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RESEARCH

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Cerebrolysin recovers diaphragmatic function and reduces injury-associated astrogliosis following a cervical spinal cord hemi-section injury in rats

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Abstract

Background Spinal cord injury (SCI) is widely considered the most disastrous medical condition. With no available treatment to date, SCI continues to cause disabilities to the patients and affect their own and their caregivers' quality of life. Cerebrolysin (CBL) is a neuropeptide preparation derived from purified brain proteins with suggested neuroprotective and neurotrophic properties. CBL showed earlier the ability to target multiple pathways that helped in the improvement of the recovery following different groups of neurological diseases and injuries, including ischemic stroke, traumatic brain injuries, and even neurodegenerative diseases. In the current study, the neuroprotective effect of CBL following a SCI is called into question using a rat model of spinal cord cervical hemi-section validated earlier by our lab and others. Using 32 rats divided into four groups randomly, CBL treatment was implemented for either 7 or 21 days duration, following the cervical spinal cord hemi-section.

Results Following the CBL treatment, rats with cervical cord hemi-section showed functional improvement of diaphragmatic muscle as recorded by electromyography (EMG). In addition, the histopathological assessment of the spinal cord showed improved neuronal viability and reduced astrogliosis at the site of the injury compared to the non-treated group. 21-day treatment showed significant improvement when compared to the shorter 7-day regimen.

Conclusion Our data suggest that CBL is capable of protecting and regenerating anterior horn motor neurons with functional recovery of diaphragmatic muscle functions in rats, suggesting the potential use of CBL for future regenerative and neuroprotective therapy following SCI.

Keywords Spinal cord hemi-section, Cerebrolysin, Electromyography, Astrogliosis

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Background

Spinal cord injuries (SCI) are widely considered as the most deleterious medical condition with no cure up to date. SCI can occur at different levels and severity, causing different forms of disabilities. Huge efforts are underway to treat SCI victims in time with suitable drugs and interventions aiming to improve or restore the spinal cord functions in an attempt to improve the quality of their lives. Cervical SCI is reported as one of the most common levels of SCI. Following a spinal cord hemisection at the cervical segment, the ipsilateral hemi-diaphragm is paralyzed due to the disruption of the rostral ventral respiratory group (rVRG) axons descending to the ipsilateral phrenic motor neurons (PN). This leaves patients with an impaired respirator function that may range from asymptomatic up to a need to mechanical ventilation support and other sensory and motor complications in addition to cardiac dysfunctions [1, 2].

For the past decade, there had been a rapid rise in investigating the possible neuroprotective and regenerative effect of neurotrophic (NTFs) and growth factors as a potential therapeutic agents following SCI [3]. Even though the results had been controversial, possibly due to the usage of different animal models, injury induction methods, and different delivery system and timing, still some of positive findings are reported paving the route for a more controlled studies to be carried out [4]. Cerebrolysin falls into the category of those suggested drugs. It contains several NTFs which are capable of stimulating neurotrophic signaling pathways: ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2) [5]. These growth factors are shown to be required for the survival and proper functioning of the neurons [6]. Cerebrolysin is approved to have beneficial effect as neuroprotective agent, via different mechanisms like preserving axonal integrity and improving vascular patency. Other mechanisms like enhancing synaptic plasticity, preserving synaptic terminals in addition to modulation of inflammatory responses following the injury were all suggested, but the exact mechanism still need to further investigated [7–9].

Khalili and colleagues have shown the therapeutic value of CBL by improving functional recovery, decreasing the mortality rate, and increasing the favorable outcome in patients of traumatic brain injury with severe disability [10]. Also in a mild closed head injury animal models, CBL treatment showed reduction of axonal injury and enhanced neurogenesis [11]. CBL resulted in functional improvement to rats with cervical spinal cord injury delivered via nanoparticles and found to prevent apoptosis of affected motor neurons and promotes functional recovery in experimentally induced spinal cord injury [8, 12]. Evaluating the enhanced spontaneous motor recovery after stroke in mice showed that CBL not only provides the rescue of motor neurons, but also enhances the regrowth of their axons with acceleration of maturation of the regenerated axons CBL [13]. In clinical practice, stroke patients with severe motor impairment receiving CBL showed a positive influence on motor network plasticity and a beneficial effect on motor recovery, suggesting CBL may have important implications for stroke rehabilitation that can be an impactful addition to the conventional therapy for patients with severe motor impairments [14].

The exact mechanism of reported neuroprotective effect of the CBL in the brain lesion is not well understood and still need further studies. However it is suggested to be achieved through multimodal pathway including neurotrophic stimulation, neuroprotection, metabolic regulation and synaptic modulation [15–18].

Cervical spinal cord injury is one of the most affected levels in the reported spinal cord injuries, and the inability of affected patients to breathe is the major cause of morbidity and mortality in human SCI patients. We thus tested the effect of using Cerebrolysin as a therapeutic agent using the rat model of cervical spinal hemi-section. We have earlier reported establishment and characterization of unilateral hemi-section of high cervical spinal cord (C2Hx) in rats beside others. The model results in an immediate cessation of ipsilateral phrenic activity and paralysis of the hemi-diaphragm [19, 20]. In the current study, we have evaluated the possible therapeutic potential of cerebrolysin in cervical hemi-section model including the functional recovery of the diaphragm, and its effect on the reported astrogliosis at the site of the injury.

Methods

Animals and treatment

The experimental animal protocol of this work was approved by the Local Ethical Committee of IACUC, Mansoura University, complying with ARRIVE guidelines and were conducted in accordance with U.K. Animals ACT, 1986. Rats were bred and housed at temperature 20–25 °C in the animal house of Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Egypt. Thirty-two female Sprague-Dawley rats of average body weight 200–250 g were used in this study. Mice were conditioned in standard cages (4 rats per cage) with an alternating 12 h light/dark cycle. Fed standard rat chow, and water was available ad libitum. Animals were randomly divided into four groups, each containing 8 rats. Group 1: control group without any intervention. Group 2: rats underwent surgical left cervical spinal hemi-section (C2Hx) without receiving

any treatment. Group 3: left cervical spinal hemi-section C2Hx is followed with treatment using Cerebrolysin for 7 days duration starting from day of surgery and Group 4: left cervical spinal hemi-section C2Hx followed with Cerebrolysin treatment for 21 days. Cerebrolysin was obtained from EVER Neuro pharma GmbH, Austria. Each rat assigned to treatment group received 2.5 ml/kg cerebrolysin intraperitoneal (ip) immediately after the surgery, and the treatment was continued daily for either 7 days (group 3) or 21 days (group 4). 28 days post-surgery, the rats were subjected to EMG recording, and samples were collected for histological and immunohistochemical assessment (Fig. 1a).

Spinal cord hemisecting

Left spinal cord hemi-section was done following [21] with slight modification. Rats were anaesthetized by ip injection of a mixture 75 mg/kg ketamine HCl (Ketamax 50 mg/ml, Troikaa Pharmaceuticals Ltd, Gujarat, India) and 10 mg/kg xylazine HCl (Xylajet 20 mg/ml, ADWIA, Egypt). They were positioned on a heated surgical plate of wax, with the nose pointing at 90° angle to the surgeon to maintain body temperature around 37.5 °C throughout the surgery. The rhomboid muscle was dissected to access the spinalis muscles (surrounding the vertebra). The spinalis muscle from C1 to C3 vertebra was retracted. The muscle around the dorsal part of the C2 vertebra with a prominent apophasis vertebra was cleaned using sterile cotton swabs; the apophasis of C2 was carefully removed. The spinal cord was exposed by dorsal left hemi-laminectomy. The dura along C2 dissected on each rostral and caudal side and the cerebral spinal fluid (CSF) is sponged up. The midline of the spinal cord was identified and a transverse left cut section under the cervical dorsal root number 2 was severed with the microscissors laterally from midline to create a left hemi-section injury. The muscles were sutured with 3/0 absorbable suture (Polysorb) as a protective layer and the back skin sutured with 3/0 nylon sutures. The wound cleaned with 10% povidone-iodine saturated sterile gauze.

Electromyography recording

For assessment of the diaphragm electrophysiology, the rats were anaesthetized with urethane 97% (1.2 gm/Kg) ip at 28th day after hemi-section, and a laparotomy was performed to expose the abdominal surface of the hemi-diaphragm ipsilateral to spinal cord hemi-section. A pair of bipolar recording electrodes was inserted into the

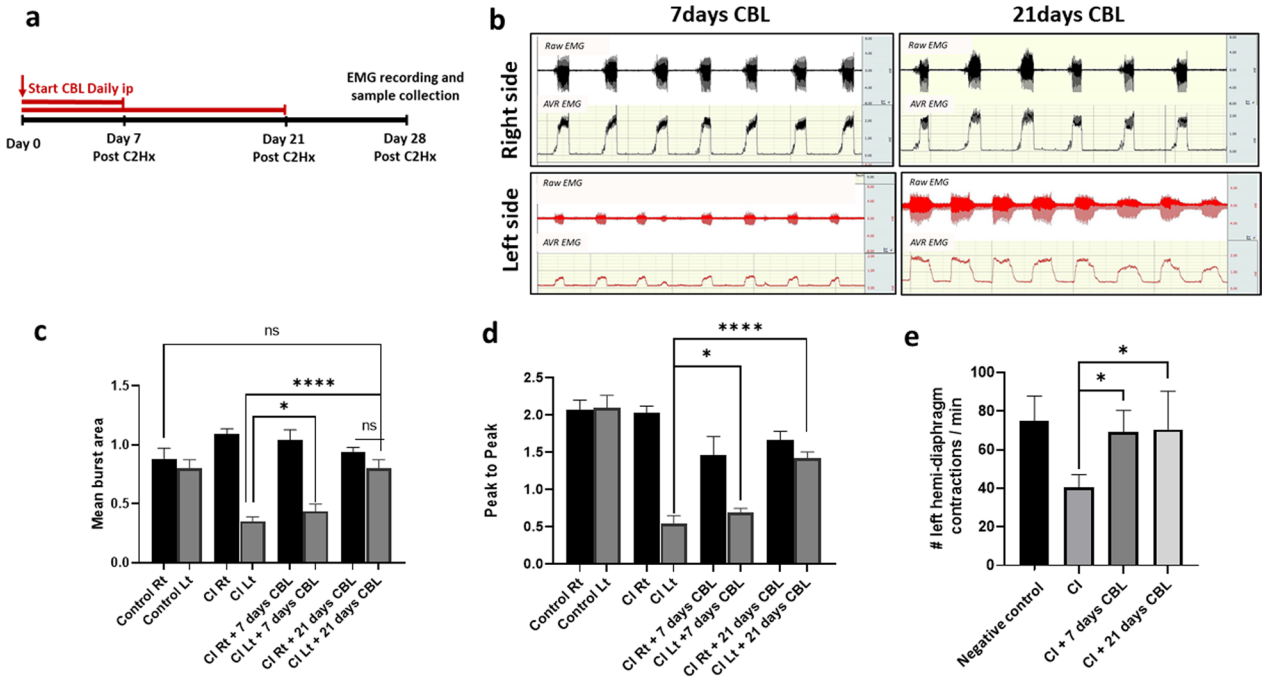

Fig. 1 Cerebrolysin treatment shows improvement of diaphragm function recorded by EMG. **a** Schematic diagram showing the workflow of experiment. **b** EMG recording for right (contralateral) and left (ipsilateral) hemi-diaphragm following C2HX. Raw (upper) and ARV (lower) EMG recording from 7 and 21 days CBL treated groups. **c** Quantification of mean burst area from four groups from right and left diaphragm. **d** Mean peak-to-peak amplitude of contractions for right and left side diaphragm from four groups. **e** Mean contraction rate of left hemi-diaphragm per minute. Graphs are presented as Median and interquartile range for **c**, and mean and ± SD for **d** and **e**. *CI* cervical injury, $n = 6–8$, one-way ANOVA is used followed with post hoc Tukey test for c and e and Kruskal–Wallis test followed with Dunn's multiple comparison test for **d**. *ns* non-significant, **p* value < 0.05. *****p* value < 0.0001

crural region of the hemi-diaphragm. Electrophysiological activity assessment of left and right hemi-diaphragm were separately conducted for each rat. Functional assessment of diaphragmatic muscle is done using Biopac system Inc. with electrode MEC110C. Mean burst area, peak to peak of each wave were recorded according to [20] and interpreted as magnitude of muscle activation, additionally, the frequency of left diaphragm contraction per minute was calculated per minute for each rat.

Histological and immunohistochemical analyses of spinal cords

Following the recording, all rats were euthanized with urethane and their vertebral column was exposed dorsally and excised at the site of the lesion as described in details in [20]. The spinal cord was removed, transversally cut and fixed in neutral buffer formalin for 48 h at room temperature (RT). Fixed samples were dehydrated with ascending concentration of ethyl alcohol (70, 80, 90 and 100%), cleared in xylene and paraffin embedded till the time of analysis. 5-µm-thick sections were obtained using a rotatory microtome (MicroTec cut 4050, Germany) and mounted on coated glass slides for H and E and cresyl violet stains, or VWR® Superfrost® Plus Micro Slide, for GFAP immunostaining. H and E stain and cresyl violet stain were done according to [22] to characterize the ipsilateral spinal tissue architecture in all groups. Additionally, the GFAP immunostaining was applied according to [23]. Briefly, spinal cord sections were deparaffinized and rehydrated. Following the routine quenching and blocking, spinal cord sections were incubated with anti-GFAP antibody (GFAP (D1F4Q) XP® Rabbit mAb #12389, Cell Signaling Technology, 1:5000) for 2 h at RT, then sections were washed using PBS for 15 min at RT. Horse-radish peroxidase substrate was applied simultaneously to all sections until the brown color is obviously detected then sections were all counterstained with hematoxylin for 3 min.

For histomorphometric analysis and quantification

Five serial sections from each group were collected for ipsilateral morphometric analyses using the image programed LAS EZ software. Cresyl violet-stained sections were used to count the total numbers of viable motor neurons and astrocytes. GFAP-stained sections were used to estimate the density to examine distribution and activity of astrocytes [24].

Statistical analysis

Statistical analysis was performed in GraphPad Prism 8.0.2. Data were tested for normality using the Shapiro–Wilks test and Kolmogorov–Smirnov test, and normally distributed data were compared using one-way ANOVA followed by post hoc Tukey test. Non-normally distributed data were compared using non parametric Kruskal–Wallis test followed with Dunn's multiple comparison test as indicated throughout the manuscript. A p-value below 0.05 was considered significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{***}p < 0.0001$.

Results

1. Cerebrolysin treatment improves the diaphragmatic function as recorded by EMG

EMG recording of both sides of diaphragm were conducted at 28 days post-surgery. A permanent paralysis of the ipsilateral hemi-diaphragm was detected following the cervical hemi-section confirming the successful cervical hemi-section injury induction. Treatment with CBL for 7 days showed a positive impact on magnitude of muscle contraction demonstrated as a significant increase of contraction power assessed by mean burst area, with less evident effect on the peak-to-peak measurement. The group that received CBL for 21 days showed a dramatic improvement of contraction magnitude, which was comparable to the EMG recording obtained from the healthy negative control group, suggesting an almost restored diaphragm muscle functionality. Diaphragm contraction rate was improved significantly in both 7- and 21-day regimen (Fig. 1).

2. Cerebrolysin treatment improved the histomorphometric feature of spinal cord at the site of injury, with recovery of viable motor neurons and reduction of astrogliosis

The light microscopic examination of serial spinal cord sections from the negative control group (Fig. 2a1, a2) shows viable motor neurons, identified with the basophilic cytoplasm and a light vesicular nucleus with prominent nucleus. Cresyl violet-stained sections reveal prominent Nissl granules which appear as basophilic structure and distributed in soma and dendrites (Fig. 2a3). The surrounding astrocytes demonstrated a relatively large light-stained nucleus, meanwhile the oligodendrocytes had dark small nucleoli and localized in close contact with neuronal body. The white matter had a fine network appearance of an intact myelinated nerve fiber which formed from central axons, unstained myelin sheath and outermost neurolemma. In the gray matter of the ventral horn and surrounding white matter of the cervical hemi-section group, variety of neurodegenerative alterations were identified (Fig. 2b1–b3). Number of viable motor neurons were significantly reduced (Fig. 2A), with the affected neurons appeared shrunken and dark eosinophilic (dead red neuron) with pyknotic nucleus or with loss of Nissl granules (chromatolysis), others were completely lost and replaced by cavities which enclosed

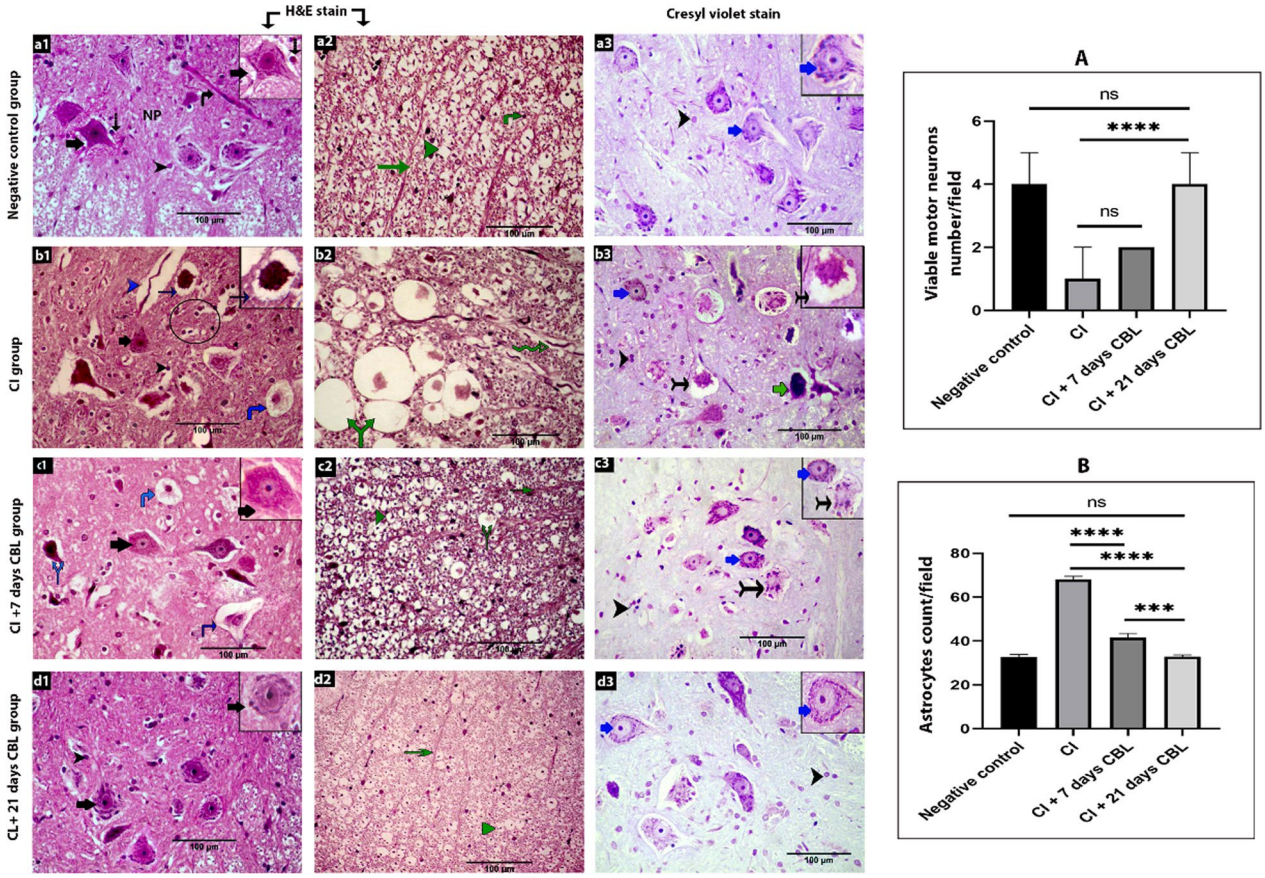

Fig. 2 CBL treatment improves histo-architecture including viable motor neurons and astrocytes count at the site of spinal cord injury. **a1**–**a3** Negative control group, **b1**–**b3** CI group**, c1**–**c3** CI + 7 days CBL group and **d1**–**d3** CI + 21 days CBL group showing: Viable multipolar neurons (black thick arrow), oligodendrocytes nucleus (discontinuous black arrow), astrocytes nuclei (black arrowhead), homogenous neuropil (NP), blood vessel (black vertical arrow), intact axon fiber (green arrowhead), intact axon tract (green arrow),viable neuron with mottled Nissl granules (blue thick arrow), dark debris of dead neurons (blue arrow), cavities enclosed remnant of degenerated neurons (vertical blue arrow), edematous blood vessels (blue arrowhead), dishomogenous and edematous neuropil (inside black circle), degenerated axons fibers (green arrow with double head),disrupted axon tracts (corrugated green arrow), neurons with chromatolysis (black tailed arrow) or with clumped Nissl granules (green thick arrow). **A** Quantification of viable motor neurons count showing improvement following 21-day CBL treatment at the site of injury and **B** Quantification of astrocytes count showing CBL treatment—both 7 and 21 days—showed reduced astrogliosis at the site of injury. Data are expressed as median and interquartile range following Kruskal–Wallis test followed with Dunn's multiple comparison test for **A**, and mean ± SEM following one-way ANOVA test followed with post hoc Tukey test for **B**

remnant of degenerated neurons. On other hand, the astrocytosis become more evident and the number of astrocytes was significantly increased compared to the negative control group with sever degeneration and demyelination in the axons leaving a wide and empty peri-axonal vacuole (Fig. 2b).

The administration of cerebrolysin for 7 days (CI + 7 days CBL group) was associated with restoration of some normal histo-architecture of the gray and white matter (Fig. 2c1–c3). Some neurons with normal histo-architectures and clear integrated Nissl granules were detected, with a trend for increased their total number which shortly failed to show a statistical significance (Fig. 2A). Moderate astrocytic infiltration was also observed; however, astrocytes count was significantly reduced than the CI group (Fig. 2B). A wide area of white matter returned the normal organization, except small separate areas that still had some vacuoles and demyelinated axons. Evaluation of sections from group that received 21 days CBL treatment demonstrated that the normal histo-architecture of the spinal cord was nearly detected (CI + 21 days CBL) (Fig. 2d1–d3). The total number of viable motor neurons and astrocytes count were nearly returned to data obtained from the healthy negative control sections, suggesting that the CBL treatment had a significant positive impact (Fig. 2A, B).

SCI is reported to be associated with astrogliosis at the site of injury [25–27]. Although the exact function of reactive astrocytes at the site on injury is not well established, we have assessed the extent of astrogliosis at the site of SCI and how CBL treatment can influence it. Astrocytes from healthy controls appeared with fine cytoplasmic processes, well-spaced from each other and with mild GFAP immune expression (Fig. 3a1–a3). Conversely, after spinal cord hemi-section injury, the astrocytes appeared with bold projections, overlapped with each other and with significant increase in GFAP expression (Fig. 3b1–b3 and A). The expression of GFAP was significantly decreased after CBL administration for either 7 days or 21 days, but the improvement was dramatic in the group that treated with CBL for 21 days, where the shape of astrocytes and the GFAP expression density in this group was nearly returned to the normal (Fig. 3d1–d3 and A).

Discussion

Cerebrolysin is a mixture of neuropeptides that mimics the action of neurotrophic and different growth factors [28]. It is considered a promising neuroprotective drug for the management of different diseases, such as stroke TBI and AD. CBL is capable of crossing the blood–brain barrier which is structurally similar to spinal cord blood–brain barrier [29]. In addition to that, there are other anatomical and pathophysiological resemblances between spinal cord and brain as both have the same meningeal covering and morphological lesions described in injuries [29].

In the current study, the therapeutic potential of CBL as a neuroprotective agent for SCI was evaluated using a cervical spinal cord hemi-section model in rats, where spinal cord hemi-section lesion causes persistent hemidiaphragm dysfunction ipsilateral to the injury 28 days post injury. Additionally, the impact of treatment duration was also tested by implementing two different

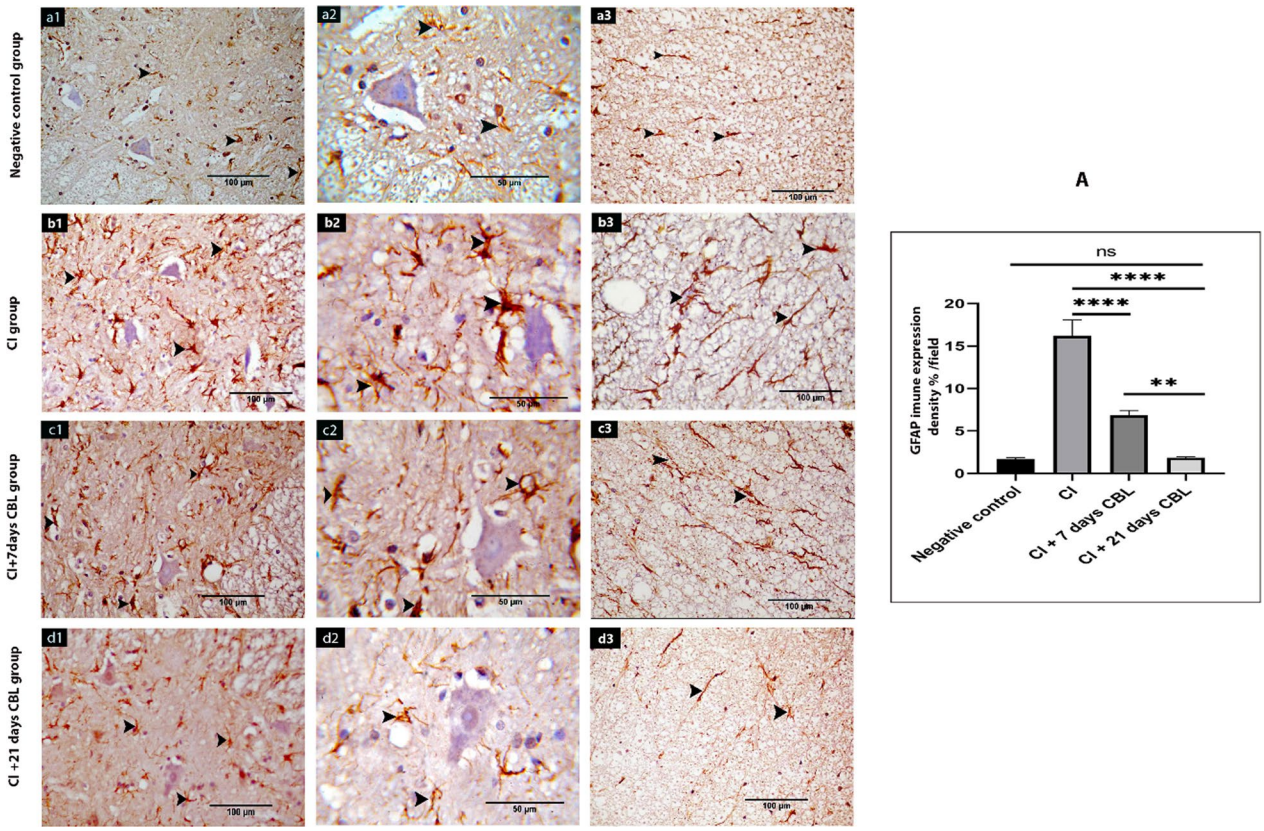

Fig. 3 CBL treatment reduces astrogliosis at the site of injury following cervical hemi-section. **a1**–**a3** Negative control group (**a2** is a higher magnification of **a1**) showing resting astrocytes with fine cytoplasmic process and mild GFAP expression. **b1**–**b3** CI group (b2 is a higher magnification of b1) showing stimulated astrocytes with bold cytoplasmic process and strong GFAP expression. **c1**–**c3** CI + 7 days CBL group (**c2** is high magnification of **c1**) showing astrocytes with moderate GFAP expression and moderate cytoplasmic process. **d1**–**d3** CI + 21 days CBL group (**d2** is a higher magnification of **d1**) showing astrocytes with fine cytoplasmic process and mild GFAP expression. **A** Quantification of GFAP immune expression density, data are expressed as mean ± SD. $n = 6–8$, one-way ANOVA test is used followed with post hoc Tukey test, *ns* non-significant, ** p value < 0.01. **** p value < 0.0001.

treatment regimens of short (7 days) compared to a longer (21 days) protocol.

Cerebrolysin treatment for 21 days, daily after C2Hx, resulted in a significant improvement of diaphragm functions following the induced spinal cord hemi-section. The improvement was detected as a significant improvement of the power and rate of breathing compared to the healthy side and the non-treated groups. Seven days regimen showed an improvement on the amplitude and power of breathing, measured by mean burst area in the curve, however when it comes to the peak-to-peak amplitude it came on the edge with p value = 0.05. However, it is worth noting that the effect of 7-day CBL treatment was evident after drug stoppage for 21 days until the day of EMG recording, indicating the long-lasting nature of CBL induced improvement, suggesting the long-term effect of the drug on both magnitude and rate of left diaphragm muscle contraction. The EMG data showed a non-explained reduction of the average contraction amplitude measured by peak-to-peak measurement for the contralateral diaphragm following CBL treatment. This need to be further assessed and reproduced to confirm the finding and the association.

Hemi-section of spinal cord, sections from the site of injury showed an evident distorted histo-architecture in the gray matter of ventral horn and white matter when tested at 28 days post-surgery. It was also associated with reduced number of viable motor neurons with evident signs of degeneration including pyknotic nuclei and chromatolysis. Those post-surgery finding were reported earlier with our group and others [30] and [31] confirmed the successful hemi-section lesion for the spinal cord. Seven days CBL treatment showed that some of the normal histo-architecture of the gray and white matter were restored, however, quantification of viable motor neurons following 7 days treatment failed to show a significant difference when compared to the non-treated groups. This is not particularly surprising considering the short treatment duration of this group. Longer treatment with CBL showed a more significant improvement for the spinal cord histo-architecture in line with the functional diaphragm improvement revealed with EMG recording. The total number of viable motor neurons significantly increased after CBL administration in this group, suggesting the potential regeneration of neurons at the site of the injury under influence of CBL corroborating other results suggesting the neuro-regenerative effect of CBL.

Further testing the influence of CBL treatment at the site of injury, our data show that CBL treatment had significantly reduced the injury induced astrogliosis at the site of SCI. The exact function of astrocytes is not very well understood with different studies suggesting both of positive and negative influence of astrogliosis [32–34], and collectively reviewed by [35]. The exact mechanism of astrocytes in neuronal protection and how exactly CBL can promote it following the SCI will need to be further investigated. However, recently, Xu et al. [36] reported that CBL treatment reduced astrogliosis and axonal injury in addition to the enhancement of neurogenesis with functional recovery in rats after closed head injury, in a complete agreement with our data.

How CBL improves the outcome of SCI as reflected on the functional assessment of the diaphragm is out of this manuscript scope. However, considering the increased numbers of motor neurons and restoring of spinal cord axons, our data support earlier findings that show CBL is capable of enhancing neurogenesis and synaptic density [7–9, 18, 37, 38].

Conclusion

Taken together, our work has led us to conclude that CBL treatment had beneficial effect on both functional diaphragm recovery and the spinal cord architecture following the SCI model in rat. There is a satisfactory agreement with the earlier data suggesting the potential protective role of CBL in other injurious models. CBL treatment had also reduced the active astrogliosis and chromatolysis associated with SCI, suggesting that the effect extends beyond neurons to also include the surrounding glia cells.

Further studies, which take molecular mechanism of CBL action into account, will need to be undertaken in the future, in addition to the further phenotype assessment including most importantly a more detailed respiratory function tests, like lung vital capacity, total lung capacity and tidal volumes will add to our understanding and accelerate the possible translational impact of CBL.

Abbreviations

CBL	Cerebrolysin
CI	Cervical injury
CNF	Ciliary neurotrophic factor
EMG	Electromyograph
GDNF	Glial cell-derived neurotrophic factor
H and E	Hematoxylin and eosin
ILGF1/2	Insulin-like growth factor 1/2
NTF	Neurotrophic factor
PBS	Phosphate buffer saline
PN	Phrenic nerve
RT	Room temperature
RVRG	Rostral ventral respiratory ganglia
SCI	Spinal cord injury

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Author contributions

SF, AR, EM, BA designed the study. SF performed the surgery and SF and SE generated and interpreted the EMG data. SR made IHC, generated figures and performed quantifications. SE, SR, BA analyzed and interpreted the manuscript

whole data. SE, SR, MA wrote the manuscript draft and AR, EM and BA critically revised it. All authors read and approved the final version of the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
The experimental animal protocol of this work was approved by the Local Ethical Committee of IACUC, Mansoura University, complying with ARRIVE guidelines and were conducted in accordance with U.K. Animals ACT, 1986.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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