cerebrolysin groups, respectively; while, in the ipsilateral hemisphere of the HIE group was $91.7 \pm 3.59 \%$, as expected, Cerebrolysin treatment decrease the brain water content in pups with HIE to $84.55 \pm 0.34 \%$ (Figure 3A), showing statistically significant differences between the HIE groups versus controls and treatment groups ($p = 0.0004$). Similar results were observed in the contralateral hemisphere, herein, brain water content was $83.12 \pm 0.5 \%$ and $83.64 \pm 0.5 \%$ in Control and Control+Cerebrolysin groups, respectively; while, in the HIE group the brain water content increased to $91.95 \pm 3.6 \%$, and Cerebrolysin treatment reduced it to $84.55 \pm 0.34 \%$ (Figure 3B), showing statistically significant

differences between the HIE groups versus controls and treatment groups ($p = 0.0002$).

On the other way, BBB permeability was evaluated using the extravasation of the NaFl tracer in the cerebral cortex. After allowing the blood circulation of NaFl for 2 h, rats with HIE showed an increase in the concentration of this tracer in the ipsilateral cortex compared with the Control ($p=0.02$) and Control+Cerebrolysin ($p=0.02$) groups; while, Cerebrolysin administration after HI injury diminished extravasation of NaFl ($p=0.0419$). In this case, the HIE group showed a statistically significant difference from the other groups ($p=0.0089$; Figure 3C). In contrast, NaFl extravasation occurred to a lesser extent in the contralateral cortex; herein, the HIE group showed a tendency to increase NaFl extravasation, which did not reach statistical significance compared to all other groups ($p = 0.07$; Figure 3D).

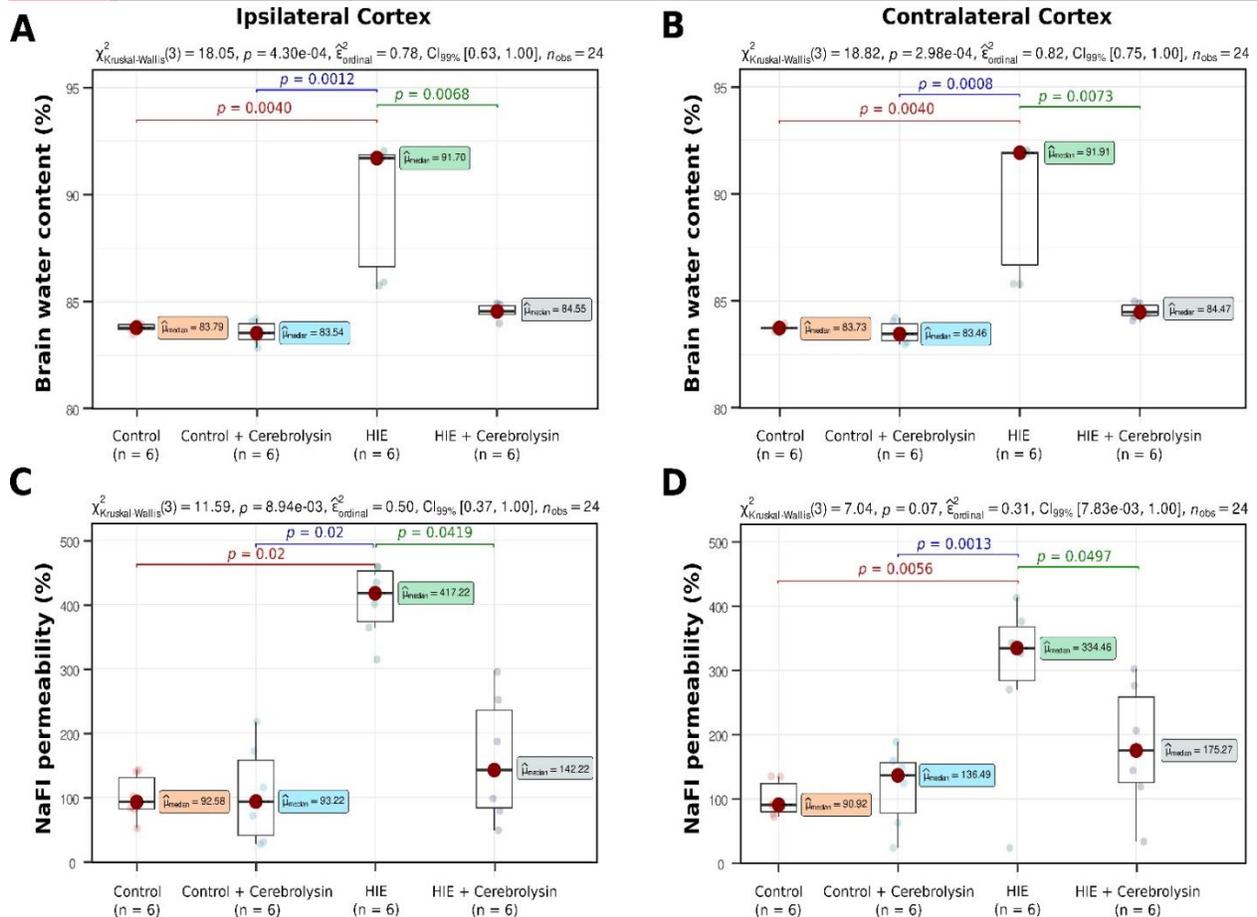


Figure 3. Therapeutic effect of Cerebrolysin on BBB permeability 7 days after HI injury. Brain water content in ipsilateral (A) and contralateral hemisphere (B). Data are expressed as median \pm SEM. NaFl permeability in ipsilateral (C) and contralateral (D) cerebral cortex. Data are expressed as median \pm SEM and normalized with respect to the control group.

3.3. SODIUM FLUORESCINE PERMEABILITY AND THE BRAIN WATER CONTENT

To determine whether there was a correlation between total brain water content and BBB permeability to NaFl, Spearman's analysis was performed for the ipsilateral and contralateral hemispheres. Our results showed that there was no correlation between the analyzed parameters, since in the ipsilateral region it was obtained that the control group had $r = 0.2571$ and $p = 0.6583$,

Control+Cerebrolysin $r = 0.1429$ and $p = 0.8028$, EHI $r = 0.0857$ and $p = 0.9194$; and HIE+Cerebrolysin $r = 0.6571$ and $p = 0.1750$; whereas, in the contralateral region, it was observed that the control group had $r = -0.1429$ and $p = 0.8028$, Control+ Cerebrolysin $r = -0.6571$ and $p = 0.1750$, EHI $r = -0.7143$ and $p = 0.1361$; and EHI+Cerebrolysin $r = 0.2000$ and $p = 0.7139$ (Figure 4).

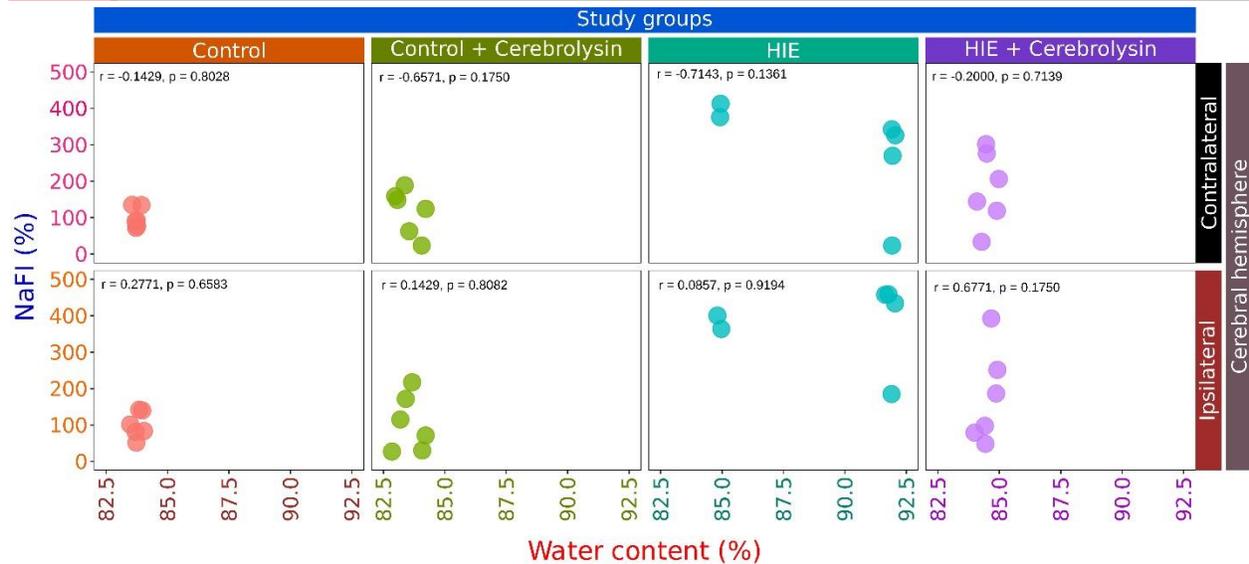


Figure 4. Correlation analysis between brain water content and NaFl permeability in ipsilateral and contralateral regions.

3.4 CHANGES IN MOTOR ACTIVITY AND COGNITIVE DEFICITS

Motor activity impairment was assessed at PD10 throughout the ambulation test, ambulation angle, front-limb suspension, grip strength, and righting reflex. In addition, gait stability was evaluated through the ambulation score and ambulation angle. The ambulation score (Figure 5A), hanging impulse (Figure 5C) and righting reflex analyses yield no statistical differences among all studied groups at PD10. Only the ambulation angle was significantly increased in the HIE group ($70.1 \pm 1.2^\circ$; $p < 0.05$)

when compared with the Control group ($60.4 \pm 0.7^\circ$) and the Control+Cerebrolysin group ($60.9 \pm 2.5^\circ$). However, the Cerebrolysin treatment significantly reduced the HIE-induced rise to control values ($60.6 \pm 2.6^\circ$, Figure 5B). In addition, the front-limb suspension test showed that HIE significantly decreased the latency time (7.9 ± 0.3 s; $p < 0.05$) compared with the Control group (12.7 ± 1.1 s) and the Control+Cerebrolysin (14.7 ± 1.9 s). Again, the Cerebrolysin treatment reset the latency time observed in HIE groups to a control value (12.7 ± 0.8 s; Figure 5D).

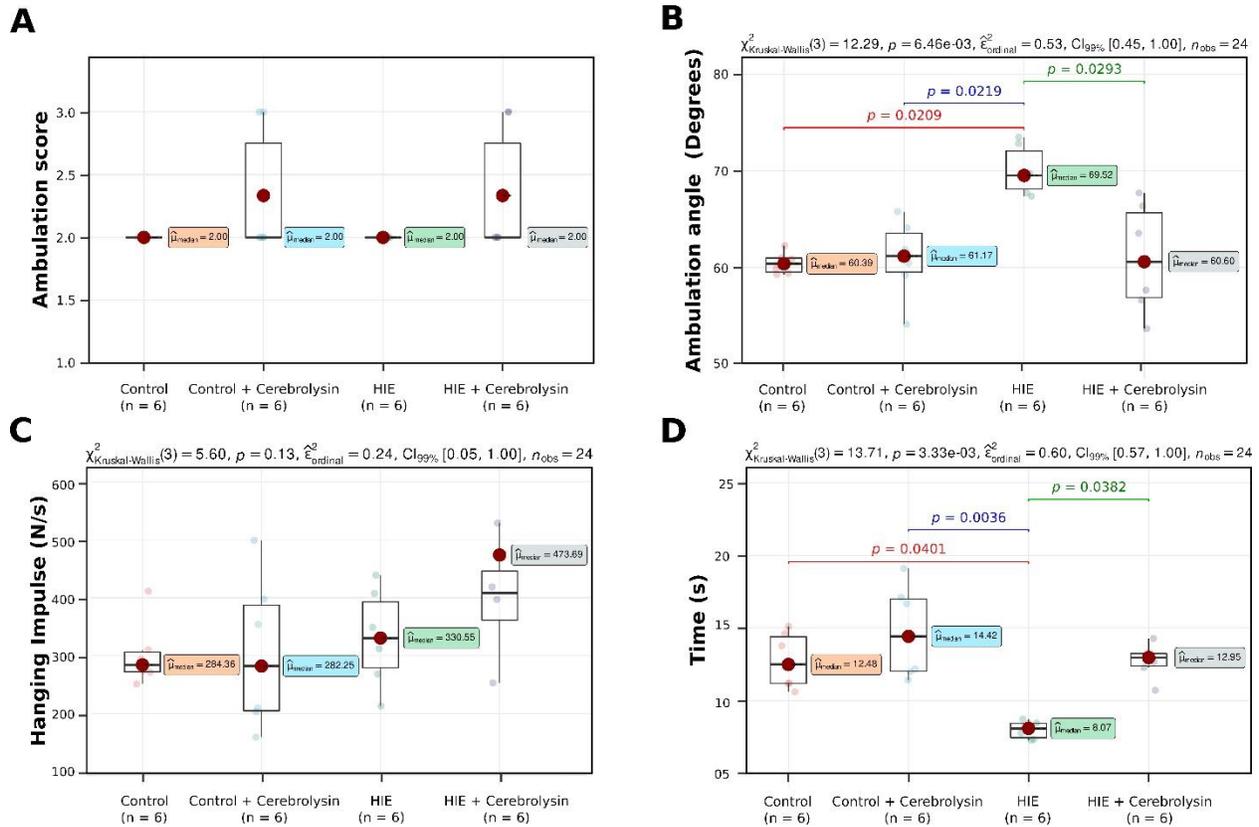


Figure 5. Therapeutic effect of Cerebrolysin on motor activity after neonatal HIE. A) Ambulation score, B) Ambulation angle, C) Hanging impulse, D) Front-limb suspension. All data are expressed as median \pm SEM.

3.5. NOVEL OBJECT RECOGNITION TEST

The novel object recognition test (NOR) was performed to determine if the therapeutic Cerebrolysin administration after a HI injury avoids the cognitive processes deficits such as memory and learning (Figure 6A). This test showed no short-term memory alterations (1 hour post-familiarization phase) in all studied groups. However, in the long-

term memory (24-hours post-familiarization phase), the HIE group showed a negative discrimination index (-0.43 ± 0.07 ; $p < 0.05$) compared with the Control group (0.32 ± 0.08) and the Control+Cerebrolysin group (0.55 ± 0.22). Again, the Cerebrolysin treatment reset the discrimination index, altered by hypoxia-ischemia (0.31 ± 0.12) (Figure 6B).

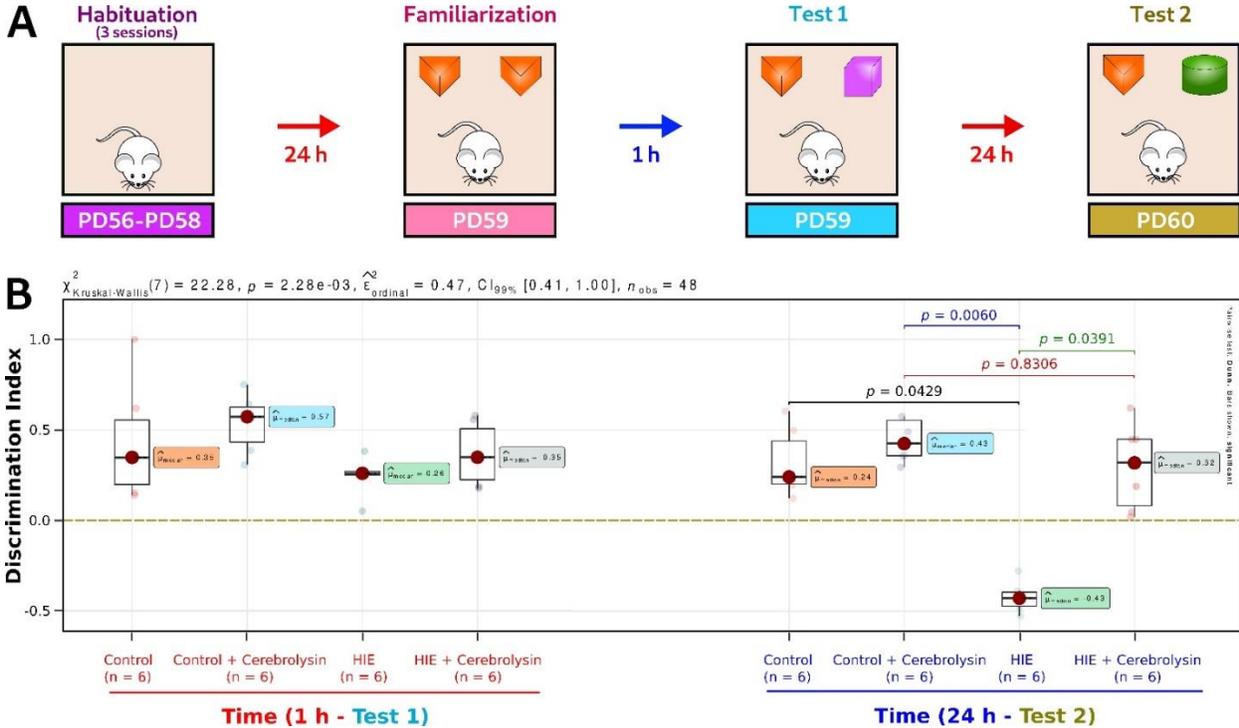


Figure 6. Therapeutic effect of Cerebrolysin on HIE-induced cognition and memory deficits evaluated by the novel object recognition test (NOR) in adulthood. A) NOR test scheme. B) Discrimination index in the test trials at 1 and 24 h post-familiarization. Data are expressed as median \pm SEM.

4. Discussion

For the first time, this work shows that the early Cerebrolysin treatment starting 24 hours after the HI injury and continued during 7 days, reversed the long-term memory acquisition deficit observed in untreated adult rats subjected to HIE. An increase in cell viability can account for the Cerebrolysin therapeutic effect because the TTC vital staining did not reveal the HIE-induced necrotic and ischemic cerebral zones in the treated animals. The cortex, hippocampus, corpus callosum, and caudate putamen were the infarct target (Figure 2A) in agreement with previous reports¹⁷⁻¹⁹. Our results also confirmed that the lesion pattern occurred mainly in the ipsilateral side of ischemic insult and showed that the contralateral side was also affected mainly in the cortical area close to ipsilateral infarction zones. The collateral affection can be caused by an extension of the persistent membrane depolarization, edema, and changes in lipid distribution from hypoxia-ischemia affected areas. This cerebral damage can cause the motor and intellectual disabilities shown here and previously reported^{19,20}.

Remarkably, the Cerebrolysin treatment prevented the infarcted area development. In the HIE group, gross brain anatomy visibly altered on day 7 post-HI injury induction, showing a wide edema zone and liquefaction (Figure 2B). The absence of these alterations by the Cerebrolysin treatment indicates

that the treatment prevents liquefaction necrosis. Previous reports have shown in adult cerebral stroke that brain tissue undergoes liquefactive necrosis by increasing the brain tissue enzymatic digestion of metalloproteinases such as MMP-2, MMP-3, MMP-8, and MMP-9, accompanied by foamy cells accumulation. These cells have intracellular and extracellular content of cholesterol crystals in the infarct zone during 4-8 weeks and are known to induce a chronic inflammatory response²¹. Therefore, the beneficial effect of Cerebrolysin on brain liquefaction can be explained by its ability to down-regulate MMP-9 expression and induce VEGF overexpression, as shown in a model of traumatic brain damage²².

In our HIE model, animals developed brain edema in the ipsilateral hemisphere until 7 days post-HI insult. Brain edema results from the loss of water homeostasis due to impaired transport in cells and blood vessels; and it is classified in cellular edema and vasogenic edema. In cellular edema there is an influx of water into the cells due to the increase in the activity of aquaporins while, in cytotoxic edema, water accumulates in the extracellular space at least in part due to impairment of the BBB^{23,24}. Cellular edema appears in the early stages of damage in HIE; accumulated evidence supports an increase in the expression of aquaporin-4 after HI injury, which is related to the influx of water into the cells, such as astrocytes. This event contributes to the

severity of the damage, as demonstrated in HIE, stroke, and intracranial hemorrhage^{25–28}. Aquaporin-4 is the most abundant water channel in the CNS and is primarily located in the perivascular foot-processes of astrocytes, glial membranes, and ependymal cells²⁹. Due to the increase in the expression of aquaporin-4 in astrocytes, astrocytic swelling occurs in HIE, which generates morphological changes, such as cell enlargement. However, in the late phases of HIE, vasogenic edema is generated caused by BBB disruption accompanied by mitochondrial damage and cell death^{26,27,30}. BBB is a dynamic and highly selective barrier that regulates the passage of hydrophilic molecules into the CNS. Its cellular components are pericytes, endothelial cells, astrocytes, and microglia which form a functional unit due to the interactions they establish with the components of the basement membrane and extracellular matrix. The effect of HI injury on the BBB generates changes in angiogenesis and permeability, which modify water transport and cell volume, and constitute the pathophysiology of brain edema in HIE; in this context, the function of the BBB plays a crucial role. In this study, Cerebrolysin administration attenuated the structural damage in the ipsilateral hemisphere. This suggests the preservation of the BBB integrity and, therefore, the decrease in water accumulation demonstrated by the total brain water content and extravasation of the NaFl tracer in the ipsilateral cortex 7 days post-HI injury (Figure 3). One of the effects of the administration of Cerebrolysin when starting the treatment 24 hours after the damage is probably the reduction in the expression of aquaporin-4, avoiding astrocytic swelling, which is an important event for water balance in the early phase, while in the late phase, Cerebrolysin would maintain the integrity of the BBB and consequently, its selectivity, as demonstrated by decreasing the permeability of NaFl in the ipsilateral cortex after continuous administration, both events contribute to reducing the appearance of cytotoxic and vasogenic edema. This effect has been documented in intracranial hemorrhage, where downregulation of aquaporin-4 in the peripheral region of the hematoma by Cerebrolysin treatment at a dose of 5 mL/kg in rats prevented astrocyte enlargement and exerted a protective effect against injury²⁸. Furthermore, Cerebrolysin maintains the BBB integrity inducing overexpression of Claudin-5, ZO-1, and occludins in the tight junction; through this mechanism, Cerebrolysin avoids the perivascular space expansion and tight junction opening to reduce brain edema²⁸. Altogether, these data indicate that Cerebrolysin in neonatal rats with HIE prevent cellular and vasogenic edema development by maintaining and recovery of the BBB selectivity and structure (Figure 3). Moreover, Cerebrolysin

administration to healthy pup rats did not affect the cell viability or modify gross brain structure. These data support the Cerebrolysin safe use in immature brains as found in different disease models such as stroke in adult animals, even in older adult rats, and cardiac neuropathy^{6,10,31–33}.

Besides, we found that HIE retarded growth development at PD60, which was not recoverable with Cerebrolysin treatment compared with the control group (Figure 2C). These results agree with other reports on growth deficits after HIE in rat pups at PD3 under 90-min hypoxia and in ischemic stroke models in adult rats where Cerebrolysin did not affect weight loss^{18,32,34}. Also, the Cerebrolysin treatment did not affect the eye-opening age compared with the control groups (Figure 2D). In addition, the HIE produced lethargy and hemiparesis evident after the injury. Given this scenario, motor activity tests were used to assess the effects of HIE and Cerebrolysin treatment. In the motor activity test, animals with HIE increased their ambulation angle to maintain gait stability, and Cerebrolysin treatment normalized this parameter. These adjustments improved the motor coordination reflected in the gait stability of HIE animals treated with Cerebrolysin (Figure 5 panels A and B), as previously shown in hypoxic models in adult animals³⁵. Also, the Cerebrolysin administration improved the front limb muscular strength decreased by HIE at PD10 (Figure 5D). This test also assesses forelimb strength, including arm and paw strength¹⁵. Furthermore, the prevention of muscle tone loss in forelimbs at PD10 agrees with the previous observation made in a stroke adult model, where the Cerebrolysin administration improves fine motor skills through IP3K/Akt pathway activation³⁵. Finally, it is worth mentioning that our HIE model did not modify grip strength, which assesses the strength of all 4 paws simultaneously, nor the righting reflex, which evaluates the vestibular system function (Figure 5C). Nevertheless, our findings are consistent with previous reports in similar HIE neonatal animal models and are in line with findings reported in patients with cerebral palsy, in which the gross motor function was improved with Cerebrolysin intramuscular therapy^{36,37}.

We propose that the Cerebrolysin treatment could stabilize the gait and improve motor skills in animals with HIE because it attenuated the structural damage in the ipsilateral hemisphere to the ischemic insult and conserved the BBB integrity. These neuroprotective effects of Cerebrolysin could result from reducing the oxidative stress and inflammatory process or activating anti-apoptotic pathways^{38–40}. Besides, the anti-apoptotic effect could explain the significant decrease in infarct volume after

Cerebrolysin administration in HIE neonatal rats, implying a more remarkable cell survival in the cerebral cortex and hippocampus, essential brain areas that participate in cognitive processes such as learning and memory.

Accordingly, the severe HIE caused cognitive deficits and markedly working memory deterioration, which were entirely reverted by the Cerebrolysin treatment (Figure 6). Cerebrolysin could improve the performance of the HIE rat in the novel object recognition test by activating different neural circuitries. This test is based on innate recognition ability that does not require reward or punishment to stimulate the exploration activity and directly depends on the activation of neural networks between the neocortex, sensorimotor cortex, hippocampus, and striatum^{41,42}. Animals treated with Cerebrolysin were able to remember the familiar object. For this reason, they explore the novel object for a longer time at 24hrs post-familiarization, indicating that their working memory is functional. In contrast, the HIE animals only recognize the familiar object in the short-term period, but not after 24hrs. This discrepancy could be explained because HIE did not affect some critical structures in the short-term recognition, such as the entorhinal and perirhinal cortex, but did alter the hippocampus, as shown by the TTC viability staining. This finding emphasizes the importance of the hippocampus in the long-term recognition processes and working memory formation that also requires other structures such as the entorhinal, perirhinal, and parahippocampal region⁴³⁻⁴⁶. Those regions are structurally and functionally preserved by the Cerebrolysin treatment in this HIE model. Furthermore, clinical studies have shown that Cerebrolysin treatment in infants suffering from HI improves communication and language development, positively impacting their social skills¹³. Our results demonstrate that the opportune Cerebrolysin treatment of neonatal HIE promotes the establishment and maintenance of learning and memory in adulthood, preventing structural and functional damage to the hippocampus. Furthermore, these data support the therapeutic use of Cerebrolysin in neonatal HIE and provide

information on long-term treatment effects. Future research is necessary to determine the molecular mechanisms involved in these effects.

5. Conclusions

Based on international administration regulation, the early, continuous, and prolonged treatment of neonatal HIE with Cerebrolysin improves some motor skills in the short term and avoids learning and memory sequels in the long term.

Conflicts of Interest:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Institutional Review Board Statement:

The protocol was registered in the Regional Hospital "Primero de Octubre" with number 1078/16, and was evaluated by the National Research Committee, Ethics Committee, and Biosafety Committee of ISSSTE, and approved with the RPI-233.2017 number on March 31, 2017. The Institutional Committee of CINVESTAV approved all experimental procedures for the Care and Use of Laboratory Animals with the number 0220-16 according to the NOM-062-ZOO-1999 Official Mexican Standard.

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