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Transcranial magnetic stimulation modifies astrocytosis, cell density and lipopolysaccharide levels in experimental autoimmune encephalomyelitis.

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ABSTRACT

Aims: Experimental autoimmune encephalomyelitis (EAE) is considered a valid experimental model for multiple sclerosis, a chronic neuroinflammatory condition of the central nervous system. Additionally, some evidence has shown that some microbial products such as the bacterial lipopolysaccharide could lead to the activation of reactive immune cells, triggering neuroinflammation. Several studies have found that transcranial magnetic stimulation (TMS) may exert a neuroprotective effect. Therefore, we aimed to assess the effect of TMS on the neuroinflammation occurring in EAE.

Materials and Methods: A total of 44 male Dark Agouti rats were used. EAE induction was performed administering subcutaneously at the dorsal base of the tail a single dose of myelin oligodendrocyte glycoprotein. Clinical evaluation of motor symptoms was performed. Brain and spinal cord were collected and analyzed for nitric oxide, bacterial lipopolysaccharide and lipopolysaccharide-binding protein. We also carried out a histologic exam, which included an astrocyte immunostaining and Nissl staining for the assessment of brain cell density and pyknotic nuclei.

Key findings: TMS effectively ameliorated motor impairment secondary to EAE. This form of magnetic field was capable of decreasing the proliferation of astrocytes as a response to the autoimmune attack, reducing the content of nitric oxide, bacterial lipopolysaccharide and lipopolysaccharide-binding protein in central nervous system. Moreover, in treated animals, brain cell density was improved and the number of pyknotic nuclei was decreased.

Significance: Transcranial magnetic stimulation modifies astrocytosis, cell density and lipopolysaccharide levels in EAE. These results suggest that TMS could be a promising treatment for neuroinflammatory conditions such as multiple sclerosis.

Keywords: Bacterial lipopolysaccharide, experimental autoimmune encephalomyelitis, neuroinflammation, nitric oxide, transcranial magnetic stimulation.

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INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is considered a valid experimental model for multiple sclerosis (MS), a chronic debilitating neuroinflammatory condition of the central nervous system (CNS), in which demyelination, gliosis and axonal loss are encountered [1]. During neuroinflammatory processes like EAE or MS, astrocytes, i.e. cells belonging to the neuroglia which participate in neuronal homeostasis, become highly active and may exert functions such as phagocytosis, inflammatory mediator production (like nitric oxide (NO)), and antigen presentation, being responsible for oxidative/nitrosative damage that leads to cellular dysfunction, progressive axonal loss and neuronal degeneration. Additionally, in relation to the pathogenesis of CNS disorders, there is growing evidence highlighting the role of commensal gut flora as contributive to the development of these conditions, especially in those closely linked to autoimmunity. In certain scenarios, both metabolites and microbial products such as the bacterial lipopolysaccharide (LPS), could lead to the activation of CNS-reactive immune cells, triggering neuroinflammatory phenomena [2].

Recently, Elzamarany et al. have shown that transcranial magnetic stimulation (TMS) may improve dexterity in MS patients [3]. In this line, some experimental studies have pointed out that extremely low-frequency electromagnetic fields (EL-EMF), a paradigm of TMS, may exert a neuroprotective effect in models of Huntington's disease, Alzheimer's disease and depression by attenuating cell loss and oxidative stress [4-8], and may induce neurogenesis via brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) [9-12]. This non-invasive therapy applies a magnetic field through the skull which induces changes in the polarization of neurons and finally some beneficial effects [13].

Therefore, based on this background, the objective of the present study was to assess the effect of TMS on motor condition and neuroinflammatory markers in a rat model of EAE.

EXPERIMENTAL PROCEDURES

Design of the study

The experiment included the following study groups: i) Control, ii) Myelin oligodendrocyte glycoprotein (MOG)-EAE, iii) EAE + Mock TMS, and iv) EAE + TMS.

Forty-four young-adult (8-week old) male Dark Agouti rats (Janvier Labs, France) weighing 190–220 g were used. Animals were housed under controlled conditions of illumination (12h light / 12h dark cycle, lights on at 08:00 h) and temperature (20–23°C), and supplied with water and food (Purina[®], Spain) *ad libitum*.

The rats were randomized into groups using computer-generated random permutations. Two major groups of animals were used in order to assess:

- i) inflammatory mediators/markers (NO, lipopolysaccharide binding protein (LBP) and LPS (n = 5; total n = 20), and
- ii) pathohistological features (Astrocyte-immunohistochemistry and Nissl staining for cell density) (n = 4 each group/staining except EAE + Mock-TMS, total n = 24).

Based on studies of the EAE time pattern [14], all animals were assessed for clinical status at day 0 (induction of EAE), 14 (onset disease and start of treatment) and 35 (relapse of disease and end of experiment). Their motor condition was evaluated according to the scale by Pérez-Nievas [15], in which: 0 = any sign, 1 = tail paralysis, 2

= hind limb paresis, 3 = hind limb paralysis, 4 = hind limb paralysis plus front limb paresis, 5 = moribund or dead. TMS was applied from day 14 to 35, day in which animals were sacrificed by decapitation, and brain and spinal cord specimens were collected.

An independent “blind” investigator who did not know the experimental group performed the evaluation of clinical status. To assure blindness, the biochemical and histological assessments were unlabeled.

The experiment was approved by the Bioethics Committee of Cordoba University and carried out according to the guidelines of the Directive of 24 November 1986 (86/609/ECC) approved by the European Communities Council and RD 53/2013 approved by Presidency Minister of Spain (BOE 08 of February of 2013).

EAE induction

EAE induction was performed at day 0 by administering subcutaneously at the dorsal base of the tail a single dose of 100 μ l with 150 μ g of MOG (fragment 35–55; Sigma, USA) in phosphate buffered saline (PBS) emulsified 1:1 in complete Freund’s adjuvant (Sigma, USA). To complete the adjuvant, 400 μ g of *Mycobacterium tuberculosis* (H37Ra, DIFCO, USA) inactivated by heat were added [15].

TMS application

The stimulation consisted of an oscillatory magnetic field in the form of a sinusoidal wave with a frequency of 60 Hz and amplitude of 0.7 mT (EL-EMF). To reproduce clinical practice, TMS was applied in the morning for two hours, once a day, five days a week (Monday-Friday), during three weeks (days 14 – 35) (modified from [16]). Rats were placed in plastic cylindrical cages designed to keep them immobile.

The two Helmholtz coils (Magnetoterapia S.A., Mexico) were positioned dorsally and ventrally to the head at a distance of approximately 6 cm between each coil and the midpoint of the head. Animals in the TMS-Mock group were handled in the same way but without receiving real stimulation.

Biochemical parameter assays

Under controlled temperature conditions, the brains and spinal cords were extracted and the corresponding homogenates immediately prepared with a mechanical homogenizer (Tempest Virtis). The buffer used for homogenization was Tris (20mM) at pH 7.4.

Spectrophotometric measures were performed using a UV-1603 spectrophotometer (Shimadzu, Japan). ELISA determinations were made using a Multimode Detector DTX-880 Beckman Coulter (Barcelona, Spain).

Nitric oxide

Total nitrite (nitrite + nitrate; NO_x), analyzed following the Griess method [17], can be used as a marker of NO production. To perform the Griess assay, all nitrates must be reduced to nitrite, and then, total nitrite is determined spectrophotometrically by Griess reaction.–The reaction is read at 540 nm and values presented in μmol/mg protein.

Lipopolysaccharide-binding protein

The assessment of LBP was performed using the LBP soluble (mouse) ELISA Kit (Enzo[®], USA). The reaction is read at 450 nm and values presented as pg/mg protein.

Bacterial lipopolysaccharide

LPS was assessed using the Pierce® LAL Chromogenic endotoxin quantification kit provided by Thermo Scientific (USA). The reaction is photometrically measured at 405-410 nm. Data are expressed as endotoxin units/mg protein.

Due to logistic reasons LPS was only studied in spinal cord.

Protein estimation

Protein levels were measured by the Bradford method, using a B6916 assay kit supplied by Sigma (USA).

Histologic studies

Based on previous studies on the effect of TMS on the brain [4, 9], striate nuclei were studied. Counts were made using a counting frame (area) of 35,500 μm^2 (40x), which was randomly placed at ten different zones. Then, the counts were averaged. Quantification was carried out in a semi-automatic manner with the software Image-Pro Plus® (Media Cybernetics, USA).

Nissl staining

Whole brains fixed in 10% buffered formaldehyde were embedded in paraffin wax, cut into 8- μm thick sections and stained with 0.025% cresyl violet (Nissl-stained). Sections were evaluated under brightfield illumination on a Leitz Orthoplan microscope (Herramientas Leitz S.L., Spain). The estimation of cell density was based on counting nuclei. Pyknosis, the irreversible condensation of chromatin in the nucleus undergoing necrosis or apoptosis, was evaluated based on nucleus morphology.

Immunohistochemistry

Astrocytes are cells characterized by expressing glial fibrillary acidic protein (GFAP), so the astrocyte population was studied using an immunohistochemical stain for the GFAP.

Once the circulatory system was washed with 0.1 M PBS at pH 7.4 and then with 4% paraformaldehyde, brain was post-fixed by immersing it in 4% paraformaldehyde in PBS overnight. After washing with PBS, the tissue was immersed in ascending concentrations (10, 20, and 30%) of sucrose overnight at 4°C and then embedded in OCT compound. Then, frozen sections of 20 µm were cut with a cryostat. After blocking the sections with 1.0% normal donkey serum plus 1.0% bovine serum albumin and 0.1% triton-X 100 in PBS, the sections of brain were incubated with goat polyclonal GFAP-specific antibody (Cat. ab53554, Abcam®, USA) 1:1000 at 4° C during 16/24 hours. Then, sections were incubated in donkey goat-specific antibody Alexa 555 (Orange) (Cat. A-21432, Life Technologies, USA) 1:1000 during two hours at room temperature. Finally, samples were counterstained with 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI) (Sigma, USA) to stain nuclei and produce blue fluorescence with ultraviolet illumination (excitation wavelength 461 nm), and then mounted in fluorescence mounting medium (S3023, Dako, Agilent Technologies, USA) and examined using a Leica DM2500 microscope (Leica Microsystems, Austria). GFAP/DAPI double positive cells were counted as astrocytes.

Statistical analysis

All results are presented as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) corrected with Bonferroni's post-hoc test was used for

comparing means in biochemical parameters as normality criteria were met after Shapiro-Wilk test. Clinical score was analyzed by Kruskal-Wallis test. In histological examinations, one-way ANOVA with Student-Newman-Keuls's method was used for cell density and one-way ANOVA with Dunn's method for immunohistochemistry (SigmaStat 3.1[®], Systat Software, USA).

The statistical significance was set at $p < 0.05$ (two-tailed test).

RESULTS

TMS ameliorated motor skills in EAE model

At day 14, all animals in which MOG was inoculated showed signs of disease, most of them presenting hind limb paresis or paralysis (Figure 1A). The motor score for control group was 0 during the whole study. Only one rat died before day 35 (EAE, Nissl-staining group). Animals receiving TMS recovered motor skills while untreated ones worsened. At day 35, TMS-treated animals presented only tail paralysis or hind limb paresis, while paralysis in non-treated rats tended to progress to front limbs (Figure 1B and 1C). As expected, no differences between EAE and EAE Mock TMS groups were found so biochemical and histologic assessments were not performed in this latter group.

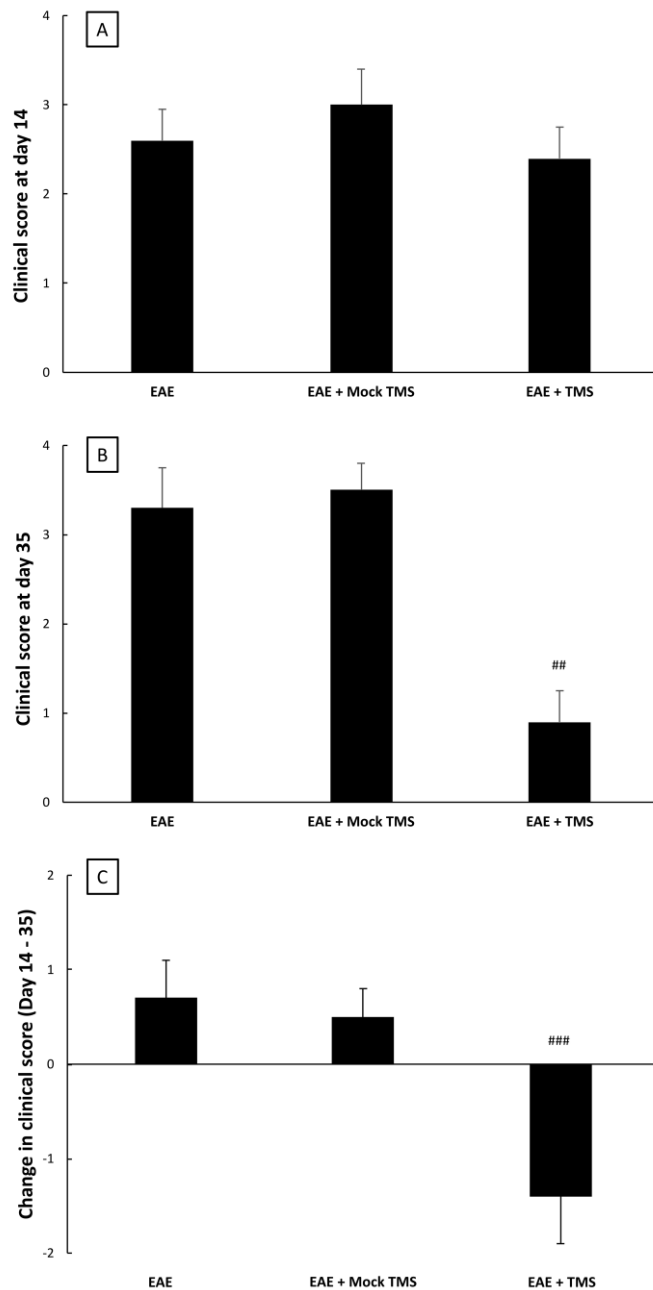


Figure 1. Representation of clinical scores. (A) Bar chart of clinical scores at day 14 (onset disease, start of treatment). (B) Bar chart of clinical scores at day 35 (relapsing disease, end of experiment). (C) Bar chart of the change in clinical scores between day 14 and 35. Bar charts represent mean \pm SD. Unless indicated by symbol, no statistical significant differences were observed between groups. ^{##} $P < 0.01$, ^{###} $P < 0.001$, compared with EAE and EAE + Mock TMS (Kruskal-Wallis test). All animals in the control group scored 0.

TMS prevented cell changes induced by MOG in EAE model

Regarding cell density, EAE led to a significant decrease in the mean nuclei number in the striatum by 22% compared with the control group, while therapy with TMS partially reverted this phenomenon (8.2%) (Figure 2). No pyknotic nuclei were observed in the control group. The treatment with TMS significantly reduced the number of pyknotic nuclei (Figure 2). Neurodegeneration was noted in this study since in pyknotic neurons that were stained darkly; no nucleus or nucleolus was visible. Other cells presented a smaller nucleus with condensed chromatin and swollen cytoplasm.

Astrocyte population was also studied. These cells were increased in the striatum by 158.0% of EAE animals (compared to Control), whereas TMS palliated astrocytosis (27.4%). No statistical significant difference was found between Control and TMS group (Figure 3). Moreover, TMS led to the normalization of the ratio between astrocytes and cell density (0.04, 0.13 and 0.05 in Control, EAE and TMS group, respectively).

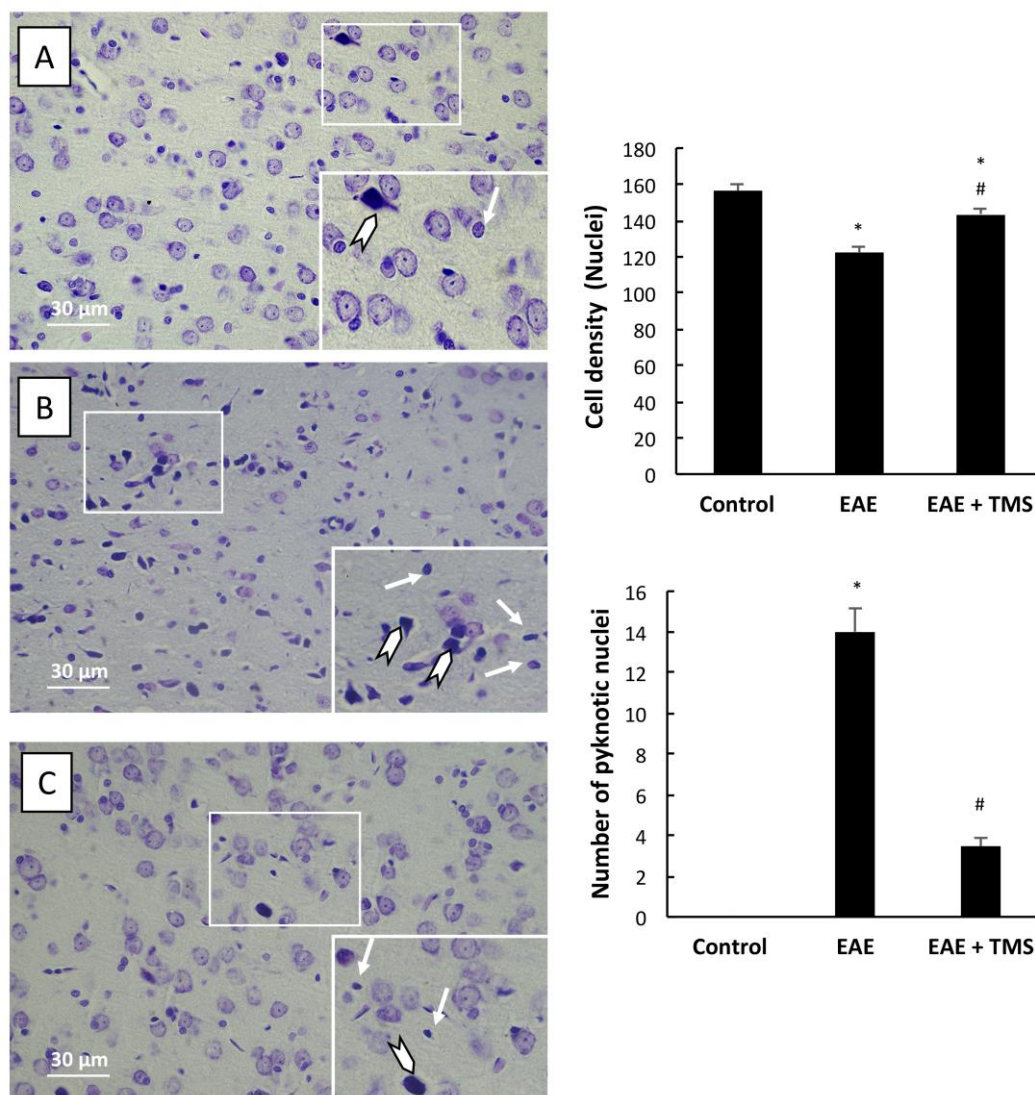


Figure 2. Evaluation of cell density and pyknotic nuclei by Nissl.

Cell density was estimated by counting nuclei. Counts were performed using a counting frame (area) of $35,500 \mu\text{m}^2$ (40x), which was randomly placed at ten different zones of the *striatum*. Then, the counts were averaged. (A) represents control group. Reactive gliosis (white arrows) and pyknotic nuclei (arrowhead) in EAE (B) is decreased after treatment with TMS (C). Bar charts represent mean \pm SD. Unless indicated by symbol, no statistical significant differences were observed between groups. One-way ANOVA with Student-Newman-Keuls's method was used. * $P < 0.05$ compared with control. # $P < 0.05$ compared with EAE.

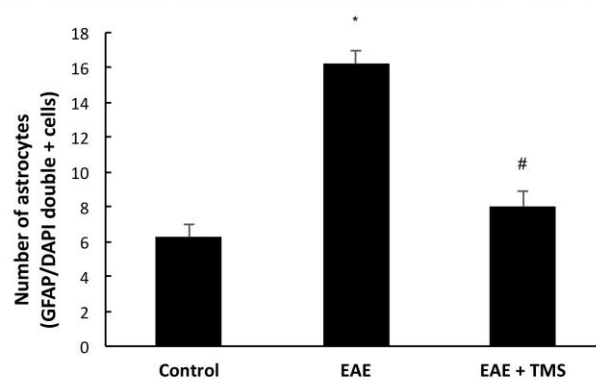
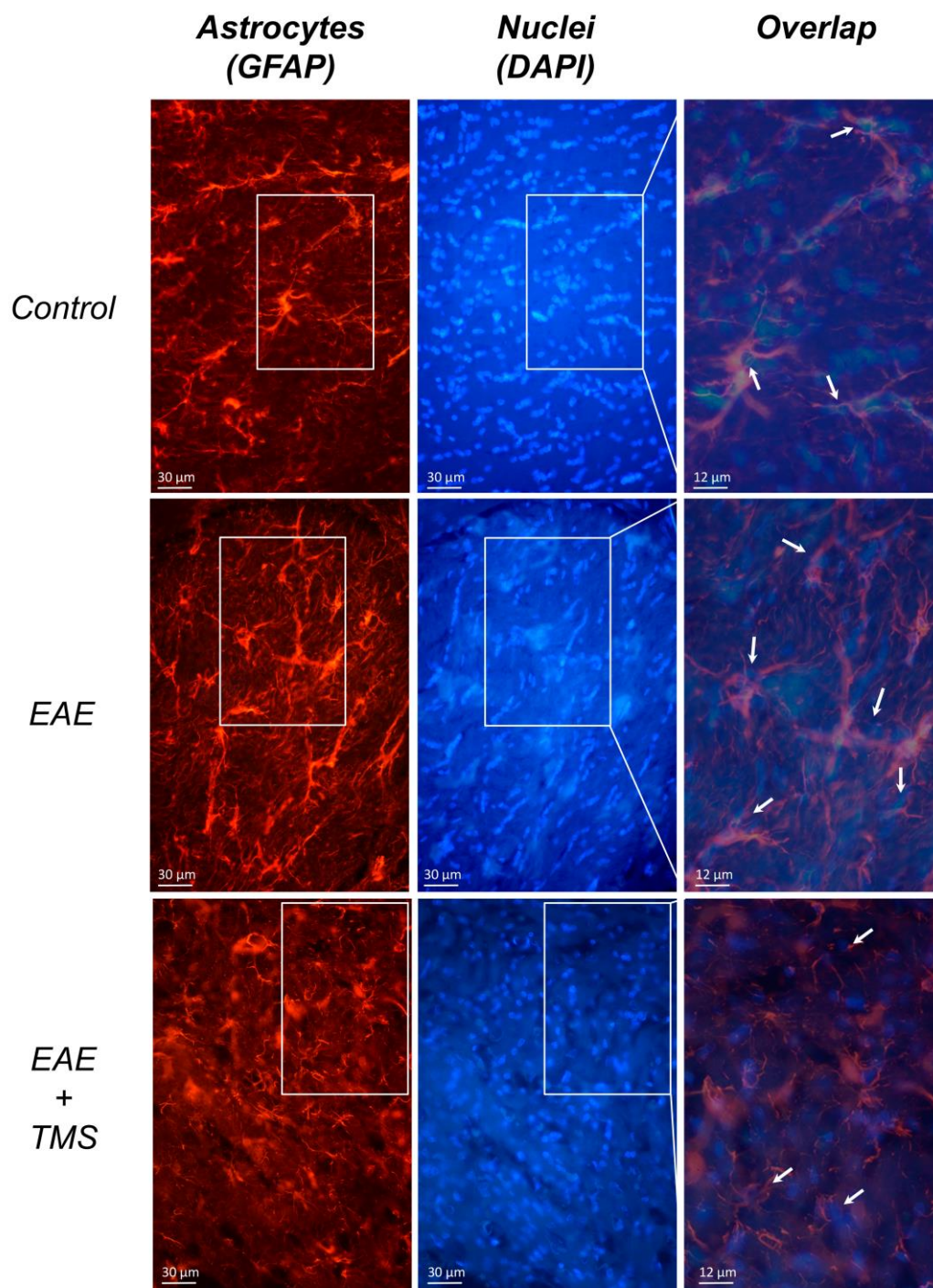


Figure 3. Immunohistochemistry staining (GFAP) for astrocytes in brain.

Cells positive for GFAP (astrocytes) and DAPI (nuclei) (GFAP/DAPI double positive) were counted as astrocytes (white arrows). Counts were performed using a counting frame (area) of 35,500 μm^2 (40x), which was randomly placed at ten different zones of the *striatum*. Then, the counts were averaged. Bar chart represents mean \pm SD. *Unless indicated by symbol, no statistical significant differences were observed between groups.* One-way ANOVA with Dunn's method was used. * $P < 0.05$ compared with control. # $P < 0.05$ compared with EAE.

Changes in NOx levels, LBP and LPS in TMS-treated EAE model

EAE increased the NOx levels in the brain and spinal cord homogenates. These increases were reverted by treatment with TMS in both tissues (Figure 4A).

Also, we analyzed the LPS and LBP levels. EAE prompted the accumulation of LBP in brain and spinal cord, and LPS in spinal cord. The accumulation of LBP and LPS in nervous tissue was diminished after treatment with TMS (Figures 4B and 4C).

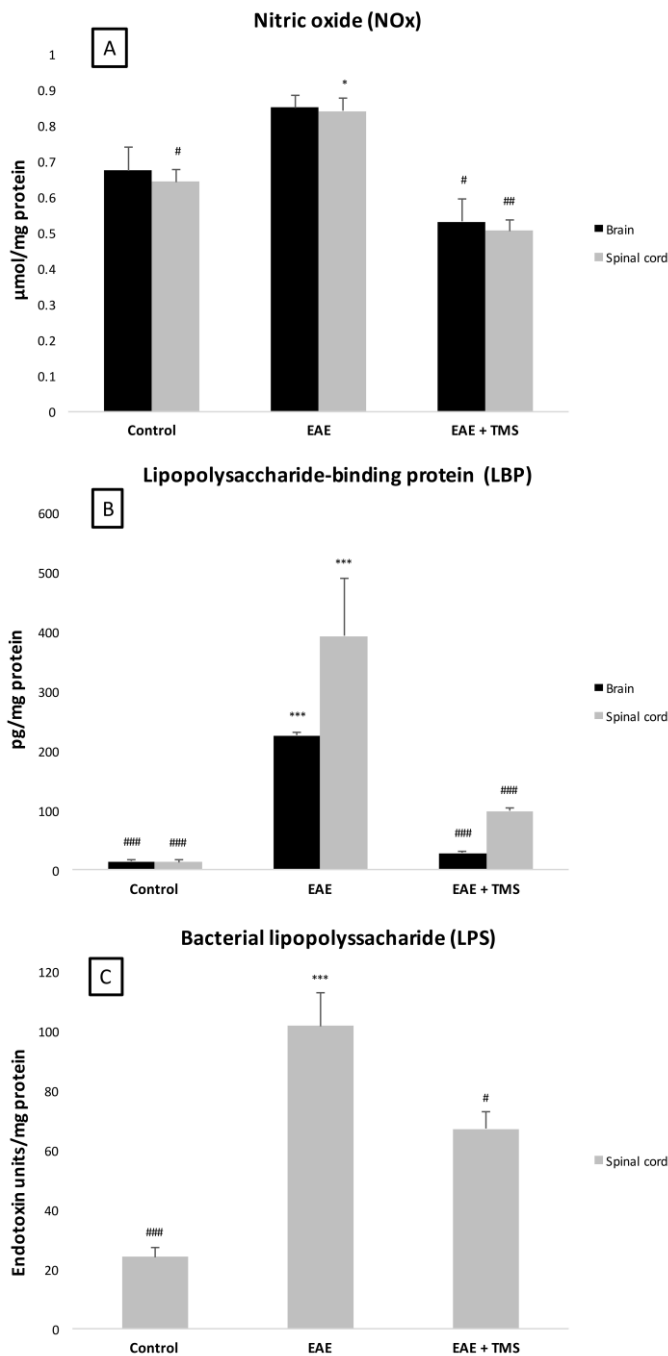


Figure 4. Biochemical parameters in brain and spinal cord. (A) Nitric oxide, (B) Lipopolysaccharide-binding protein (LBP), and (C) Bacterial lipopolysaccharide (LPS). Bar charts represent mean \pm SD. Unless indicated by symbol, no statistical significant differences were observed between groups. One-way ANOVA with Bonferroni test was used. $*P < 0.05$, $***P < 0.001$ compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P <$

0.001 compared with EAE. The whole brain was used for the evaluation of these parameters.

DISCUSSION

The present study assesses for the first time the effect of TMS against neuroinflammation on EAE, a MS-like experimental model.

From a clinical point of view, the model of MOG-EAE consistently induced motor impairment. The main finding of this research has been to demonstrate that TMS could ameliorate motor symptoms secondary to EAE. Once this was shown, we carried out several biochemical determinations and histologic studies to shed light on the mechanisms and pathways underlying this therapeutic effect of TMS.

Neuroinflammation is undoubtedly a crucial factor in the pathogenesis of EAE and MS. In that process, astrocytes appear to play an important role. Astrocytes mediate the production of NO, a pro-inflammatory and pro-oxidative molecule closely involved in the pathophysiology of MS [18], although NO may be also produced by other many pathways. Moreover, these cells have been proposed responsible for oxidative/nitrosative damage in oligodendrocytes and neurons in MS [18]. Our results using GFAP-immunohistochemical staining demonstrate that an increase in the number of astrocytes as a response to the autoimmune attack is produced in the brain during EAE, while TMS is capable to mitigate this phenomenon. This finding emphasizes that: i) astrocytes play an important role in the immune response to EAE, ii) the beneficial effect of TMS may be due, at the least, to an anti-inflammatory-like effect, and iii) TMS could be a promising treatment for neuroinflammatory conditions such as MS.

NO is an endogenous gas produced by mammalian cells which is involved in many physiological and pathological processes. Previous works revealed that NO

concentrations are raised in the cerebellum and spinal cord of animals with EAE [19], and in serum from patients with MS [20]. In addition, NO content seems to correlate with the severity of symptoms of EAE [21, 22]. According to our data, EAE can induce increase in NO_x that may be counteracted by TMS in possible relation to an inhibition of the inducible nitric oxide synthase (iNOS) [23]. Moreover, several studies have demonstrated that reactive oxygen species and consequently oxidative stress contribute to the pathologic lesions and symptoms encountered in EAE [24-26]. In our opinion, in addition to the pro-inflammatory function of NO, an overproduction of this mediator in an environment full of reactive oxygen species such as superoxide anion (O₂⁻) will favor their combination into peroxynitrite (ONOO⁻) triggering a subsequent deleterious state of nitrosative stress which would aggravate the condition.

Also in relation to the pathways mediating neuroinflammation, both metabolites and microbial products such as the LPS may lead to the activation of CNS-reactive immune cells [2]. LPS induces the synthesis of the LBP, and forms an LPS–LBP complex that binds to CD14 [27]. Moreover, some studies using EAE models have demonstrated that germ-free mice develop notably attenuated EAE in contrast to conventionally colonized mice [28]. We analyzed variations in levels of LPS and its carrier protein, LBP, as a way to evaluate inflammation in this model. Rats with EAE presented higher LBP and LPS values in CNS than healthy subjects, alterations that were palliated by treatment with TMS. A similar variation of LBP was observed in patients with MS after treatment with natalizumab [26]. We did not determine LPS in brain, however, based on the parallelism observed in brain and spinal cord for NO and LBP, there is no reason to expect that LPS in brain behaves differently than in spinal cord.

Our results after the evaluation of LBP and LPS also allow us to shed light on the possible collaboration between intestinal bacteria and the immune system in the pathophysiology of this experimental model. Supporting this thesis, Nouri et al. [29] demonstrated that disruption of intestinal homeostasis may be an early and immune-mediated event in EAE. Moreover, other studies have showed the capacity of LPS to mediate trafficking and migration of a population of gut-derived antigen-presenting cells to CNS-associated lymphoid tissue [30]. Therefore, activated immune cells carrying LPS-LBP may be responsible for the concentration of these products in CNS. The mechanisms by which TMS may decrease the content of LPS-LBP in CNS remain unexplained, nonetheless, this fact might be in relation to a lesser inflammatory cell infiltration.

Inflammation usually leads to biochemical alterations resulting in cell malfunction and finally death [31]. By Nissl staining, we observed a reduced cell density and an increase of pyknotic nuclei in the *striatum* of animals with EAE, changes partially reverted after treatment with TMS. The reduced number of total cells in EAE could be explained as being due to possible cell loss secondary to cell dysfunction accompanying neuroinflammation. Our group previously reported a similar effect of TMS in the *striatum* of rats induced with 3-nitropropionic acid, a model of Huntington's disease [4, 9]. In those studies, TMS led to a significant diminution in lactate dehydrogenase (LDH) and Caspase-3, supporting the thesis that TMS may palliate cell loss by exerting an anti-necrotic and anti-apoptotic effect.

In addition to a neuroprotective effect that could mitigate necrosis and apoptosis, and improve cell viability [4, 6, 8, 9], there is some evidence that TMS may induce neurotrophic/neurogenetic phenomena which might collaborate in the increased cell density observed after treatment with TMS. Some studies using a model of

Huntington's disease reported that TMS was capable of reducing cell loss and even caused recovery of cell density, probably due to a neurotrophic/neurogenetic effect, which was evidenced by elevation of the neurotrophins BDNF and GDNF [4, 9]. In line with this possible reparative effect of TMS, Sherafat et al., using the same protocol of TMS as we, reported that this therapy could potentiate proliferation and relocation of neural stem cells, enhancing myelin repair in an experimental model of white matter demyelination [11]. Other experimental studies using bromodeoxyuridine (BrdU) and NeuN to label immature and mature neurons, respectively, have also demonstrated that TMS may stimulate the differentiation and migration of adult brain stem cells (located mainly in the subventricular zone of the hippocampus) to damaged areas [10, 12], presenting these new neurons similar spontaneous postsynaptic potentials and electrophysiologic properties to adult ones [32].

Nonetheless, in the present experiment, we did not perform any specific immune staining for neurons so we cannot definitively shed light on the actual identity of the cells presenting in the brains of animals treated with TMS. In spite of that, founded on the normalization of the ratio astrocyte/cell density, we might speculate that activated microglial cell may not be fully responsible for the improvement observed in cell density after TMS.

CONCLUSIONS

TMS effectively ameliorates motor impairment in EAE. This form of magnetic fields is capable of modifying the astrocyte response, reducing the content of NO, LBP and LPS in CNS, as well as improving cell density.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

REFERENCES

1. Constantinescu CS, Farooqi N, O'Brien K and Gran B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *British journal of pharmacology* 2011;164:1079-106.
2. Wang Y and Kasper LH. The role of microbiome in central nervous system disorders. *Brain, behavior, and immunity* 2014;38:1-12.
3. Elzamarany E, Afifi L, El-Fayoumy NM, Salah H and Nada M. Motor cortex rTMS improves dexterity in relapsing-remitting and secondary progressive multiple sclerosis. *Acta Neurol Belg* 2016;116:145-50.
4. Tunez I, Drucker-Colin R, Jimena I, et al. Transcranial magnetic stimulation attenuates cell loss and oxidative damage in the striatum induced in the 3-nitropropionic model of Huntington's disease. *Journal of neurochemistry* 2006;97:619-30.
5. Tunez I, Montilla P, del Carmen Munoz M, Medina FJ and Drucker-Colin R. Effect of transcranial magnetic stimulation on oxidative stress induced by 3-nitropropionic acid in cortical synaptosomes. *Neuroscience research* 2006;56:91-5.
6. Tasset I, Drucker-Colin R, Pena J, et al. Antioxidant-like effects and protective action of transcranial magnetic stimulation in depression caused by olfactory bulbectomy. *Neurochem Res* 2010;35:1182-7.

7. Tasset I, Perez-Herrera A, Medina FJ, et al. Extremely low-frequency electromagnetic fields activate the antioxidant pathway Nrf2 in a Huntington's disease-like rat model. *Brain stimulation* 2013;6:84-6.
8. Liu X, Zuo H, Wang D, et al. Improvement of spatial memory disorder and hippocampal damage by exposure to electromagnetic fields in an Alzheimer's disease rat model. *PloS one* 2015;10:e0126963.
9. Tasset I, Medina FJ, Jimena I, et al. Neuroprotective effects of extremely low-frequency electromagnetic fields on a Huntington's disease rat model: effects on neurotrophic factors and neuronal density. *Neuroscience* 2012;209:54-63.
10. Podda MV, Leone L, Barbati SA, et al. Extremely low-frequency electromagnetic fields enhance the survival of newborn neurons in the mouse hippocampus. *The European journal of neuroscience* 2014;39:893-903.
11. Sherafat MA, Heibatollahi M, Mongabadi S, et al. Electromagnetic field stimulation potentiates endogenous myelin repair by recruiting subventricular neural stem cells in an experimental model of white matter demyelination. *Journal of molecular neuroscience : MN* 2012;48:144-53.
12. Arias-Carrion O, Verdugo-Diaz L, Feria-Velasco A, et al. Neurogenesis in the subventricular zone following transcranial magnetic field stimulation and nigrostriatal lesions. *Journal of neuroscience research* 2004;78:16-28.
13. Medina FJ and Tunes I. Mechanisms and pathways underlying the therapeutic effect of transcranial magnetic stimulation. *Reviews in the neurosciences* 2013;24:507-25.
14. Luque E, Ruz-Caracuel I, Medina FJ, et al. Skeletal muscle findings in experimental autoimmune encephalomyelitis. *Pathology, research and practice* 2015;211:493-504.

15. Perez-Nievas BG, Garcia-Bueno B, Madrigal JL and Leza JC. Chronic immobilisation stress ameliorates clinical score and neuroinflammation in a MOG-induced EAE in Dark Agouti rats: mechanisms implicated. *Journal of neuroinflammation* 2010;7:60.
16. Drucker-Colin R, Verdugo-Diaz L, Mendez M, et al. Comparison between low frequency magnetic field stimulation and nerve growth factor treatment of cultured chromaffin cells, on neurite growth, noradrenaline release, excitable properties, and grafting in nigrostriatal lesioned rats. *Molecular and cellular neurosciences* 1994;5:485-98.
17. Ricart-Jane D, Llobera M and Lopez-Tejero MD. Anticoagulants and other preanalytical factors interfere in plasma nitrate/nitrite quantification by the Griess method. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2002;6:178-85.
18. Miljkovic D and Spasojevic I. Multiple sclerosis: molecular mechanisms and therapeutic opportunities. *Antioxidants & redox signaling* 2013;19:2286-334.
19. Ljubisavljevic S, Stojanovic I, Pavlovic D, et al. Correlation of nitric oxide levels in the cerebellum and spinal cord of experimental autoimmune encephalomyelitis rats with clinical symptoms. *Acta neurobiologiae experimentalis* 2012;72:33-9.
20. Nazliel B, Taskiran D, Irkec C, Kutay FZ and Pogun S. Serum nitric oxide metabolites in patients with multiple sclerosis. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia* 2002;9:530-2.
21. Farias AS, de la Hoz C, Castro FR, et al. Nitric oxide and TNFalpha effects in experimental autoimmune encephalomyelitis demyelination. *Neuroimmunomodulation* 2007;14:32-8.

22. Danilov AI, Jagodic M, Wiklund NP, Olsson T and Brundin L. Effects of long term NOS inhibition on disease and the immune system in MOG induced EAE. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2005;13:188-95.
23. Reale M, De Lutiis MA, Patruno A, et al. Modulation of MCP-1 and iNOS by 50-Hz sinusoidal electromagnetic field. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2006;15:50-7.
24. Qi X, Lewin AS, Sun L, Hauswirth WW and Guy J. Mitochondrial protein nitration primes neurodegeneration in experimental autoimmune encephalomyelitis. *The Journal of biological chemistry* 2006;281:31950-62.
25. Dasgupta A, Zheng J, Perrone-Bizzozero NI and Bizzozero OA. Increased carbonylation, protein aggregation and apoptosis in the spinal cord of mice with experimental autoimmune encephalomyelitis. *ASN neuro* 2013;5:e00111.
26. Escribano BM, Medina-Fernandez FJ, Aguilar-Luque M, et al. Lipopolysaccharide Binding Protein and Oxidative Stress in a Multiple Sclerosis Model. *Neurotherapeutics* 2016.
27. Bannerman DD, Paape MJ, Hare WR and Sohn EJ. Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge. *J Dairy Sci* 2003;86:3128-37.
28. Lee YK, Menezes JS, Umesaki Y and Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 2011;108 Suppl 1:4615-22.
29. Nouri M, Bredberg A, Westrom B and Lavasani S. Intestinal barrier dysfunction develops at the onset of experimental autoimmune encephalomyelitis, and can be induced by adoptive transfer of auto-reactive T cells. *PloS one* 2014;9:e106335.

30. Ochoa-Reparaz J, Mielcarz DW, Begum-Haque S and Kasper LH. Gut, bugs, and brain: role of commensal bacteria in the control of central nervous system disease. *Ann Neurol* 2011;69:240-7.
31. Kahl KG, Zielasek J, Uttenthal LO, et al. Protective role of the cytokine-inducible isoform of nitric oxide synthase induction and nitrosative stress in experimental autoimmune encephalomyelitis of the DA rat. *Journal of neuroscience research* 2003;73:198-205.
32. Arias-Carrion O, Hernandez-Lopez S, Ibanez-Sandoval O, et al. Neuronal precursors within the adult rat subventricular zone differentiate into dopaminergic neurons after substantia nigra lesion and chromaffin cell transplant. *Journal of neuroscience research* 2006;84:1425-37.