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Exploring the therapeutic potential of Cerebrolysin-loaded exosomes in multiple sclerosis; an experimental study on neuroprotection and immune modulation

Mohammad Hassan Omrani[1], Sina Khodakarimi[1], Hanieh Beyrampour-Basmenj[2], Mahnaz Talebi[3], Naeimeh Akbari-Gharalari[4,1], Abbas Karimi[5], Zeynab Aliyari-Serej[6], Maryam Eyvazi[7], Ahmad Mehdipour[8], Abbas Ebrahimi-Kalan[1,3*]

[1]Department of Neurosciences and Cognition, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Department of Medical Biotechnology, School of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Neurosciences Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Neurophysiology Research Center, Cellular and Molecular Medicine Research Institute, Urmia University of Medical Sciences, Urmia, Iran
[5]Department of Molecular Medicine, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[6]Department of Applied Cell Sciences, School of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[7]Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[8]Department of Tissue Engineering, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

***Correspondence to**
Abbas Ebrahimi-Kalan, Email: abbasebra@gmail.com, Ebrahimiab@tbzmed.ac.ir

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Abstract

Introduction: Multiple sclerosis (MS) is a complex immune-mediated disorder of the central nervous system (CNS) characterized by inflammation and degeneration, currently lacking a cure. The blood-brain barrier (BBB) challenges of delivery of therapeutic agents to affected neural tissues. Exosomes, with low-immunogenicity and the ability to cross biological barriers, offer a promising avenue for targeted delivery of neurotrophic factors (NTFs) to support neuronal survival and regeneration. This study investigated the therapeutic potential of stem cell-derived exosomes (SCDEs) and Cerebrolysin-loaded exosomes (CLE) in an experimental autoimmune encephalomyelitis (EAE) model, a preclinical paradigm for MS.

Objectives: The co-delivery of NTFs and exosomes aimed to modulate the immune response and promote remyelination and neuroprotection in EAE.

Materials and Methods: An EAE modeling was induced in C57BL/6 mice in an experimental study, treated with SCDE, CBL, or CLE by intraperitoneal administration. Assessments included clinical scoring, gene expression analysis in splenocytes, and cytokine quantification.

Results: Results showed that the CLE group exhibited significant improvement in behavioral manifestations compared to SCDE and CBL groups. CLE treatment stimulated survival signaling pathways, influencing EAE progression, and suppressed pro-inflammatory transcription factors while upregulating anti-inflammatory factors in splenocytes. CLE significantly reduced pro-inflammatory cytokine interferon-gamma (IFN-γ) in serum samples, suggesting a neuroprotective effect by targeting pathways and overcoming BBB limitations.

Conclusion: The observed immune modulation and neural protection in the CLE group suggest its potential for MS treatment. Further investigations are needed to elucidate mechanisms and explore clinical applications. The study highlights the promise of CLE-based therapy in addressing MS pathology.

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Introduction

Around 2.8 million people based on recent data, in worldwide suffer from multiple sclerosis (MS), a long-lasting inflammatory condition that damages the neuronal myelin and is driven by immune system activity (1). MS is a complex disease involved by both genetic and environmental factors (2). Immune cells, particularly autoreactive CD4+ T cells, which secrete interferon-gamma (IFN-γ) and interleukins (IL-1, IL-17), are central to the pathogenesis of MS, as they play a significant role in central nervous system (CNS) invasion (3). Despite extensive research, the precise mechanisms underlying MS remain incompletely understood. Although various therapeutic strategies have been developed to manage MS, a definitive cure is still elusive (4). Mesenchymal stem cells (MSCs) exhibit anti-inflammatory, neuroprotective, and immunomodulatory properties, alongside self-renewal and multilineage differentiation capabilities (5). These traits make MSCs a highly potential option for addressing neurodegenerative disorders, including MS, spinal cord injuries, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (6). However,

Key point

This study explores the potential of cerebrolysin-loaded exosomes (CLE) for treating multiple sclerosis (MS) using a preclinical model. CLE demonstrated significant improvement in disease symptoms, promoting neuroprotection by modulating immune responses and reducing pro-inflammatory factors. These results indicate that CLE may represent a valuable treatment option for MS, justifying additional research into its potential clinical uses.

challenges such as potential tumorigenicity and the lack of standardized protocols for assessing the safety, efficacy, and bio-distribution of MSCs hinder their clinical application (7). Stem cell-derived exosomes (SCDEs) are nano-sized lipid-based vesicles that serve as natural delivery vehicles with low immunogenicity and the ability to transport bioactive molecules, maintain their stability in the bloodstream (Figure 1), and cross the blood-brain barrier (BBB) (8,9). Recent investigates have shown that MSCs exosomes can inhibit the proliferation of activated T cells by inducing cell cycle arrest (10). Neurotrophic factors (NTFs) play a crucial role in stimulating adult neurogenesis via autocrine and paracrine signaling. They are engaged in multiple processes related to the regeneration of neural and glial cells, such as managing neuro-inflammation, providing neuroprotection, facilitating regeneration, and supporting re-myelination (11). However, due to their relatively large molecular size (ranging from 10 to 35 kDa) and hydrophilic nature, NTFs face challenges in crossing the BBB and exhibit suboptimal pharmacokinetic properties when delivered systemically (12). Cerebrolysin (CBL), a peptide mixture derived from porcine brain tissue, represents an approved combination of NTFs. This blend contains NTFs, small peptides, neuropeptide-like elements, and free amino acids that replicate the effects of natural NTFs in water-based solutions (13). The effects of CBL on neurogenesis, neuroplasticity, and neuroprotection have been investigated in various neurodegenerative conditions (14). In experimental autoimmune encephalomyelitis (EAE) models, CBL has been shown to reduce inflammation and enhance motor recovery. The BBB remains a significant barrier to drug delivery, limiting the penetration of most therapeutics to approximately 5%-10% (15). The inability of many potent therapeutic agents to effectively cross the BBB underscores the need for innovative and clinically viable drug delivery systems (16). Recent research has highlighted exosomes as an ideal drug delivery platform, owing to their favorable characteristics (17). Consequently, studies focusing on SCDEs are of substantial interest (18).

Objectives

The aim of this study was to evaluate changes in the morphology, function, and biochemistry of EAE after intraperitoneal (i.p.) administration of SCDE and Cerebrolysin-loaded exosomes (CLE).

Materials and Methods

Materials

Female C57BL/6 mice have been widely used for modeling of MS in an experimental study and were obtained from the Pasteur Institute in Tehran, Iran. For Modeling of MS, complete Freund's adjuvant (Sigma-Aldrich, USA), peptide named myelin oligodendrocyte glycoprotein (MOG_{33-35}; SICBD), and Bordetella pertussis toxin (List Biological Lab, USA) were sourced to establish an accepted model named: EAE. Dulbecco's modified eagle medium (DMEM), Pen-Strep, FBS, Trypsin/EDTA were provided by GIBCO (Darmstadt, Germany). Ultra-filtration membrane was bought from Merck Millipore (Darmstadt, Germany). For the consideration of gene expression, the TAKARA real-time PCR (polymerase chain reaction) kit and Invitrogen TRIzol™ Reagent were conducted.

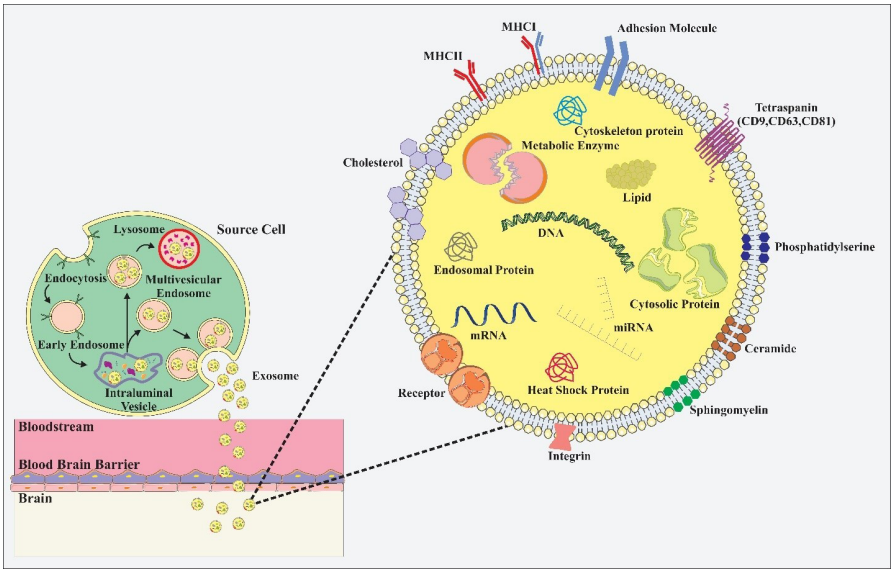

Figure 1. Biogenesis of exosomes and their ability to cross the blood-brain barrier (BBB). This schematic illustrates the process of exosome formation and their unique capacity to penetrate the BBB, a critical feature for the delivery of therapeutic agents to the central nervous system.

The DuoSet ELISA Development Kits for IFN (R&D) were used for conducting the ELISA (enzyme-linked immunosorbent assay) test. To analyze the entire slide, ImageJ software (version 1.32j, NIH, USA) was utilized. Exosomes characterized by dynamic light scattering (DLS) Nano Sizer (Nano-ZS; Malvern, UK) and; JEOL GX 400 D spectrometer and scanning electron microscopy (SEM) was performed on FEI Inspect-F (Philips, Eindhoven, NL) operated at 20 kV. All statistical analysis was performed using GraphPad Prism version 9.

Experimental animals

Here, aged 6-8 weeks female C57BL/6 mice, weighing between 19-21 grams, were maintained in a standard condition, with 6-8 mice per cage. The mice were randomly assigned to five experimental groups. The first group served as the negative control and received phosphate-buffered saline (PBS) (n=8). The second group (EAE) acted as the positive control (n=8). The third group (CBL) was treated with CBL at a dosage of 5 mg/kg/day following EAE induction. The fourth group consisted of EAE-induced mice that were treated with SCDE at a dose of 2.15 mg per mouse (n=8). The fifth group (CLE) comprised EAE-induced mice receiving CBL-loaded exosomes (n=8). All treatments were administered by i.p. injection.

Preparation of Cerebrolysin-loaded exosomes

MSC isolation

To isolate MSCs, C57BL/6 mice were sacrificed, and their femur bones were carefully dissected by cutting at the joints, with the ends excised. The bone marrow was flushed out using a 26-gauge syringe until the bones appeared pale. The collected cells were subsequently grown in a low-glucose DMEM environment enriched with fetal bovine serum (FBS) (heat-inactivated), penicillin, and streptomycin. This incubation lasted for 72 hours at 37 °C with an atmosphere of 5% CO_2. To eliminate non-adherent cells, the medium was aspirated, the cells were washed with PBS, and fresh complete medium was added. The culture medium was changed every three days, and MSCs from passages 3 to 5 were utilized for isolating exosomes (19).

Exosome isolation

The MSCs were transferred to T-75 flasks (passages 3–5) and allowed to grow until they achieved 70% confluency. Afterward, the cells were washed three times with PBS and placed in low-glucose DMEM without FBS for a duration of 72 hours. The conditioned media were then collected and processed through a series of centrifugation steps: first at 300 g for 10 minutes, followed by 1000 g for 20 minutes, and finally at 10 000 g for 30 minutes. The resulting solution was filtered using membrane filters with a pore size of 0.22 µm. Subsequently, the clarified sample underwent ultracentrifugation at 100 000 g for two hours to isolate the exosomes, after which the supernatant was removed. The exosome pellet was then re-suspended in PBS (20).

Characterization and measurement of exosome size

The size of exosomes was assessed through DLS. Each exosome sample underwent a dilution, where 100 µL was mixed with PBS at a 1:10 ratio, resulting in a total volume of 1 mL. This diluted solution was then placed in a cuvette, and three separate measurements were conducted for every sample. Furthermore, the exosome pellet obtained from ultracentrifugation was reconstituted and preserved in a 2.5% glutaraldehyde-PBS solution. After fixing, the sample was rinsed with PBS twice, dehydrated using an ethanol-based method, air-dried, and subsequently examined for morphological characteristics with a scanning electron microscope.

Loading of Cerebrolysin

A solution combining exosomes and CBL was created with a final volume of 100 µL and allowed to incubate at 37 °C for 20 minutes. After this incubation period, the solution underwent sonication using a water bath sonicator with the following parameters: 20% amplitude, six cycles of 30 seconds on followed by 30 seconds off, totaling three minutes, including a two-minute cooling phase between each cycle. Once sonication was complete, the mixture was again incubated at 37 °C for an hour before being subjected to ultracentrifugation at 100 000 g for two hours to isolate the exosomes as a pellet.

Induction of EAE

EAE was induced in mice following a well-established protocol. On day 0, which was designated as the day of immunization, EAE was initiated through a subcutaneous injection of recombinant MOG_{33-35} emulsified in complete Freund's adjuvant. Additionally, 300 ng of pertussis toxin, dissolved in 100 µL of PBS, was administered intraperitoneal on day 0 and again on day 2 post-immunization. The mice were clinically scored on a daily basis, with evaluations of their weight and overall health. A clinical scoring system was utilized each day, ranging from 0 to 7. A score of 0 meant no clinical signs were present, while a score of 1 indicated a slight reduction in tail tonicity. A score of 2 represented complete paralysis of the tail, and a score of 3 denoted an unusual gait accompanied by a limp tail. A score of 4 indicated paralysis in the hind legs, and a score of 5 reflected hind limb paralysis along with partial immobility of the hind body. A score of 6 represented paralysis in both the hind and forelimbs, while a score of 7 signified death or a severely ill state. These results were then compared to those of the control group to evaluate the effects of the treatments (21).

Real-time polymerase chain reaction (PCR)

To evaluate the impact of SCDE and CLE treatments on immune cells, we analyzed the expression of genes

Table 1. Primer sequences for quantitative real-time PCR

Primer	Forward primer	Reverse primer	Size of product (bp)
Foxp3	AGCAGTGTGGACCGTAGATGA	GGCAGGGATTGGAGCACTT	77
GATA3	AAGCTCAGTATCCGCTGACG	GTTTCCGTAGTAGGACGGGAC	86
Tbx21	AACCGCTTATATGTCCACCCA	CTTGTTGTTGGTGAGCTTTAGC	96
MMP-9	GCAGAGGCATACTTGTACCG	TGATGTTATGATGGTCCCACTTG	229
Rorc	CGCGGAGCAGACACACTTA	CCCTGGACCTCTGTTTTGGC	167
Actb	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	154

responsible for encoding transcription factors, specifically GATA3, T-box transcription factor (TBX21), RAR-related orphan receptor gamma (Rorc), forkhead box P3 (FOXP3), and matrix metalloproteinase 9 (MMP9) through quantitative real-time PCR (qPCR). Messenger RNA (mRNA) was isolated and purified from splenocytes using TRIzol™ Reagent, and complementary DNA (cDNA) was synthesized using RevertAid Reverse Transcriptase according to the manufacturer's instructions. For the qPCR analysis, SYBR Green PCR master mix was employed. The PCR reactions were performed on a Roche LightCycler PCR System under the defined conditions: a primary denaturation at 95 °C for 15 minutes, in continue 45 cycles of denaturation at 95 °C for 15 seconds, and annealing/extension at 60°C for 50 seconds, utilizing specific primer sequences as listed in (Table 1). The beta-actin (Actb) gene served as the reference gene, and the –ΔΔCt method was utilized to calculate relative changes in gene expression levels.

ELISA assay

Under sterile conditions, serum samples were obtained from mice. The lymphocytes were then suspended in RPMI medium enriched with 10% fetal calf serum at a density of 2.5×10^6 cells/mL and incubated in 24-well plates. These lymphocytes were then stimulated with soluble Leishmania antigens at a concentration of 40 µg/mL. The plates were incubated at 37 °C in a 5% CO_2 environment for 72 hours. Following incubation, the supernatants were collected and transferred into 500 µL tubes, which were stored at -70 °C until further analysis. The subsequent ELISA was performed according to the manufacturer's instructions.

Statistical analysis

The analysis of data was performed utilizing GraphPad Prism software. Findings are reported as means accompanied by their respective standard deviations (SD). For datasets that exhibited a normal distribution, comparisons between groups were carried out using one-way ANOVA, followed by the Bonferroni post hoc test. Conversely, when the data did not adapt to a normal distribution, the Kruskal-Wallis non-parametric test was applied for the evaluation of qualitative data. A significance threshold of $P < 0.05$ was established as the criterion for statistical significance.

Results

Solation and characterization of MSCs and exosomes

Dynamic light scattering

The results indicated that the exosomes exhibited a stable and uniform size distribution, with an average diameter measuring 58.33 nm, as illustrated in (Figure 2A) through the evaluation of the size distribution and zeta potential of the microvesicles by DLS.

Scanning electron microscopy

The morphology of the exosomes was examined using SEM. Exosome samples were fixed in 5% glutaraldehyde for two hours, placed on APTES-treated silicon wafers, and allowed to adhere for 1 hour. The specimens underwent a thorough washing process with PBS, followed by dehydration using a sequential ethanol gradient consisting of 20%, 50%, 70%, 90%, 95%, and finally 100%. Subsequently, after being dried at the critical point and coated with a layer of gold, the exosomes were examined (22). Specifically, exosomes (1 µg) were re-suspended in 1 mL of filtered PBS at pH 7.4, as illustrated in (Figure 2B).

Gene expression and Cerebrolysin-loaded exosomes

Quantitative analysis of gene expression for key transcription factors, including FOXP3, TBX21, GATA3, Rorc, and MMP9, was performed in both control and experimental groups using real-time PCR. They are depicted in (Figure 3A-E). The reduced expression of TBX21 in the treatment groups indicates a decrease in Th1 cell levels, leading to lower production of IFN-γ. Additionally, the expression of the pro-inflammatory gene Rorc was significantly suppressed in the treatment groups, particularly in the EAE+EXO+CBL group. The CBL, SCDE and CLE groups also showed reduced the expression of MMP9, which contributes to better disease management. Conversely, there was a consistent upregulation of GATA3 and FOXP3 expression across all treatment groups, correlating with a reduction in EAE symptoms.

Cerebrolysin-loaded exosomes and interferon-gamma expression

The early onset of the symptoms in EAE is primarily driven by the IFN-γ expression, a key cytokine produced by Th1 cells, which are crucial inflammatory mediators in autoimmune diseases such as MS. Our findings demonstrated that both the CBL and CLE treatment

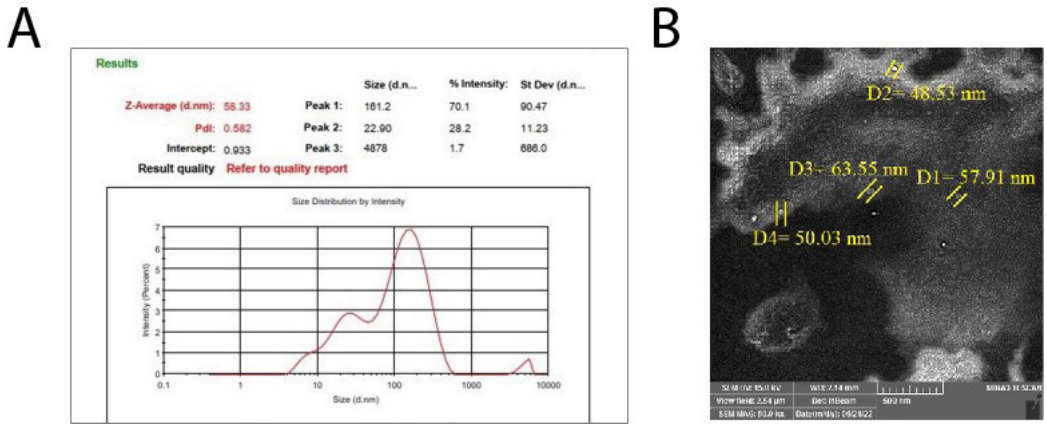

Figure 2 (A and B). Characterization of exosomes using dynamic light scattering (DLS) and scanning electron microscopy (SEM). The figure presents the size distribution and morphology of the isolated exosomes, as determined by DLS and SEM analyses. The DLS data show a consistent size distribution with an average diameter of approximately 58 nm, and the SEM images reveal the characteristic cup-shaped structure of the exosomes.

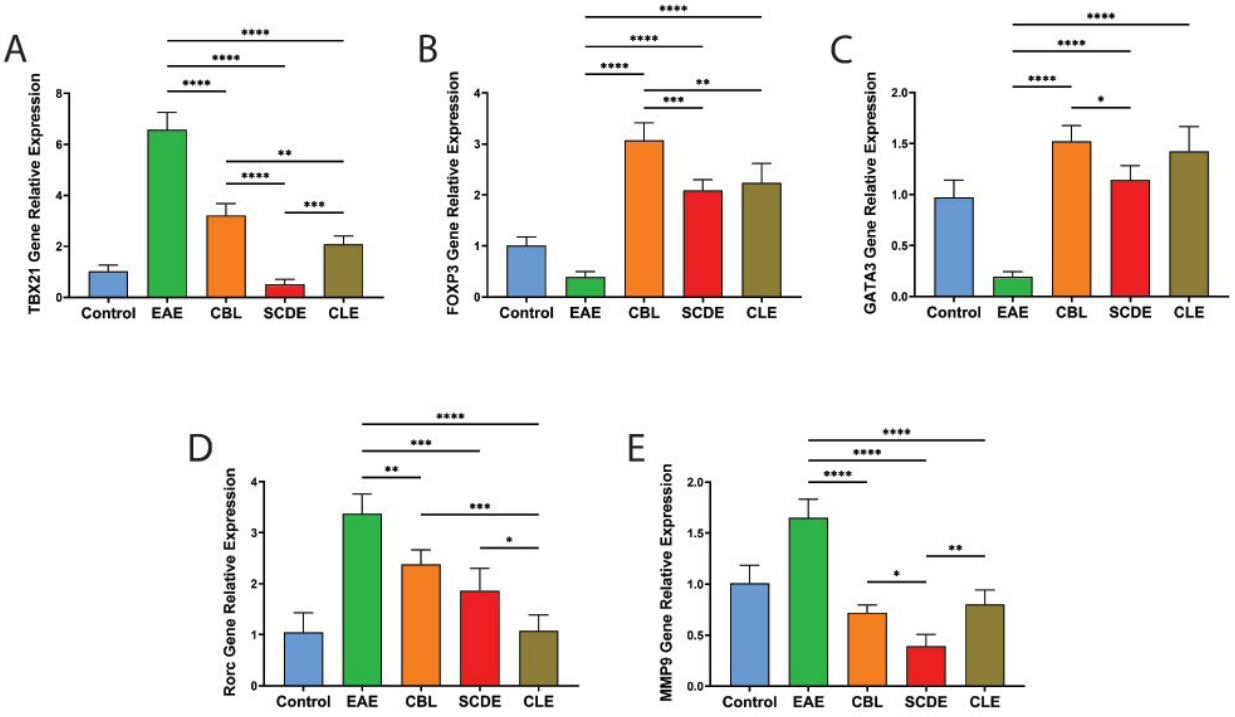

Figure 3 (A-E). Modulation of gene expression in splenocytes by cerebrolysin-loaded exosome (CLE). This figure displays the relative expression levels of key transcription factors involved in the regulation of inflammatory and anti-inflammatory responses, including TBX21, GATA3, FOXP3, Rorc, and MMP9, in the splenocytes of the different treatment groups. The data demonstrate that the CLE treatment significantly suppressed the expression of pro-inflammatory factors, such as Rorc, while upregulating the anti-inflammatory markers GATA3 and FOXP3, compared to the EAE control group. Significance levels are indicated as follows: (*p ≤ 0.05), (**$P \leq 0.01$), (***$P \leq 0.001$), and (****$P \leq 0.0001$).

groups exhibited a significant reduction in IFN-γ expression. This suppression of the pro-inflammatory cytokine corresponded with a marked alleviation of early-stage disease symptoms. As shown in (Figure 4), IFN-γ expression decreased significantly, from 600 pg/mL to about 200 pg/mL in CBL group.

Assessment of locomotion activity following EAE induction

To determine the efficacy of rearing activity as an indicator of EAE progression and severity, we utilized a scoring system to evaluate locomotion. The maximum score on this scale was 7, with a score of 7 indicating mortality due to the disease; however, none of the mice in our study reached this extreme outcome. Significant increases in clinical scores were observed in EAE induced mice beginning on day 12 post-immunization, with rising paralysis and complete tail limp becoming apparent between days 18 and 21. Hind limb paralysis was noted between days 21 and 23. Treatment with CBL began on days 13 and 19 for the SCDE and CLE groups, respectively. By day 24 post-

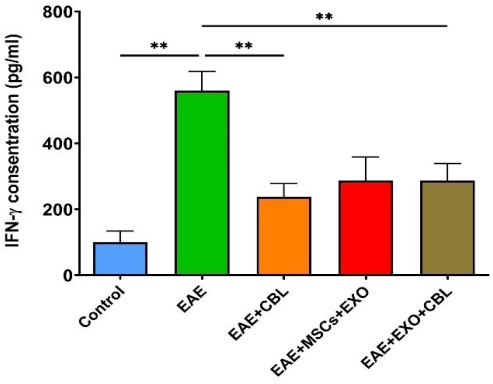

Figure 4. Reduction of IFN-γ production in serum by cerebrolysin-loaded exosome (CLE). The graph depicts the changes in serum IFN-γ levels across the control and treatment groups. The CLE treatment group showed a marked reduction in IFN-γ expression compared to the EAE control group, suggesting an attenuated Th1-mediated inflammatory response. Significance levels are indicated as follows: (*$P \leq 0.05$), (**$P \leq 0.01$).

immunization, the animals were sacrificed. The results indicated that the CBL and CLE groups had lower clinical scores compared to the control group, as illustrated in (Figure 5A).

Discussion

Considering the inflammatory and degenerative nature of MS, the ability of NTFs to traverse the BBB may benefit neuronal survival in MS. This study aimed to investigate the use of exosomes as carriers for NTFs across the BBB. SCDEs contain various anti-inflammatory and neuroprotective agents, which may play a role in modulating immune responses, promoting re-myelination, and supporting neuroprotection in neurodegenerative diseases (23). However, many mentioned factors have short half-lives in in-vivo, poor pharmacokinetic properties, and limited BBB penetration. These factors restrict their effectiveness due to proteolytic degradation, clearance mechanisms, and binding by peripheral tissue components. We hypothesized that co-delivery of NTFs using exosomes could effectively modulate immune activity and enhance re-myelination in MOG-induced EAE in C57BL/6 mice. Treatments with CBL, SCDE, and CLE were administered intraperitoneal on days 13 and 19 post-immunization (corresponding to the onset and peak of clinical symptoms, respectively). The results indicated that treated groups had significantly lower clinical scores compared to the EAE group, suggesting that SCDE and CLE have protective effects on neurons. The favorable properties of exosomes, including enhanced stability in circulation, biocompatibility, low immunogenicity, and effective cellular uptake, facilitate the delivery of NTFs to neural cells in the CNS (24). NTFs such as CNTF, GDNF, NGF, and BDNF have been shown to play protective roles in MS by reducing neuronal cell death and promoting neurogenesis and brain function in various brain diseases, including closed head injury and stroke (25). CBL has been reported to promote neuroprotection and neurogenesis through the upregulation of factors like NGF and BDNF (26). BDNF, which is translated as proneurotrophin (pro-BDNF), can be cleaved into its mature form. Mature BDNF binds to high-affinity receptor tyrosine kinase B (TrkB), modulating survival signaling transduction that may help reverse neurodegenerative diseases (27). Elevated levels of pro-BDNF in circulating lymphocytes and inflammatory cells at CNS lesion sites in MS patients suggest that administering mature BDNF could prevent apoptosis (28). Neurological function tests conducted daily to assess the efficacy of CBL, SCDE, and CLE revealed that the treated groups had substantially lower clinical scores compared to the EAE control group, indicating significant improvements in performance. This suggests that exosome-based delivery systems can enhance neuroprotection and behavioral outcomes in MS models. Th1 and Th17 cells are primarily implicated in

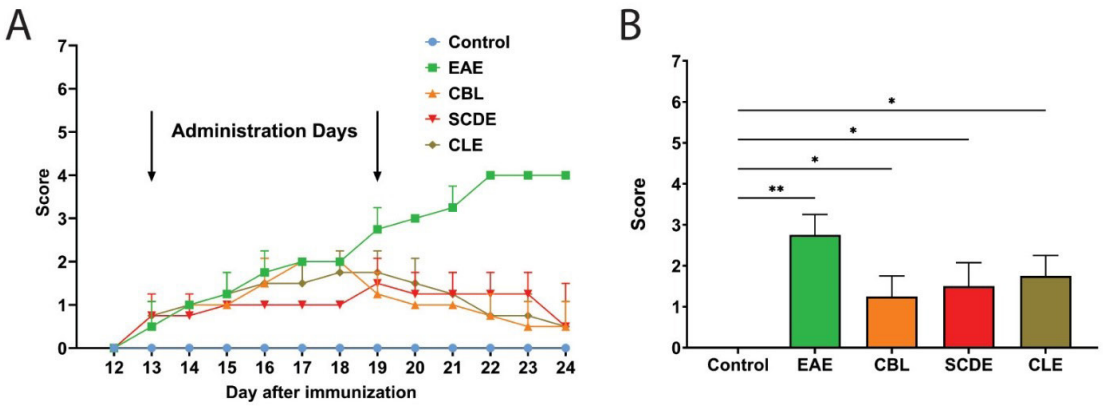

Figure 5 (A-B). Improved locomotor function in the EAE model with cerebrolysin-loaded exosome (CLE) treatment. The figure presents the longitudinal assessment of clinical scores, which reflect the severity of the disease in the different treatment groups. Panel A shows the time course of the clinical scores, demonstrating that the CLE group exhibited lower scores compared to the EAE control, indicating reduced disease severity. Panel B compares the clinical scores on a specific day, further highlighting the improved locomotor function in the CLE-treated mice compared to the EAE control, even in clinical score of CLE and SCDE groups compare to EAE, alteration is significant. The data are presented as the mean ± standard error of the mean for a sample size of 8. Statistical significance is denoted as follows: (*$P \leq 0.05$) and (**$P \leq 0.01$).

MS pathogenesis due to their production of IFN-γ and interleukin-17, respectively (29). Autoreactive CD4+ T cells enter the brain parenchyma via VLA-4 and LFA-1 receptors (30), secreting pro-inflammatory cytokines such as IFN-γ, TNF-alfa, IL-17, IL-21-23. These cytokines act as chemoattractants, promote phagocytosis, and activate microglia and astrocytes, contributing to CNS inflammation and damage (31). To evaluate the impact of treatments on pro-inflammatory and anti-inflammatory cells, transcription factors were assessed in splenocytes isolated 24 days after immunization. RORγt, which is crucial for Th17 cell differentiation, was significantly reduced in the CLE group compared to other groups (32). Foxp3 expression, essential for T regulatory (Treg) cell function and self-tolerance, was increased by CBL and CLE compared to the EAE and control groups (33). GATA3, a transcription factor critical for Th2 differentiation, T cell development, and NK cell function, was also upregulated by CBL and CLE (34). Conversely, TBX21, which encodes the T-bet transcription factor crucial for Th1 cell commitment and IFN-γ production, was decreased by SCDEs compared to other groups (35). Additionally, MMP9, which facilitate the influx of inflammatory cells and the breakdown of the BBB, were significantly reduced in SCDE-treated mice.

Study Highlights

What is the current knowledge?

- Multiple sclerosis (MS) prevalence: Nearly 2.8 million people worldwide suffer from MS.
- MS characteristics: It is a chronic inflammatory and immune-mediated CNS
- Role of Th cells: Th cells, particularly autoreactive CD4+ T cells, are crucial in CNS invasion and disease progression.
- MS treatment challenges: Existing treatments cannot cure MS; precise mechanisms remain elusive.
- Potential of MSCs: MSCs exhibit anti-inflammatory, neuroprotective, and immune-modulatory properties.

What is new here?

- Use of SCDE and CLE: The study evaluates the effects of stem cell-derived exosomes (SCDEs) and CLE in an MS model.
- Enhanced delivery system: Exosomes provide a novel drug delivery mechanism, improving stability, biocompatibility, and BBB penetration.
- Gene expression modulation: CLE treatment reduced pro-inflammatory gene expression and increased anti-inflammatory markers.
- IFN-γ reduction: Significant reduction in IFN-γ levels, indicating decreased Th1-mediated inflammation.
- Improved clinical outcomes: Lower clinical scores in EAE mice treated with SCDE and CLE indicate better neuroprotection and functional recovery.

Conclusion

In summary, this research emphasizes the role of immunomodulatory effects of exosomes, particularly SCDEs, in modulating the immune response. SCDEs demonstrated potential in suppressing pro-inflammatory cytokines, reducing the differentiation of pathogenic Th17 cells, and increasing regulatory T cell levels. Additionally, CLE treatment resulted in a significant reduction in IFN-γ expression, indicating effective suppression of pro-inflammatory signaling. These findings support the therapeutic potential of exosomes, especially SCDEs and CLEs, in treating MS. Further research into the mechanisms underlying the immunomodulatory effects of exosomes is crucial for developing innovative treatments for autoimmune and neurodegenerative disorders. This study underscores the significant impact of exosomes on immune regulation and advocates for their continued exploration in clinical applications.

Limitations of the study

One of our constraints was the unified NTFs as CBL, instead of utilization of NTF separately. It is recommended that future research utilized them separately.

Authors' contribution

Conceptualization: Abbas Ebrahimi-Kalan, Mohammad Hassan Omrani, Mahnaz Talebi, Abbas Karimi.
Data curation: Abbas Ebrahimi-Kalan, Hanieh Beyrampour-Basmenj, Abbas Karimi.
Formal analysis: Abbas Ebrahimi-Kalan, Sina Khodakarimi, Ahmad Mehdipour.
Funding acquisition: Abbas Ebrahimi-Kalan.
Investigation: Abbas Ebrahimi-Kalan, Mahnaz Talebi, Zeynab Aliyari-Serej.
Methodology: Abbas Ebrahimi-Kalan. Mohammad Hassan Omrani, Houman Kahroba, Zeynab Aliyari-Serej.
Resources: Sina Khodakarimi, Hanieh Beyrampour-Basmenj, Naeimeh Akbari-Gharalari.
Supervision: Abbas Ebrahimi-Kalan.
Validation: Abbas Ebrahimi-Kalan, Mahnaz Talebi, Abbas Karimi.
Visualization: Abbas Ebrahimi-Kalan.
Writing—original draft: Mohammad Hassan Omrani, Sina Khodakarimi, Naeimeh Akbari-Gharalari.
Writing—review and editing: Abbas Ebrahimi-Kalan, Mohammad Hassan Omrani, Sina Khodakarimi, Naeimeh Akbari-Gharalari.

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Ethical issues

The research protocol received approval from the Faculty of Advanced Medical Sciences at Tabriz University of Medical Sciences (Ethical code#IR.TBZMED.VCR.REC.1398.24). All experimental procedures adhered to the regulations set by the Research Ethics Committee of the university and the Iranian Ethical Guidelines for animal research. Furthermore, all animal experiments were carried out in accordance with protocols approved by the United States National Institutes of Health (NIH, 1978). Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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