Improvement in functional recovery with administration of Cerebrolysin after experimental closed head injury

Laboratory investigation

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Object. Cerebrolysin is a unique peptide preparation that mimics the action of neurotrophic factors. This study was designed to investigate the effects of acute treatment of experimental closed head injury (CHI) in rats with Cerebrolysin on neurological function.

Methods. Adult male Wistar rats (n = 60) were subjected to impact acceleration–induced CHI. Closed head injured rats received intraperitoneal injection of saline (n = 30) or Cerebrolysin (2.5 ml/kg, n = 30) starting 1 hour postinjury and administered once daily until they were killed (2 or 14 days after CHI). To evaluate functional outcome, the modified neurological severity score (mNSS), foot fault, adhesive removal, and Morris water maze (MWM) tests were performed. Animals were killed on Day 14 (n = 20) after injury, and their brains were removed and processed for measurement of neuronal cells, axonal damage, apoptosis, and neuroblasts. The remaining rats (n = 40) were killed 2 days postinjury to evaluate cerebral microvascular patency by fluorescein isothiocyanate (FITC)–dextran perfusion (n = 16) and to measure the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase–9 (MMP-9) by using real-time reverse transcriptase-polymerase chain reaction (RT-PCR, n = 8) and by immunohistochemical analysis (n = 16).

Results. At 14 days post-CHI, the Cerebrolysin treatment group exhibited significant improvements in functional outcomes (the adhesive removal, mNSS, foot-fault, and MWM tests), and significantly more neurons and neuroblasts were present in the dentate gyrus (DG) (p < 0.05) compared with the saline-treated group (p < 0.05). At 2 days post-CHI, the Cerebrolysin group exhibited a significantly higher percentage of phosphorylated neurofilament H (pNF-H)–positive staining area in the striatum (p < 0.05), a significant increase in the percentage of FITC-dextran perfused vessels in the brain cortex (p < 0.05), a significant increase in the number of VEGF-positive cells (p < 0.05), and a significant reduction in the MMP-9 staining area (p < 0.05) compared with the saline-treated group. There was no significant difference in mRNA levels of MMP-9 and VEGF in the hippocampus and cortex 48 hours postinjury between Cerebrolysin- and saline-treated rats that sustained CHI.

Conclusions. Acute Cerebrolysin treatment improves functional recovery in rats after CHI. Cerebrolysin is neuroprotective for CHI (increased neurons in the dentate gyrus and the CA3 regions of the hippocampus and increased neuroblasts in the dentate gyrus) and may preserve axonal integrity in the striatum (significantly increased percentage of pNF-H–positive tissue in the striatum). Reduction of MMP-9 and elevation of VEGF likely contribute to enhancement of vascular patency and integrity as well as neuronal survival induced by Cerebrolysin. These promising results suggest that Cerebrolysin may be a useful treatment in improving the recovery of patients with CHI.

(1)This article contains some figures that are displayed in color online but in black-and-white in the print edition.
months after the injury.33 Several biochemical derangements are responsible for secondary injury, including perturbation of cellular calcium homeostasis, increased free radical generation and lipid peroxidation, mitochondrial dysfunction, inflammation, apoptosis, upregulation of MMP-9, and diffuse axonal injury.28,60,65 However, most TBI clinical trials that target a single pathophysiological pathway have failed.42 To date, there is no effective pharmacological therapy available for TBI.36 Thus, it is likely that successful therapy may require targeting multiple injury pathways.

Recent studies indicate that growth and neurotrophic factors, including BDNF, GDNF, NGF, CNTF, and IGF, administered alone or in combination for experimental brain and spinal cord injury, improve functional outcome.35,55 Cerebrolysin is a mixture of low-molecular-weight neuropeptides derived from purified brain proteins by standardized enzymatic proteolysis, with proposed neuroprotective and neurotrophic properties similar to naturally occurring growth and neurotrophic factors.48 For example, BDNF not only stimulates axonal sprouting40 but also enhances the proliferation and differentiation of neural progenitor cells in the DG of the hippocampus after focal stroke,64 which may contribute to the functional recovery. Other studies suggest that Cerebrolysin has a neuroprotective effect, limiting neuronal dysfunction, maintaining the structural integrity of neurons under detrimental conditions,64 promoting neurogenesis, and improving functional outcome after stroke in rats.60 The efficacy and safety of Cerebrolysin have been demonstrated in recent clinical trials, including those for stroke, TBI, and Alzheimer disease.16 These results indicate that Cerebrolysin is a promising multifunctional neuropeptide preparation for acute and chronic neurological diseases and injuries. Until now, only one preclinical study investigated the effects of acute Cerebrolysin treatment in a stab injury model of TBI in rats.86 Closed head injury is the leading cause of death and disability in young patients in industrialized countries.54 However, the efficacy of Cerebrolysin treatment for CHI has not been investigated. Here, we used a well-established clinically relevant Marmarou impact acceleration rat model to induce CHI, and we investigated the effect of Cerebrolysin on neurological function and the mechanisms underlying its therapeutic activity.

Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Health System.

Closed Head Injury Model

The Marmarou impact acceleration device (Custom Design & Fabrication South, LLC) was used to induce CHI in rats in this study.38 Male Wistar rats weighing 380–430 g (Charles River Breeding Co.) were used in our experiments. The animals went through an appropriate period (1 week) of quarantine and acclimation. Anesthesia in the rats was initially induced with 4% isoflurane and maintained with 1.0%–1.5% isoflurane in 70% N2O and 30% O2 throughout the surgical period via a 14-gauge endotracheal intubation tube (Rat ET tube, Hallowell EMC) where animals were mechanically ventilated using small animal ventilator (Model 683, Harvard Apparatus). Rectal temperature was maintained at 37 ± 0.5°C throughout the surgical procedure using a feedback-regulated water heating system. The skin over the cranial vault was shaved and swabbed with Betadine and 70% alcohol. A 2-cm midline incision was made using a scalpel, and the skull was exposed using 2 cotton-tipped applicators to gently push the periosteum of the skull to the most lateral edges of the incision, applying gentle pressure to absorb bleeding and dry the exposed skull. The scored side of the stainless steel disk (10 mm in diameter and 3 mm in thickness) was coated with cyanoacrylic glue and mounted on the parietal bone midline between bregma and lambda, straddling the sagittal suture. After the glue dried for 5 minutes, the animal was placed prone on the foam bed, under the hollow Plexiglas tube, and was secured by strapping surgical tape over the dorsal surface attached to either side of the Plexiglas bed. Closed head injury was induced by dropping the cylindrical column of segmented brass (450 g) through the Plexiglas tube from a distance (2 m) onto the disc fixed to the skull vault of the animal. Rebound impact was prevented simply by sliding the Plexiglas box (foam bed) containing the animal away from the tube immediately following the initial impact. To prevent skull fractures, a small stainless steel helmet-disk was placed on the rodent skull while the animal was supported by the foam bed. The metal helmet was removed and the incision was closed with sterile 4-0 suture. Rats that died on impact and those with skull fractures were excluded from the study.

Experimental Groups and Treatment

For the 14-day survival study, 20 rats were divided into 2 groups (10 rats/group): the CHI + Cerebrolysin group and the CHI + saline (vehicle) group. Cerebrolysin or saline at 2.5 ml/kg was administered intraperitoneal once daily for 14 days, starting at 1 hour after CHI. Animals treated with saline (vehicle) were used as a control group. All rats were allowed to survive 14 days after CHI. For the 2-day survival study, 40 rats were divided into 2 groups (20 rats/group): the CHI + Cerebrolysin group and the CHI + saline (vehicle) group. Among the 20 rats in each group, 8 were used for immunostaining, 8 for FITC-dextran perfusion, and 4 for gene expression. Cerebrolysin at 2.5 ml/kg or saline was administered intraperitoneal once daily starting at 1 hour after CHI. All rats were allowed to survive 2 days after CHI.

Evaluation of Neurological Outcome

Morris Water Maze Test. All functional tests were administered by investigators blinded to the treatment status. To detect spatial learning impairments, a recent version of the MWM test was used.11 The procedure was modified from previous versions40 and has been found to be useful for spatial memory assessment in rodents with brain injury.11 Animals were tested during the last 5 days (that is, 10–14 days after CHI) before sacrifice. Data col-
Acute Cerebrolysin treatment for closed head injury

lection was automated using the HVS Image 2020 Plus Tracking System (US HVS Image), as described previously.\(^9\) The advantage of this version of the water maze is that each trial takes on the key characteristics of a probe trial because the platform is not in a fixed location within the target quadrant. In the traditional version of the MWM test, the position of the hidden platform is always fixed and is relatively easy for rodents to find. With the modified MWM test used in our study, the platform is relocated randomly within the correct quadrant with each training trial. The rodents spend additional time searching for the platform within the target quadrant; therefore, each trial effectively acts as a probe trial. The advantage of this protocol is that rodents find the platform purely and extensively by reference to extramaze spatial cues, which improves the accuracy of spatial performance in the MWM.\(^1\)

Foot-Fault Test. To evaluate sensorimotor function, the foot-fault test was carried out before TBI and at 1, 7, and 14 days after TBI. The rats were allowed to walk on a grid. With each weight-bearing step, a paw might fall or slip between the wires and, if this occurred, it was recorded as a foot fault.\(^4\) A total of 50 steps were recorded for both left and right forelimbs and hindlimbs.

Modified Neurological Severity Score Test. Neurological functional measurement was performed using the mNSS test.\(^10\) The test was carried out on all rats preinjury and on Days 1, 7, and 14 after TBI. The mNSS is a composite of the motor (muscle status and abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex tests and has been used in previous studies.\(^36\) Neurological function was graded on a scale of 0–18 (normal score 0; maximal deficit score 18). In the severity scores of injury, 1 point is awarded for each abnormal behavior or for the lack of a tested reflex. Thus, the higher score, the more severe is the injury.

Adhesive Removal Test. To evaluate the somatosenory deficits during the 14-day recovery after CHI, the adhesive removal test was administered before and after surgery.\(^9\) Two small pieces of adhesive-backed paper dots of equal size (113.1 mm\(^2\)) were used as bilateral tactile stimuli occupying the distal-radial region of each forelimb. The time to remove each stimulus from forelimbs was recorded during 3 trials per day. Individual trials were separated by at least 5 minutes. Before surgery, the animals were trained for 3 days. Then, this test was conducted before CHI (baseline), and at 1, 7, and 14 days after CHI. The mean time required to remove both stimuli from limbs was recorded.

Tissue Preparation

The rats were anesthetized with chloral hydrate administered intraperitoneally and were perfused transcardially with saline solution, followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Rat brains were removed and immersed in 4% paraformaldehyde for 4 days. Using a rat brain matrix (Activational Systems, Inc.), each forebrain was cut into 2-mm-thick coronal blocks for a total 7 blocks from bregma 5.2 mm to bregma –8.8 mm per animal.\(^47\) The tissues were embedded in paraffin, and a series of 6-µm-thick slides were cut.

Immunohistochemical Analysis

To examine the effect of Cerebrolysin on neuroblasts in the DG, immature neurons were detected by immunostaining with an anti-DCX antibody (1:200, Santa Cruz Biotechnology). To evaluate the protective effect of Cerebrolysin on neuronal death and axonal damage, immunostaining was performed with mouse anti-NeuN monoclonal antibody (1:300, MAB 377, Chemicon) and pNF-H (1:1000, Covance SMI 31R) antibodies, respectively. In situ apoptotic cells were detected by the TUNEL method (ApopTag Peroxidase in Situ Apoptosis Detection Kit, Millipore). The TUNEL method is based on the specific binding of terminal deoxynucleotidyl transferase to 3’-OH ends of DNA and the ensuing synthesis of polydeoxynucleotide polymer. The staining was performed according to the procedures provided by the manufacturer. The MMPs are proteases that cleave the extracellular matrix, including major components of the basal lamina and tight junctions between endothelial cells. This proteolytic cleavage results in BBB disruption and edema.\(^28\) Vascular endothelial growth factor plays an important role in neurovascular remodeling after TBI.\(^58,59\) For measurement of brain tissue VEGF and MMP-9, immunostaining was performed using goat anti-VEGF (1:200; sc-1836, Santa Cruz) and goat anti-MMP-9 (1:200; sc-6840 Santa Cruz) antibodies, respectively.

Measurement of Microvascular Patency

To examine the patency of cerebral microvessels, FITC-dextran (2 × 10\(^6\) molecular weight, Sigma, 50 mg per rat) was administered intravenously to rats 2 days after CHI. Rats were killed 5 minutes after FITC-dextran injection. Three coronal sections (100 µm, including striatum and hippocampus), corresponding to coronal coordinates for bregma –1 to 1 mm and –2.5 to –4.5 mm from each rat, were digitized and analyzed using the MCID system, as described previously.\(^30\) Data are presented as the numbers of FITC pixels divided by the total numbers of pixels within the field of view, expressed as a percentage.

RNA Preparation and RT-PCR

To examine the effect of Cerebrolysin on expression of genes that mediate microvascular integrity and neuronal damage, mRNA levels of Ang1, VEGF, and MMP-9 were measured by real-time RT-PCR analysis. Total RNA was isolated from brain cortex and hippocampus tissues using RNeasy Mini kit (catalog no. 74104, Qiagen). Genomic DNA was digested by performing on-column DNase digestion using RNeasy-free DNase set (catalog no. 79254, Qiagen). Quantitative RT-PCR was performed using SYBR Green real-time PCR method, in which power SYBR Green PCR master mix (catalog no. 4367659, Invitrogen) was used. The RNA concentration and purity were assayed using an ultraviolet-visible recording spectrophotometer (Shimadzu). One microgram of total RNA was used in 20 µl of reverse transcript reaction using SuperScript III First-Strand system...
for RT-PCR kit (catalog no. 18080–051, Invitrogen). Two microliters of RT reaction product was used in 20 µl of quantitative RT-PCR. Quantitative RT-PCR was performed on an ABI ViiA 7 PCR instrument (Applied Biosystems) using default program parameters provided by the manufacturer for 40 cycles. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during PCR. Each sample was tested in duplicate, and samples obtained from 4 animals for each group were used for analysis of relative gene expression using the 2−ΔΔCT method.32 Analysis of relative gene expression data was done using real-time quantitative PCR and the 2−ΔΔCT method.

The following primers were used in RT-PCR: MMP-9-F 5ʹ-CCACCGAGCTATCCACTCAT-3ʹ, MMP-9-R 5ʹ-GTCCGGTTTCAGCATGTTTT-3ʹ; Ang1-F 5ʹ-TGTGGTGGTGGCTGGAT-3ʹ, Ang1-R 5ʹ-CTTGGGCCTTG-GATCATAGTG-3ʹ; VEGF-F 5ʹ-TGCTGTCCTGGGACTGG-3ʹ, VEGF-R 5ʹ-CGGCGCTTGCCGAGTTCT-3ʹ; GAPDH-F 5ʹ-AGAGAGAGGCCCTCAGTTGCT-3ʹ, GAPDH-R 5ʹ-TGTGAGGGAGATGCTCAGTGT-3ʹ.

**Cell Counting and Quantitation**

For measurement of NeuN–, VEGF–, and TUNEL-positive cells, and pNF-H- and MMP-9-positive areas, fields of view from coronal sections of the rat brain encompassing the striatum and hippocampus were digitized using a ×20 objective via the MCID computer imaging analysis system (Fig. 1). The numbers of apoptotic cells, NeuN-positive cells, VEGF-positive cells, or the positive area of pNF-H and MMP-9 in the brain regions were counted throughout each field of view. The TUNEL-, VEGF-, and NeuN-positive cells were expressed in square millimeters. Cell counts were performed by observers blinded to the individual treatment status of the animals. All counting was performed on a computer monitor to improve visualization and in 1 focal plane to avoid oversampling. For pNF-H and MMP-9 staining, the positive area was measured and expressed as the percentage of area.

**Statistical Analysis**

All data are presented as the mean ± SD. An independent-samples t-test was used for testing immunostaining between the saline- and Cerebrolysin-treated groups. Data on foot fault, adhesive removal, mNSS, and spatial learning were analyzed by ANOVA for repeated measurements followed by the post hoc Student-Newman-Keuls test to compare the difference between the Cerebrolysin-treated and saline-treated groups. Statistical significance was set at p < 0.05.

**Results**

**Cerebrolysin Administration Is Associated With Significantly Reduced Sensorimotor Deficits After CHI**

To evaluate sensorimotor function recovery, adhesive removal, foot fault, and mNSS tests were performed before and after CHI. Compared with the saline group, Cerebrolysin significantly reduced adhesive removal time from Days 7 to 14 after CHI (Fig. 2A, p < 0.05) and also decreased foot-fault frequency from Days 1 to 14 after CHI (Fig. 2B, p < 0.05). The higher the mNSS, the worse the neurological function. No functional deficits were found in rats prior to CHI (Fig. 2C). However, rats receiving Cerebrolysin at 1 hour after CHI had significant improvement in the mNSS (that is, reduced mNSSs) compared with saline controls at Days 7 and 14 (p < 0.05).

**Cerebrolysin Administration Is Associated With Significantly Improved Spatial Learning After CHI**

To detect spatial learning deficits, the modified MWM test was used in the present study. The more time the rat spends in the correct quadrant, the better
the spatial learning. As shown in Fig. 2D, from Days 10 to 14 after CHI, the time spent in the correct quadrant by saline-treated rats gradually increased over time without a significant difference compared with Day 10 (p > 0.05). Cerebrolysin-treated rats showed significant improvement in spatial learning performance compared with the saline group from Days 11 to 14 after CHI (p < 0.05). The less time it takes for the rat to reach the hidden platform in the MWM compared with the saline group from Days 11 to 14 after CHI. There was no significant difference in swim speed between the groups, indicating that motor deficits did not contribute to cognitive deficits. There were 10 rats/group. D = day; Pre = preinjury. Data represent the mean ± SD. *p < 0.05 vs saline.

Cerebrolysin Administration Is Associated With Significantly Reduced Neuronal Loss in the DG and CA3 After CHI

Anti-NeuN staining was performed to explore the protective effect of Cerebrolysin on neuronal cells in the DG and CA3. The number of NeuN-labeled cells per square millimeter was significantly higher in the Cerebrolysin-treated group than in the saline group after CHI when examined at 2 days (7222 ± 1005 vs 5503 ± 1060 for DG, p < 0.05; 2538 ± 629 vs 1706 ± 184 for CA3, p < 0.05) and 14 days (Fig. 3, p < 0.05) post-CHI. There was no significant neuronal cell loss found in other regions, including the cortex, CA1, thalamus, and striatum in both groups.

Cerebrolysin Administration Is Associated With Significantly Reduced Axonal Damage in the Striatum After CHI

The protective effect of Cerebrolysin of axonal damage after CHI was assessed by anti–pNF-H staining. Phosphorylated neurofilament H is the major neurofilament in axons and dendrites. Neurofilament dephosphorylation and proteolysis have been implicated as a pathogenic mechanism underlying cell death after CHI. Cerebrolysin treatment significantly increased the percentage of the pNF-H-positive area in the striatum compared with the saline-treated group when examined at 2 days (57.8% ± 2.2% vs 43.3% ± 1.8%, p < 0.05) or 14 days post-CHI (Fig. 4, p < 0.05). There was no significant difference observed in other brain areas including the cortex, DG, CA3, CA1, corpus callosum, and thalamus between these groups.

Cerebrolysin Administration Did Not Significantly Decrease Apoptotic Cells After CHI

To evaluate the effect of Cerebrolysin on apoptosis, TUNEL staining was carried out. TUNEL staining showed apoptotic cells with typical dark brown round or oval apoptotic bodies. Few scattered apoptotic cells were
Fig. 3. Cerebrolysin effect on neuronal loss after CHI. **Left:** NeuN staining. Cerebrolysin significantly reduces neuronal cell loss in the DG and CA3 region examined at 14 days compared with the saline-treated group. **Right:** Bar graph showing the number of neuronal cells. There were 10 rats/group. CT = cortex; ST = striatum; TH = thalamus. Bar = 50 μm. *p < 0.05 vs saline. Data represent the mean ± SD.

Fig. 4. Cerebrolysin effect on axonal damage in the striatum 14 days after CHI. **Left:** Phosphorylated neurofilament H staining. Compared with the saline-treated group, Cerebrolysin treatment significantly increases pNF-H-positive area in the striatum. **Right:** Bar graph showing the percentage of the pNF-H-positive area. There were 10 rats/group. CC = corpus callosum. Bar = 50 μm. *p < 0.05 vs saline. Data represent the mean ± SD.
present in the brain regions studied. There was no significant difference in the apoptosis of brain regions between Cerebrolysin treatment and the saline control examined at 2 days (data not shown) or 14 days (Fig. 5, p > 0.05) after CHI.

**Cerebrolysin Administration Is Associated With Significantly Increased Neuroblasts in the DG After CHI**

Neuroblasts express DCX. Figure 6 showed that Cerebrolysin treatment significantly increased DCX-positive cells in the DG compared with the saline-treated group 14 days after injury (p < 0.05).

**Cerebrolysin Administration Is Associated With Significantly Increased Patency of Cerebral Microvessels After CHI**

FITC-dextran was administered intravenously to measure cerebral microvascular patency at 2 days after CHI. Cerebrolysin treatment significantly increased the percentage of FITC-dextran–perfused vessels in the brain cortex compared with saline treatment after CHI (Fig. 7, p < 0.05). There was no significant difference detected in other brain regions in FITC-dextran–perfused vessels.

**Cerebrolysin Administration Is Associated With Significantly Decreased MMP-9 Expression After CHI**

Matrix metalloproteinases are capable of degrading or modifying almost all components of the extracellular matrix. Increased MMP-9 expression may contribute to BBB disruption, brain edema, and synapse loss after CHI.28 There was a significant reduction in the percentage of the MMP-9–positive area in many brain regions after Cerebrolysin treatment compared with saline treatment examined on Day 2 after CHI (Fig. 8, p < 0.05).

**Cerebrolysin Administration Is Associated With Significantly Increased VEGF Expression After CHI**

Vascular endothelial growth factor has neuroprotective effects and promotes angiogenesis and neurogenesis after brain injury.58,67 To detect the effect of Cerebrolysin on promoting VEGF expression, VEGF staining was performed. There was a significant increase in the VEGF-positive cells in many brain regions after Cerebrolysin treatment compared with saline treatment examined 2 days after CHI (Fig. 9, p < 0.05).

**Cerebrolysin Administration Does Not Alter mRNA Levels of Ang1, MMP-9, and VEGF 2 Days After CHI**

Ang1, VEGF, and MMP-9 mediate microvascular integrity and neuronal damage.41,62 RT-PCR analysis did not detect significant differences in mRNA levels of Ang1, MMP-9, and VEGF in the hippocampus and cortex between Cerebrolysin- and saline-treated rats with CHI when animals were killed 2 days postinjury (Fig. 10, p > 0.05).

**Discussion**

Our present study provides evidence that Cerebrolysin treatment initiated 1 hour after injury and repeated once daily for 14 days promotes neurological functional recovery in rats after CHI. Cerebrolysin treatment is associated with significantly improved behavioral benefits,
including sensorimotor function and spatial learning, as evaluated by foot fault, adhesive removal, and the mNSS and MWM tests. The improvement of functional recovery by acute Cerebrolysin treatment may be related to its neuroprotective effect of neurons (increased neurons in the DG and the CA3 regions of the hippocampus) and retention of axonal integrity in the striatum (significantly increased percentage of pNF-H–positive tissue) and maintenance in cerebral microvascular patency (increased FITC-dextran perfusion area). Reduction of MMP-9 and elevation of VEGF likely contribute to enhancement of vascular patency and integrity as well as neuronal survival induced by Cerebrolysin.

The CHI model used in this study has been demonstrated to cause BBB damage and brain edema. A previous study demonstrated that early intervention with Cerebrolysin reduces the BBB permeability and diminishes brain edema and other brain pathology, which contributes to recovery of sensorimotor function after TBI induced by a stab cerebral injury. In the present study, Cerebrolysin treatment significantly increased the percentage of FITC-dextran–perfused vessels in the brain cortex, indicating that Cerebrolysin enhances the microvascular patency of the brain after CHI. This beneficial effect of Cerebrolysin may result from a combined action of multiple neurotrophic factors, such as CNTF, IGF-1, and GDNF. Upregulation of MMP-9 contributes to the BBB damage and synaptic loss after experimental TBI. Clinical studies show that MMPs may promote BBB opening and hemorrhage secondary to brain injury in patients. Vascular endothelial growth factor is an important regulator of hypoxia-inducible angiogenesis and vascular permeability. In addition, VEGF provides neuroprotection and increases neurogenesis in several models of experimental TBI. Our present study indicates that the beneficial effect of acute Cerebrolysin treatment for CHI is likely associated with both reduction of MMP-9 and elevation of VEGF as shown by immunostaining. However, there were no significant differences in mRNA levels of VEGF and MMP-9 between Cerebrolysin- and saline-treated CHI rats at 2 days postinjury in this study. Since we cannot exclude the possibility that mRNA levels are increased prior to or after the 2-day time, further investigations are warranted to evaluate expression of these genes at various time points post-CHI. Alternatively, the effect of Cerebrolysin on MMP-9 and VEGF protein levels could be induced at translational levels. It is well established that miRNAs regulate protein levels by binding to the 3’ untranslated region of genes. Thus, if we do not detect the effect of Cerebrolysin on transcription of

Fig. 6. Cerebrolysin effect on neuroblasts in the DG 14 days after CHI. Upper: DCX staining was used to detect neuroblasts in the DG. Compared with the saline-treated group, Cerebrolysin treatment significantly increases the DCX-positive neuroblasts in the DG. Lower: Bar graph showing the number of DCX-positive cells. There were 10 rats/group. Bar = 50 μm. Data represent the mean ± SD.
these genes it would be important to investigate whether Cerebrolysin affects miRNAs. In addition, our laboratory has demonstrated a pivotal role of miRNAs in rats after stroke, and investigation of the effects of Cerebrolysin on miRNA expression may be valuable and provide fundamental insight into mechanisms of therapeutic action.

The CHI model we used in the present study is a clinically relevant rat model to mimic human diffuse TBI caused by falls or motor vehicle accidents. Substantial neuronal cell damage is observed in the brain especially in the CA3 region of the hippocampus after CHI if the injury is severe enough (for example, 450 g × 2 m). Our data that acute Cerebrolysin treatment starting 1 hour after CHI significantly decreases neuronal cell loss in the DG and CA3 support a neuroprotective effect of Cerebrolysin. Preclinical studies have shown that acute treatment with Cerebrolysin reduces cerebral infarction in rats after transient ischemia while delayed administration of Cerebrolysin promotes neurological functional recovery without reducing lesion volume. These data suggest that, in addition to the neuroprotective effect, Cerebrolysin has the capacity to promote brain repair after brain injury. Cerebrolysin has been demonstrated to reduce inflammation, stabilize calcium homeostasis, and prevent neuronal death in different in vitro and in vivo models of brain ischemia. Furthermore, delayed Cerebrolysin treatment initiated 24 hours after stroke onset did not affect infarct volume but significantly improved functional outcome in rats. This benefit may result from Cerebrolysin-induced promotion of neural stem cell proliferation and neurogenesis. In the adult rodent brain, new neurons can be generated in discrete regions including the SGZ of the DG of the hippocampus. The hippocampus is a structure intimately involved in learning and memory where neurogenesis plays a critical role. Preclinical studies have revealed that TBI induces neurogenesis in the SGZ in the rat and mouse, while treatments that enhance neurogenesis promote cognitive function after TBI. Cerebrolysin promotes adult hippocampal progenitor cells to differentiate into neurons in vitro and enhances neurogenesis in a rodent model of Alzheimer disease. Newly generated neurons in the SGZ are capable of projecting axons to the CA3 region, which may contribute to maintenance of spatial learning and memory in normal rats and improve functional recovery in injured adult rats. The 14-day survival data showed that DCX-positive cells were found in the DG in the saline and Cerebrolysin treatment groups, while Cerebrolysin treatment significantly increased DCX-positive cells in the DG. DCX is a protein expressed by neuronal pre-
cursor cells and immature neurons. Cerebrolysin has a direct effect on promoting migration of neural progenitor cells harvested from the SVZ of rats. The present study suggests that Cerebrolysin enhances the formation of neuroblasts in the SGZ. Neuroblasts can migrate to the granular layer where newborn neurons become mature and integrate into neural circuitry.

Decreasing cell apoptosis is a marker for neuroprotection. However, in the present study, there is no significant difference in apoptosis measured by TUNEL staining in the brain regions at 2 and 14 days after CHI between Cerebrolysin- and saline-treated groups. The studies from different investigators indicate that the time course of apoptosis in the brain is quite different after CHI. This may result from different conditions including the severity of injury, brain regions studied, postinjury time points, and methods used for apoptosis assay. Significant apoptosis measured by TUNEL staining occurred at 12 and 24 hours in rats injured with the Marmarou weight-drop device. DNA fragmentation measured by the random oligonucleotide-primed synthesis assay was significantly increased with the maximum level occurring at 10 hours after CHI with the same model we used, while DNA fragmentation measured by DNA electrophoresis was maximal at 3 days in the same model. Thus, investigating the effect of Cerebrolysin on apoptosis at different time points after CHI, possibly with several methods, is warranted. Nevertheless, improved cognitive function, significantly more NeuN-stained neurons present in the DG and CA3 region, and more DCX-stained immature neurons in the DG of Cerebrolysin-treated CHI rats imply that Cerebrolysin may be a valuable treatment for CHI. Further investigation is necessary to determine the effects of Cerebrolysin on long-term functional recovery.

Previous studies demonstrated that brain injury induces the expression of growth-promoting factors, which evoke limited axonal regeneration and may, at least in part, contribute to spontaneous functional recovery after brain injury. Cerebrolysin induces sprouting in neurons of the central and peripheral nervous systems. Our present data demonstrate that the Cerebrolysin treatment group showed a significant increase in the percentage of the pNF-H–positive area in the striatum compared with the saline group. These data also show that Cerebrolysin provides a neuroprotective effect on axonal damage in the striatum in rats after CHI. The CHI induced by impact acceleration injury is mainly characterized by diffuse axonal injury with heterogeneous neuronal injury within both axons and soma in various brain regions. Our findings that Cerebrolysin reduces axonal damage in the striatum and cell loss in the hippocampus after TBI indicate the regional heterogeneity of both diffuse injury and therapeutic effects.

Neurotrophic factors are promising for the treatment of CNS diseases. Since neurotrophins do not cross the BBB, these proteins cannot be used for neuroprotection.

**Fig. 8.** Cerebrolysin effect on MMP-9 expression after CHI. **Left:** MMP-9 may degrade the integrity of the BBB. Compared with the saline-treated group, Cerebrolysin treatment significantly decreases MMP-9 expression after CHI in many brain regions. **Right:** Bar graph showing the percentage of the MMP-9–positive area. There were 8 rats/group. Bar = 50 μm. Data represent the mean ± SD.
Acute Cerebrolysin treatment for closed head injury

using intravenous administration. Acute brain injuries induced by stroke and trauma damage the BBB, which may assist factors in entering injured brain. A common delivery method is the invasive brain cannulation for neurotrophin administration. Direct and indirect evidence indicate that low-molecular-weight Cerebrolysin, which contains many neurotrophic factor–like peptides, is able to cross the BBB. Cerebrolysin is well tolerated without severe side effects and may be effective when administered in patients who have suffered stroke or TBI.6

Conclusions

Collectively, our findings demonstrate that early Cerebrolysin treatment followed by repeated dosing provides neuroprotection and promotes brain repair, which may contribute to improved functional outcomes, indicating that Cerebrolysin is a potential therapy for patients with acute TBI. Obviously, further study on the optimal dose, administration route, therapeutic window, and long-term effects of Cerebrolysin on functional recovery is warranted.

Disclosure

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Author contributions to the study and manuscript preparation include the following. Conception and design: Xiong, Y Zhang, Chopp, ZG Zhang. Acquisition of data: Xiong, Y Zhang, Meng. Analysis and interpretation of data: all authors. Drafting the article: Xiong, Y Zhang. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Xiong.
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Acute Cerebrolysin treatment for closed head injury


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