ORIGINAL PAPER

Cerebrolysin increases motor recovery and decreases inflammation in a mouse model of autoimmune encephalitis

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Abstract

Multiple sclerosis (MS) is a complex chronic neurodegenerative disease that involves an abnormal autoimmune response directed against the brain, nerves and spinal cord; it is considered the most frequent cause of neurological disability, because MS-associated inflammatory lesions can affect a wide range of systems to a varying degree and may cause a plethora of neurological comorbidities and symptoms. The symptoms are quite variable from patient to patient and depend on the spatial distribution of the central nervous system (CNS) lesions, but usually involve sensory disturbances, cognitive deficits, unilateral vision loss, bladder dysfunction, ataxia, fatigue, double vision, weakness of the limbs and intestinal disorders. Experimental autoimmune encephalitis (EAE) mouse model reproduces the pathological features of the human disease, and is a widely used model used for studying the pathology and different treatment options in the preclinical stage. In this study, we aimed to evaluate the motor function, as well as the degree of demyelination and inflammatory changes in the brains of mice immunized for the myelin oligodendrocyte glycoprotein (MOG)$_{35-55}$, and treated with Cerebrolysin. Animals were randomly assigned to one of the three groups: (*i*) EAE untreated group (*n*=10), (*ii*) EAE treated group (*n*=10), and (*iii*) control group (*n*=5), and their motor dysfunction was followed until the clinical pathology begun to improve. We also analyzed histologically and immunohistochemically the lesions in the optical nerves, cervical spinal cord and medulla. Our results showed higher deficit scores for untreated animals compared to treated animals. After harvesting the tissue, we have first evaluated the density of myelin in the optical nerves, cervical spinal cord and medulla and we found significant differences between treated and untreated groups of animals. We continued to investigate the structure of the CNS parenchyma by evaluating the intensity and morphology of the neuronal cytoskeleton and microglia by immunohistochemical stainings. Although larger animal groups are necessary, this is the first pilot study to investigate the use of a neurotrophic factor as a putative treatment option for a MS model.

Keywords: multiple sclerosis, Cerebrolysin, myelin, microglia, neurofilaments.

Introduction

Multiple sclerosis (MS) is a disease of the central nervous system (CNS) that involves an abnormal auto-immune response directed against the brain, nerves and spinal cord. Experimental autoimmune encephalitis (EAE) mouse model reproduces the pathological features of the human disease, and is a widely used model used for modeling the pathology and different treatment options in the preclinical stage [1]. EAE is an autoimmune disease mediated by T-helper cells, characterized by infiltration of the CNS with T-helper cells and monocytes, associated with local inflammation. EAE has been a powerful study tool for MS pathogenesis as well as for potential therapeutic interventions [2]. MS is characterized by axonal demyeli-nation in the CNS, due to an immune response [3]. In EAE model, there is a progressive paralysis that begins with the tip of the tail and then extends to the rest of the body. The most common method of inducing EAE is to inject an antigen consisting of myelin protein fragments together with an adjuvant [4]. Similar to MS patients, mice with this disease present demyelination, together with acute and chronic inflammation [5].

EAE mouse model was initially induced more than 60 years ago by active immunization with spinal cord homogenates [6]. Research data has led to the discovery of numerous encephalitogenic peptides and the mice remain the most used animal species, in part due to the wide availability of transgenic mice and knockout models available for targeted functional studies. Depending on the species of mice used in the study, EAE can be initiated by immunization with CNS homogenate myelin basic protein (MBP), proteolipid protein (PLP) encephalitis epitopes (PLP$_{139-151}$, PLP$_{178-191}$), myelin oligodendrocyte protein (MOG)$_{92-106}$ or MOG$_{35-55}$ in complete Freund's adjuvant emulsion. In the C57BL/6 (H-2b) mouse model, EAE is induced with MOG$_{35-55}$, clinically presenting with a form of progressive chronic disease [7].

A plethora of neuroprotection mechanisms have been proposed in the treatment of stroke and degenerative CNS diseases, with Cerebrolysin being a standardized complex of neurotropic factors extracted from porcine brain and consisting of low small peptides (<10 kDa) and free amino acids [8–12].

In the present study, we aimed to evaluate the motor function, as well as the degree of demyelination and inflammatory changes in the brains of MOG_{35-55}-immunized animals, after the treatment with Cerebrolysin.

⌗ Materials and Methods

Animals and EAE induction

The study was performed on 25 C57BL/6 female mice of 10–11 weeks of age (19.61 ± 1.57 g) ("Cantacuzino" National Institute of Research and Development for Microbiology and Immunology, Bucharest, Romania). The animals were kept in groups of three, in a controlled 12-hour light/12-hour dark environment, and had access at will to water and a normal diet with pellets until the day before the initiation of the experiments. Mice were arbitrarily set to one of the three groups: (*i*) EAE untreated group (*n*=10), (*ii*) EAE treated group (*n*=10), and (*iii*) control group (*n*=5). This protocol was approved by the local Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania (203/24.10.2017).

Immunization kits based on MOG_{35-55}/complete Freund's adjuvant (CFA) emulsion pertussis toxin (PTX) (code EK-2110) were purchased from Hooke Laboratories, Inc., Lawrence, MA, USA, and the working protocol was based on the recommendations of the producer [13–15].

Briefly, in the first day of immunization, the animals received initially the MOG_{35-55} antigen emulsion as two subcutaneous injections (0.1 mL/site) on the upper and lower back areas; at two hours after the antigen administration, the first PTX dose was injected intraperitoneally [150 ng diluted in 0.1 mL glycerol/phosphate-buffered saline (PBS)] [16]. Next day, the second PTX dose was administrated in the same concentration and localization. Control animals received saline injections equivalent in volume and injection time with the treatment regimens.

The animals were then followed for 30 days, and during this time their motor dysfunction was scored according to the following EAE scoring guide [17]: 0 (no alteration of the motor function), 0.5 (limp of the tail's tip), 1 (complete limp tail), 1.5 (limp tail and hind paws impairment), 2 (limp tail and weakness of the hind paws), 2.5 (completely unable to move one paw, but movement in the other paw), 3 (limp tail and paralysis of both hind paws or of a front paw and a hind paw), 3.5 (limp tail and paralysis of the hind paws, moreover the animal keeps its hind paws together on one side of the body), 4 (limp tail, hind paw paralysis and front paw paresis), 4.5 (hind paws paralysis and front paws paresis, the animal cannot move within the cage and it is not watchful), 5 (the mouse just rolls or is found dead). After the beginning of the treatment, all the animals were fed with freshly prepared 2% agarose–glucose pellets.

The treated animal group started to receive a daily intraperitoneal dose of Cerebrolysin of 5 mL/kg, starting with the second day after recording an EAE score higher than 0. Non-treated animals received a daily saline injection of equivalent volumes.

Tissue processing and immunohistochemistry

After 30 days of survival, the animals were deeply anesthetized, hearts were perfused with saline, 10% neutral buffered formalin (NBF), and then the telencephalon, brainstem, cervical spinal cord, and eyes were harvested and further fixed in NBF for two days, at room temperature.

All tissues were next processed for paraffin embedding and cutting on a HM355S rotary microtome equipped with a waterfall-based section-transfer system (Thermo Scientific Inc., Walldorf, Germany). All blocks were cut as 4 μm-thick sections and further utilized for histochemical staining as well as immunohistochemistry (IHC).

Besides the classical Hematoxylin–Eosin (HE) staining, seriate sections were processed for Luxol fast blue staining for myelin identification. Basically, the sections were deparaffinized, re-hydrated to 95% ethanol, and then incubated in a 0.1% Luxol fast blue solution, overnight, at 60^0C, differentiated in 90% ethanol, re-hydrated to distilled water, counterstained for 3 minutes in 0.2% Nuclear red solution, then de-hydrated, cleared, and mounted with a xylene-based mounting medium.

For IHC, the sections were rehydrated in graded alcohol series, subjected to antigen retrieval by microwaving in citrate buffer pH 6 for 20 minutes, incubated in 1% hydrogen peroxide for 30 minutes, and kept for another 30 minutes in 3% skimmed milk in PBS. The sections were next incubated at 4°C for 18 hours with the primary antibody [goat anti-ionized calcium-binding adaptor molecule 1 (Iba1), Abcam, 1:1000; rabbit anti-neurofilaments (NF), Abcam, 1:500], and the next day the signal was amplified for 30 minutes with a specific antispecies peroxidase polymer detection system adsorbed for mouse immunoglobulins (Nichirei Bioscience, Tokyo, Japan). The signal was lastly detected with 3,3'-Diaminobenzidine (DAB) (Dako, Glostrup, Denmark) and the slides were coverslipped in DPX (Sigma-Aldrich, St. Louis, MO, USA) after a Hematoxylin staining. Negative control slides were obtained by omitting the primary antibodies.

Image analysis

All the slides were imaged at 20× and 40× objectives on a Nikon 90i microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands) equipped with a Prior OptiScan ES111 motorized stage (Prior Scientific, Cambridge, UK), a Nikon DS-Ri2 complementary metal-oxide semiconductor (CMOS) 16 Mp color camera and the Nikon NIS-Elements Advanced Research imaging and control software.

From Luxol fast blue stained sections and IHC processed slides, intensity and area of the signals were extracted as integrated optical densities (IODs) in Image ProPlus AMS software (Media Cybernetics, Bethesda, MD, USA). In order to assess the subtle morphology of the neuronal cytoskeleton and of the microglia, we have calculated the fractal dimension (FD) of the signal given by IHC based on the binary images corresponding to the thresholded signal of interest. FDs were calculated by

the box-counting algorithm, as the slope of the regression line for the *log-log* plot of the counting box size and the number of counts in Image ProPlus.

Finally, all resulting behavioral and imaging data were plotted in Microsoft Excel as average ± standard error of the means (SEM), and statistical differences were sought using a Student's *t*-test. A value of *p*<0.05 was considered to be statistically significant.

Results

The clinical assessment of the animals revealed the beginning of hind limb deficits starting with 8–12 days after EAE immunization protocol (Figure 1, A and B), with increasing scores up to the 14th day after the beginning of the initial impairments, after which the scores begun to drop, illustrating the oscillating evolution of the disease with the known relapsing/remitting pattern (Figure 1C). In the untreated animal group (*n*=10), two animals did not develop any signs of disease, while in the treated animal group (*n*=10), three animals did not develop the disease; all these animals were not considered further on in the study or within the statistical analysis. A granulomatous reaction developed under the back skin of the animals, on the places where the antigen emulsion was injected, however this did not occur in any of the five animals that did not develop EAE clinical changes.

In order to mimic the human pathology, we have begun the daily treatment with Cerebrolysin in the treatment group, in the next day after the onset of the smallest neurological motor deficit. Comparing the average score values for treated animals with those for untreated animals, we observed a small but significant difference between these two groups, with higher deficit scores for untreated animals, Student's *t*-test, *p*<0.001 (Figure 1C). The treatment, however, did not lead to a steep decrease of the score, with the oscillating pattern being preserved, but did lead to lower scores on the same slope-pattern. The maximum-recorded score was of 3, and no animal died during the clinical follow-up.

After harvesting the tissue, we have first evaluated the density of myelin by assessing the integrated optical density of the red, green and blue (RGB) signature for the Luxol fast blue pigment (Figure 2). The optical nerves of both treated and untreated animals showed reduced myelin staining compared to controls, but only for the treated animals group this difference was not significant (Figure 2, A–C). There seem to be no difference between different regions of the same optic nerve. For the cervical spinal cord, again only untreated animals showed a notable difference compared to the control group (Student's *t*-test, *p*<0.05), while the treated group exhibited values extremely close to the control group. For the medulla, however, both treated and untreated groups showed significantly lower densities compared to the control, without any difference in between them (Figure 2, A, D and E). In all analyzed regions, there were no classical demyelinating plaques, as for the human pathology. These measurements were all done in the inner areas of the medulla/spinal cord, as these areas showed more distinct identifiable fibers, compared to the more compact myelin columns under the meninges.

We continued to investigate the structure of the neuropil by evaluating the intensity and morphology of the neuronal cytoskeleton, as viewed by IHC for NF (Figure 3). Intensity of the staining (IOD) revealed a clear-cut decrease for untreated animals compared to the treated group (Student's *t*-test, *p*<0.01) for both the spinal cord and the medulla (Figure 3, A, C–F). For the treated animals, there was no significant difference between the two anatomical regions, while the IOD values were significantly lower in the spinal cord for the untreated group (Student's *t*-test, *p*<0.05). When we analyzed the morphology of the NF, FD showed much higher values for the treated animals, in their spinal cords, compared to the untreated group (Student's *t*-test, *p*<0.01) (Figure 3B). Interestingly, however, there was no difference between the two animal groups for the medulla. As for the Luxol fast blue, all these measurements were done in the inner areas of the medulla/spinal cord, as there was a less dense meshwork of cytoskeletal scaffolds to be assessed.

Lastly, we were interested to evaluate the intensity staining and morphology of the microglia, based on the Iba1 immunostaining (Figure 4) in non-submeningeal areas. For the spinal cord, the intensity of the signal actually increased for the treated animals' group (Student's *t*-test, *p*<0.05), while for the medulla, the signal was drastically increased only for the treated animals (Student's *t*-test, *p*<0.01) (Figure 4A). While the intensity of the signal, namely IOD, showed statistically significant variations, the morphology of the microglia seemed to be unchanged, with no significant difference between treated/untreated animals (Figure 4, B–D). This leads to the hypothesis that the treatment reduced the overall inflammation, but not the morphology of the microglia.

Discussions

MS is a complex chronic neurodegenerative disease that involves CNS and is believed to be autoimmune; it is mediated by autoreactive lymphocytes that cross the blood-brain barrier and enter the CNS, where they cause local inflammation [18]. Worldwide, approximately 2.5 million people are affected by this disease, with the most affected age group being young people aged 20 to 40 years old [19]. In the United States and Canada, MS is highly prevalent with estimated incidence rates between 177 and 350 per 100 000 [20]. The higher frequency of MS is seen on women, which are twice as severely affected as men [21]. MS is considered the most frequent cause of neurological disability, because MS associated inflammatory lesions can affect a wide range of systems to a varying degree and may cause a multitude of neurological symptoms and comorbidities [22].

The symptoms depend on the spatial distribution of the CNS lesions and are quite variable from patient to patient, but usually involve sensory disturbances, cognitive deficits, unilateral vision loss, bladder dysfunction, double vision, ataxia, fatigue, weakness of the limbs and intestinal disorders [23].

Since the mid-1800s have been described aspects of MS pathology, and Cruveilhier observed the presence of MS lesions at the level of the CNS [24].
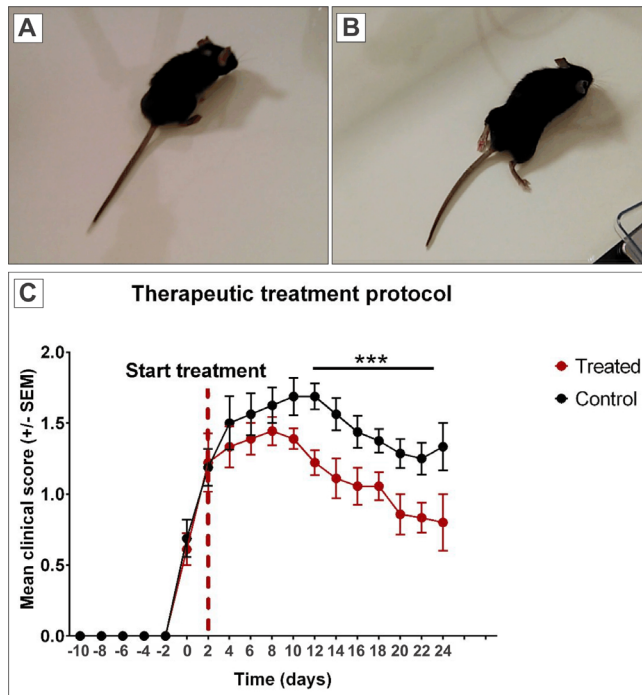
Figure 1 – *The extent of motor deficits in EAE animals ranged from a limp tail (A) to a limp tail with partial hind limb paralysis (B). Overall, when administrated at the second day after a higher than 0 EAE score has been recorded, Cerebrolysin-treated group showed improved motor recovery compared to untreated animals (***p<0.001). Giving the fact that not all animals developed at least a score of 0.5 at the same moment, the graph averages the EAE scores from the same moment of the onset of the disease, and respectively of the treatment (C). Error bars represent standard error of the means (SEM). EAE: Experimental autoimmune encephalitis.*
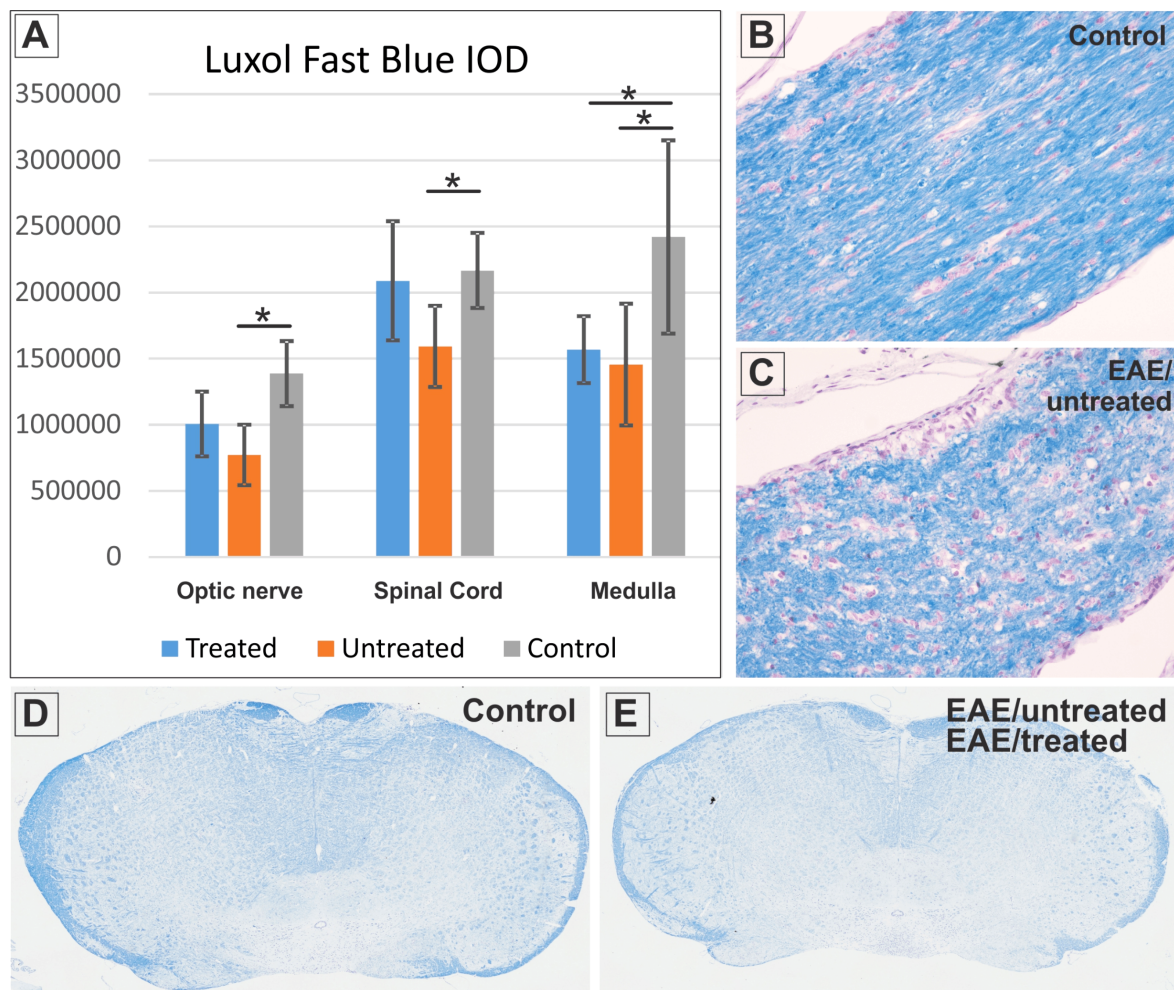

Figure 2 – *Myelin density staining, assessed as the integrated optical density (IOD) of the staining. For the optic nerves and the spinal cords, there was no statistical difference (*) in myelin density when compared to control non-EAE animals (A–C). Both treated and untreated EAE models showed lower myelin densities compared to controls, for the medulla (A, D and E). Error bars represent standard error of the means (SEM). Luxol fast blue staining: (B and C) 200×; (D and E) 10× scan. EAE: Experimental autoimmune encephalitis.*
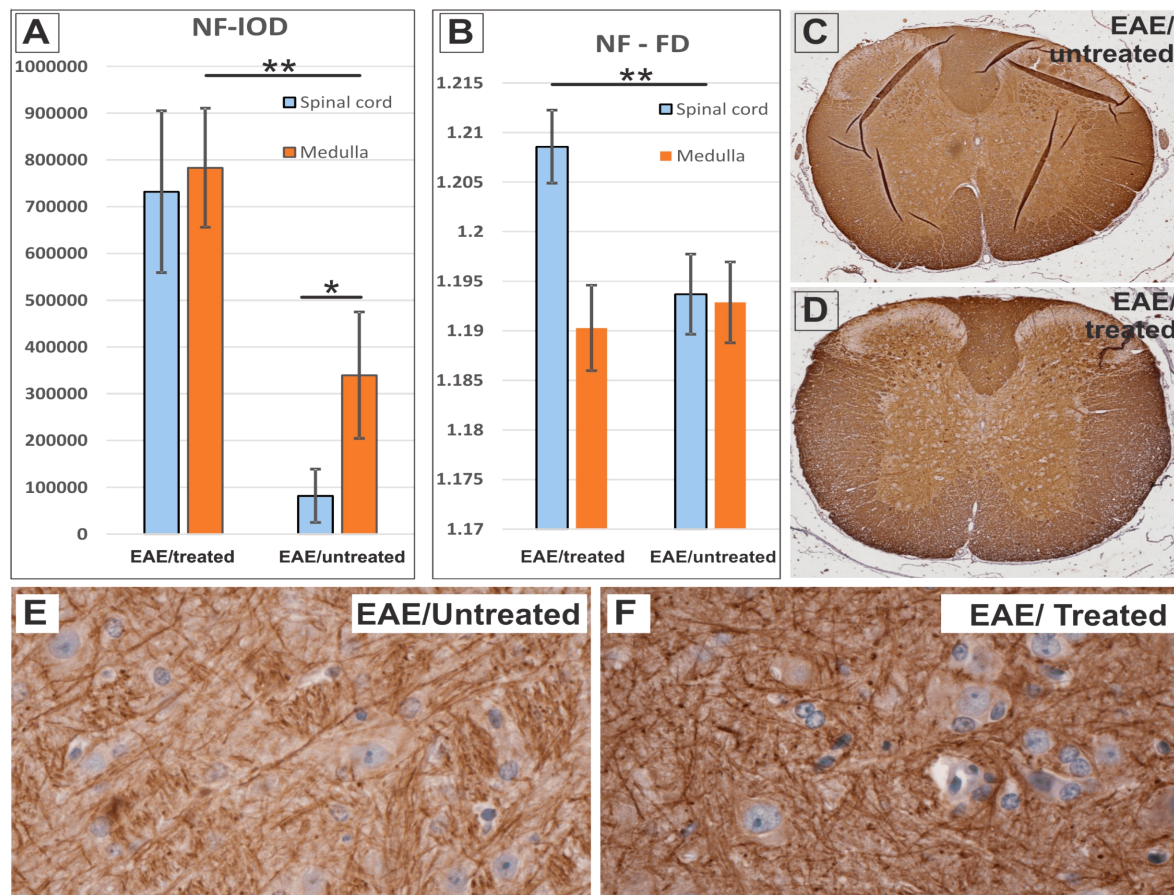
Figure 3 – *Neuronal cytoskeleton integrity. IOD of the immunostaining revealed a staining density decrease for the treated animals compared to untreated animals (**p<0.01) (A, C–F). For the spinal cord only, the morphology of the neurofilaments (NF) remained more complex compared to untreated animals (p<0.01) (B). Error bars represent standard error of the means (SEM). Immunohistochemistry for neurofilaments: (C and D) 200×; (E and F) 10× scan. IOD: Integrated optical density; FD: Fractal dimension; EAE: Experimental autoimmune encephalitis.*
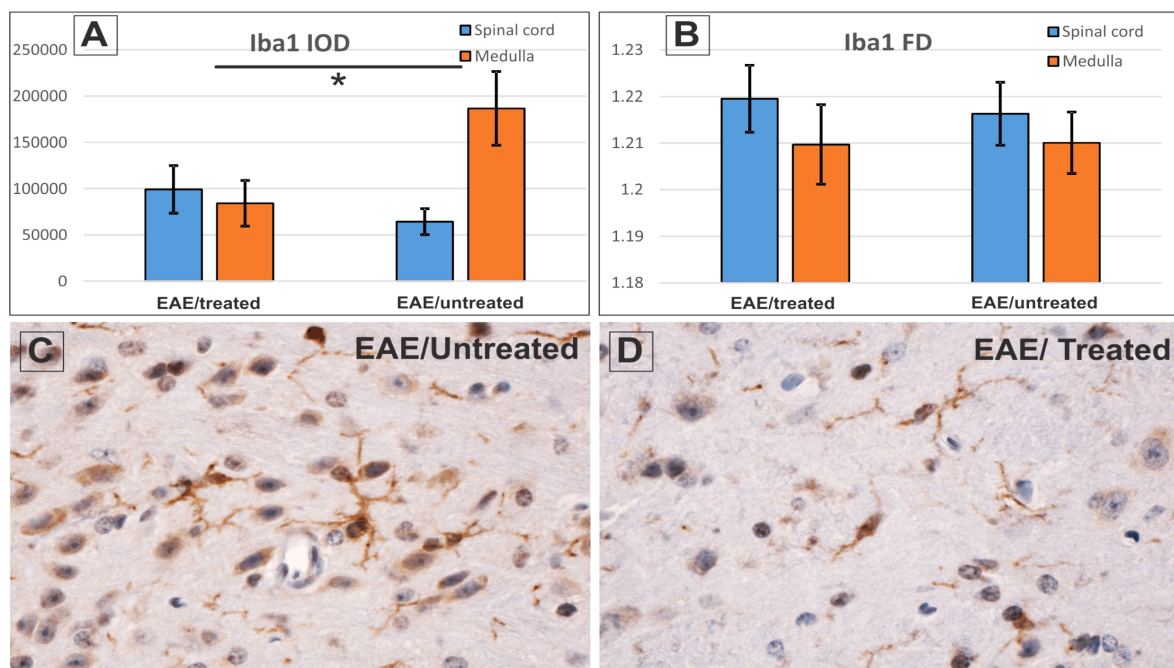

Figure 4 – *Microglia density and morphology. IOD for the treated animals showed a by-phasic pattern, with lower values for the spinal cord, while medulla density was much higher in treated animals (*p<0.05) (A, C and D). Microglia morphology, as illustrated by the fractal dimension (FD), was not different between the two animal groups and anatomical regions (B). Error bars represent standard error of the means (SEM). Immunohistochemistry for Iba1: (C and D) 200×. IOD: Integrated optical density; EAE: Experimental autoimmune encephalitis; Iba1: Ionized calcium-binding adaptor molecule 1.*

In any case, the first exhaustive description of the disease was given in the 1860s by Charcot, which underlined the need for more different attacks in the correct depiction of MS [25]. Initially, approximately 85% of the patients present with a relapsing-remitting form, in which attacks are separated by remission periods. There can be a neurological deficit in remission or not, and this form can persist for years. Anyway, of all these patients, about 50% will move to a progressive secondary form in which deficits of the disease accumulate without recurrence. Approximately 15% of all patients have a primary progressive form, in which the disease progressively worsens after the first onset, without remission [26]. At the tissue level, MS is depicted by demyelination, gliosis, axonal lesions and neuronal loss. Neurodegeneration that affects both white matter and grey matter is seen from the first stages of the disease [27].

In mice, EAE can be induced either by passive transfer of encephalitogenic T-lymphocytes or by active immunization with protein or peptides [28]. In both cases, the immunogen is obtained from the CNS's own proteins like as MBP, PLP or MOG. Immunization of SJL/J mice with PLP immunodominant epitope ($PLP_{139-151}$) induces a recurrent-remissional disease form [29], whereas the disease induced by the immunoglobulin peptide MOG_{35-55} in C57BL6/J mice tends to be a chronic type of disease [30]. There is an atypical form of EAE in some cases. This occurs especially in models obtained by the transfer of reactive T-lymphocytes [31].

Although not all MS therapeutic strategies have been developed with this model, all current immunomodulatory drugs approved by the *Food and Drug Administration* (FDA) are somewhat effective in treating EAE, a strong indicator that EAE is a model extremely useful for studying potential treatments for MS [2]. In EAE, clinical symptoms depict inflammation mainly located in the spinal cord, while EAE-induced MOG affects also the optic nerve [32]. Neuroinflammatory features translate into the infiltration of T- and B-lymphocytes, macrophages, and the same as by the constitution of focal demyelinating plaques in CNS, comparable to the pathology observed in MS [33]. T-lymphocytes are the key players in EAE and MS pathology. It is largely accepted that EAE is started by T-helper (Th) lymphocytes that break self-tolerance systems and link to myelin *via* T-cell receptors. In the subsequent stages of the disease, two T cells have been deemed responsible with maintaining the autoimmune response: Th1-secreting interferon gamma (IFNγ) and Th17-secreting interleukin-17 (IL-17) cells, which cooperate with antigen exhibiting cells and recruit effector cells: monocytes/macrophages and microglia in inflamed zones of development (especially in perivascular areas of the CNS and meninges), the phagocytic activity of these cells being bluntly responsible for demyelination [28, 34].

A large group of neurotrophic factors involved in survival, growth, and differentiation of the neurons and glia have been identified and tested on different neurodegenerative and cerebrovascular diseases [8, 35–37]. Cerebrolysin is a standardized extract of porcine origin, composed of low-molecular-weight neuropeptides and free amino acids, and has been utilized in many human and animal model clinical trials for ischemic stroke with variable results, mostly due to differences between the animal models and the human pathology, or to anatomical and histological differences [8, 38, 39]. In the present study, we aimed to assess the utility of Cerebrolysin treatment in a proven EAE mouse model, utilizing a 5 mL/kg daily dose of Cerebrolysin. First of all, we showed that the clinical deficit is clearly alleviated in the treated animals, compared to controls, although the treatment did not induce an immediate improvement but maintained the oscillatory pattern of the disease at a lower level.

The interactions and sequence of the various pathological processes leading to the histological lesions observation in MS are still insufficient understood. It is as well discussed if neuroinflammation or demyelinating are primary or secondary circumstances in the evolution of the disease [18]. We showed here that demyelination is reduced in Cerebrolysin-treated animals in both the optic nerves and at the level of the cervical spinal cord, but without significant differences for the medulla. Integrity of the cytoskeleton, as investigated by IHC for NF, showed statistically significant increased densities for treated animals, for both spinal cord and medulla regions.

There are currently several pathophysiological aspects of EAE with distinct clinical presentation paradigms depending on the immunization pathway used, animal species and protein/peptides. Thus, different paradigms were applied to study disease progress and specific histopathological features relevant to MS and to analyze the mechanisms of potential therapeutic processes.

$PLP_{139-151}$ was detected as an immunodominant epitope for EAE in the SJL model [40, 41] and the first clinical evidence of the disease could appear 12–18 days after one immunization. More than half of the immunized mice shoed evidences of relapse after recovery from the initial onset. Of note, recurrent EAE could also be determined by the passive transfer of T-reactive lymphocytes to $PLP_{139-151}$. Passive transfer evolved in a combination of forms of the disease, some animals developed a form of remission and relapsing and others developed monophasic disease [42]. Mice immunized with $PLP_{139-151}$ developed splenic lymphocyte responses at $PLP_{178-191}$ and these T-reactive lymphocytes at $PLP_{178-191}$ could induce EAE in adoptive transfer [40].

In the present work, we addressed to the final players in the immune chain leading to the myelin loss, which is the activated microglia. For the medulla regions, the density of the microglia decreased for the treated animals, while, interestingly, the cellular density seemed to increase in the spinal cords of the treated animal. More questions still remain, as the morphology of the microglia, as illustrated by the fractal dimension value of the cellular silhouettes, did not seem to change when comparing the treated model with the EAE-untreated animals. This suggests that the treatment influences subtle changes related to the number of the inflammatory cells, but not so much the final morphology of these monocytic cells.

In a seminal study, Mendel et al. [43] analyzed the ability of several MOG-derived peptides to caused EAE in B6 mice. Significantly, it was related that MOG_{35-55} determines a chronic form of the disease that does not resolute. Although this claim was independently confirmed

[30], it is also possible that the MOG$_{35-55}$ peptide dose may influence the course of the disease. Berard *et al.* [44] reported that whereas a large dose of peptide and adjuvant (300 μg MOG$_{35-55}$, 4 mg/mL *Mycobacterium tuberculosis* adjuvant, 300 ng PTX) causes a non-remising EAE form in mice B6, a small dose (50 μg MOG$_{35-55}$, 0.5–1 mg/mL of *M. tuberculosis* adjuvant, 200 ng PTX) induces recurrent-remising disease. These findings suggest that dosage under specific environmental conditions and careful titration of peptides may be required to standardize the form of disease arising after immunization with MOG$_{35-55}$. EAE-induced MOG$_{35-55}$ allowed analysis of the role played by autoimmunity in the CNS. Accumulated evidence reveals that CD8+ lymphocytes have a function in MS pathology. CD8+ T-lymphocytes exceed lymphocyte counts CD4+ in MS lesions by up to 10 to 1 [45], these T-lymphocytes can easily be discovered in the peripheral blood of MS patients [46]. So that, together these data postulate that CD8+ T-lymphocytes multiply in reply to myelin antigens and can circulate in inflammatory places in the context of CNS autoimmunity. Ford & Evavold [47] separated peripheral immune cells from mice immunized with MOG$_{35-55}$, reactivated them with peptides, and then transplanted CD8+ lymphocytes to receiver animals. They established that CD8+ T-lymphocytes are able to induce EAE in recipient animals as well as their CD4+ homologues [48].

⌕ Conclusions

In this animal model, the pathology seems to mostly involve the cervical spinal cord, rather the medulla, and Cerebrolysin seems to alleviate some of the pathological denominators like demyelination, neuronal cytoskeleton loss and microglia density. A moderate protection also seems to appear at the level of the optic nerve, where this EAE model shows a degree of demyelination, compared to control animals.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Laura Emilia Toader, Bogdan Cătălin and Ionica Pirici contributed equally to this manuscript.

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