Edaravone attenuates disease severity of experimental auto-immune encephalomyelitis and increases gene expression of Nrf2 and HO-1

Running title: Edaravone in experimental auto-immune encephalomyelitis

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Abstract

The aim of this study was to evaluate therapeutic potential of edaravone in the murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) and to expand the knowledge of its mechanism of action. Edaravone (6 mg/kg/day) was administered intraperitoneally from the onset of clinical symptoms until the end of the experiment (28 days). Disease progression was assessed daily using severity scores. At the peak of the disease, histological analyses, markers of oxidative stress (OS) and parameters of mitochondrial function in the brains and spinal cords (SC) of mice were determined. Gene expression of inducible nitric oxide synthase (iNOS), nuclear factor erythroid 2–related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha was determined at the end of the experiment. Edaravone treatment ameliorated EAE severity and attenuated inflammation in the SC of the EAE mice, as verified by histological analysis. Moreover, edaravone treatment decreased OS, increased the gene expression of the Nrf2 and HO-1, increased the activity of the mitochondrial complex II/III, reduced the activity of the mitochondrial complex IV and preserved ATP production in the SC of the EAE mice. In conclusion, findings in this study provide additional evidence of edaravone potential for the treatment of multiple sclerosis and expand our knowledge of the mechanism of action of edaravone in the EAE model.

Keywords: experimental autoimmune encephalomyelitis, edaravone, mitochondrial dysfunction, Nrf2/HO-1 pathway, oxidative stress

Introduction

Multiple sclerosis (MS) is an autoimmune neurological disease characterized by chronic inflammation of the central nervous system (CNS), resulting in a range of physical, mental, or even psychiatric symptoms [1]. Despite outstanding progress in the development of novel therapeutic agents for MS in recent years, we are still far from discovering an ultimate drug for MS. Current disease-modifying therapies aim to prevent the inflammatory damage to the CNS, but their severe adverse effects urge new, safe therapeutic approaches.

MS is characterized by auto-reactive IFN-gamma-producing Th1 and IL-17-producing Th17 effector cells and other immune cells (CD8+ T cells, B cells) that penetrate the CNS and damage the myelin sheath [2]. The inflammation in the CNS also results in activation of microglia, which produce pro-inflammatory mediators and elicit demyelination and axonal loss [3]. Another crucial feature of MS is oxidative stress (OS) emerging from the uncontrolled generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), in addition to the mitochondrial dysfunction and energy deficit [4]. Inflammation and OS are intricately linked processes; even a new term "OxInflammation" has been suggested to depict the vicious cycle of chronic inflammation and OS [5]. Pro-inflammatory mediators promote generation of ROS and RNS, but on the other side, reactive species also favor the pro-inflammatory response, creating a vicious cycle that is difficult to break [5]. Additionally, prolonged microglia activation impairs activation of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α), a major regulator of mitochondrial biogenesis and energy metabolism [6]. PGC-1α downregulation has been linked to an augmented level of mitochondria - derived ROS, whereas its upregulation was associated with protection of neural cells against OS [4,7,8]. A plethora of players in this complex network of "OxInflammation" are mediated by the Kelch-like ECH - associated protein 1 - Nuclear factor erythroid 2 related factor 2 - Antioxidant response element (Keap1/Nrf2/ARE) signaling pathway, a master regulator of antioxidant and phase II

detoxification genes [8]. Heme oxygenase-1 (HO-1) is a representative downstream enzyme of this pathway, which generates carbon monoxide, biliverdin, and iron ions, apart from removing toxic heme. HO-1 inducers exert favorable effects in MS, through the protection against OS, and inflammation and regulation of apoptosis [8].

Edaravone is an amphiphilic free radical scavenger, which is currently approved for the treatment of amyotrophic lateral sclerosis (ALS) in the USA and Japan and for the treatment of acute-phase cerebral infarction in Japan [9]. Mechanism of action of edaravone is not completely understood, but its neuroprotective effects have primarily been ascribed to ability to scavenge peroxynitrite [9]. Lately, the treatment with edaravone has been associated with the activation of Nrf2 pathway in various animal models of the CNS disorders [10-12]. Edaravone was also reported to ameliorate clinical severity in the model of experimental autoimmune encephalomyelitis (EAE), the most commonly used murine model of MS [13]. However, the Nrf2 pathway expression was not investigated in this study. Edaravone attenuated lymphocytes infiltration to the CNS and reduced expression of induced NO synthase (iNOS) in the microglia in spinal cords (SC) of EAE mice. Further research is needed to assess the potential of edaravone in MS treatment. The aim of this study was to evaluate the therapeutic potential of edaravone in the EAE model and to expand the knowledge of its mechanism of action. We hypothesized that the effect of edaravone on mitochondrial functions might be involved in its therapeutic effects.

Materials and Methods

Animals and induction of EAE

Mice were housed in the animal facility of Institute of Pharmacology, First Faculty of Medicine, Charles University in Prague. They had free access to a standard granulated diet and water *ad*

libitum. The mice were housed in standard environmental conditions: light (12 h light and 12 h dark); temperature (22±2°C); relative humidity (50±10%). All experiments were approved by the Ministry of Education, Youth and Sports of the Czech Republic under the number MSMT-9445/2018-8.

The EAE was actively induced in conventional inbred female C57Bl/6J mice (9-13 weeks old) by immunization with myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide (Prospec, Rehovot, Israel) and complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* H37Ra (Sigma-Aldrich, Prague, Czech Republic). The MOG/CFA emulsion was prepared by connecting two glass syringes to a 3-way connector and passing the solutions through the connector. The emulsion was injected subcutaneously in two 50 µl doses. In total, 100 µg of MOG peptide per mouse was administered. To facilitate the transfer of lymphocytes into the CNS, 300 ng of *pertussis* toxin (List Biologicals, Campbell, California, USA) in 200 µl of phosphate buffer saline (PBS) was injected intraperitoneally two hours and two days after the EAE induction [14].

The mice were monitored daily for the signs of EAE, which were scored as follows: 0 - no signs of clinical disease; 0.5 – partially limp tail; 1- limp tail; 2 -loss in coordinated movement; hind limb paresis; 2.5 - one hind limb paralyzed; 3 - complete paralysis of hind legs; 3.5 - hind limbs paralyzed, weakness in forelimbs; 4 - paralysis of hind and fore legs; 5 – dead [15].

All mice were observed until they showed mild EAE symptoms, such as a partially limp tail (clinical score 0.5) and were then divided into ET (which received edaravone) and CG1 (which received the vehicle) groups until the end of the experiment.

Treatments

The mice were randomly allocated to one of the following groups:

- Group evaluating therapeutic potential of edaravone (ET group): dose of 6 mg/kg/day intraperitoneally (this dose was previously shown to be the effective dose in the EAE model [13]);
- Control group 1 (CG1 group): mice treated with a vehicle from the onset of EAE symptoms until the end of the experiment;
- Control group 2 (CG2 group): mice treated with the vehicle, but not immunized with MOG/CFA.

Pilot study before the main experiments has indicated that the peak of the EAE disease in our lab is on the 6th day after the beginning of the clinical symptoms. Therefore, on day 6 after the onset of clinical symptoms, half of the mice were sacrificed by a rapid decapitation. Spinal cords and brains were removed and subsequently used for histological analysis, qPCR for gene expression, determination of markers of OS and parameters of mitochondrial function. The rest of the mice were observed for clinical scores until day 28 after the immunization, when they were sacrificed. Brains and SCs were harvested and used for qPCR analysis for gene expression.

Histological analyses

Samples of the vertebral column with SC were collected and fixed with 4% formaldehyde. Twenty-four hours later, and SC was carefully removed from the vertebral canal. Material was divided into three segments encompassing cervical, thoracic and lumbar part of the spinal cord and embedded into paraffin wax. Sections (7μm thick) were stained with hematoxylin-eosin staining. Images were captured at Leica DMLB microscope with MC170 HD camera (Leica Microsystems, Wetzlar, Germany) [16,17]. The inflammation was scored as previously described [16,18]. The inflammation extent was scored as follows: 0=no inflammation evident; 1=small number of inflammatory cells; 2=numerous infiltrating cells; 3=extension of

perivascular cuffing into adjacent tissues (widespread infiltration) [16]. The final score for each experimental animal was obtained by evaluating each of the three segments on 4 sections separated from each other by approximately 100μm (12 sections per animal).

qPCR for gene expression

SCs and brains were stored in RNAlater™ Stabilization Solution at -20ºC for subsequent qPCR analysis. Total RNA was extracted using TRI reagent (Sigma-Aldrich, Prague, Czech Republic). cDNA was synthesized from RNA using an M-MLV Reverse Transcriptase (Top Bio, Prague, Czech Republic). cDNA served as a template for amplification of target genes, as well as the housekeeping gene β-actin (Actb gene, forward primer: CTGTCGAGTCGCGTCCACC, reverse primer: TCGTCATCCATGGCGAACTGG) by the quantitative real-time PCR with SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, USA), using the manufacturer's instructions. Target genes were Nrf2 (forward primer: CGCCAGC-TACTCCCAGGTTG, reverse primer: GGGGATATCCAGGGCAAGCG) and HO-1 (forward primer: GAGCCGTCTCGAGCATAGCC, reverse primer: ATCCTGGGG-CATGCTGTCGG), iNOS (forward primer: ATGGACCAGTATAAGGCAAGC; re-verse primer: GCTCTGGATGAGCCTATATTG) and PGC1-α (forward primer: GGCTGGTTGCCTGCATGAGT, reverse primer: CCAACCAGAGCAGCACACTCT). The expression of target genes was calculated by comparing the relative levels after normalization to β-actin expression.

Determination of oxidative stress markers

The extent of lipid peroxidation (as Thiobarbituric Acid Reactive Substances (TBARS)) and conjugated dienes (CD) were assessed in the SCs, and brains homogenates as previously

described [19]. All chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic). SCs and brains homogenates (10%) were prepared in the buffer (0.2 M Tris–HCl pH 7.4 + 0.002 M EDTA-Na_2 + 0.025 M sucrose) and centrifuged at 4000 rpm.

Total protein assessment

The total amount of protein in all samples used in this study (for OS markers and mitochondria function parameters) was determined by the Bradford method [20] using the Bio-Rad protein assay (Bio-Rad, Prague, Czech Republic).

Mitochondrial functions

Isolated purified mitochondria were used for determination of the electron transport chain (ETC) complexes activity (I-IV), mitochondrial respiration linked to the ETC complexes I and II, as well as adenosine triphosphate (ATP) production. All chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic).

Isolation of mitochondria from the brains and spinal cords

Preparation of mitochondria was performed as described previously [21]. Mitochondrial isolation buffer (pH 7.4) consisted of 200 mM mannitol, 75 mM sucrose, 5 mM HEPES, 0.1% BSA, 1mM EDTA. Freshly prepared mitochondria kept on ice were used for respirometry and ATP formation; the ETC complexes activity was measured with frozen mitochondria stored at − 80 °C.

Activity of respiratory chain complexes

Isolated mitochondria were resuspended in the hypotonic buffer (25 mM potassium phosphate, 5 mM MgCl2, pH 7.2) and ultrasonicated three times to reach the maximum enzymatic activity. Each independent measurement had a corresponding control. Samples were measured in a total reaction volume of 3 mL at 30 °C. The activity of the ETC complexes and CS was measured spectrophotometrically using a GENESYS 180 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts , USA). Activity of the complexes I, II/III and IV was measured by the methods as previously described [22-24].

Mitochondrial respiration

The mitochondrial respiration medium (MiR05) consisted of 110 mM sucrose, 60 mM K+-lactobionate, 20 mM taurine, 3 mM MgCl2·6H2O, 10 mM KH2PO4, 0.5 mM EGTA, 1 g/L, and 20 mM HEPES, and was adjusted to pH 7.1 with KOH.

The following stock solutions were used for respirometry: 2 mM malate, 5 mM pyruvate, 10 mM succinate, 1.25 mM ADP, 0.75 mM MgCl2, 2 µM rotenone in ethanol, and 2.5 µg/mL antimycin A in ethanol. Substrates and inhibitors were used at the final concentrations described previously [22]. Drug-induced changes in mitochondrial respiration were measured by high-resolution respirometry to detect changes in the oxygen consumption rate of isolated mitochondria as described previously using an Oxygraph-2k (Oroboros Instruments Corp, Innsbruck, Austria) and a TIP2k automatic titration-injection micropump [22].

ATP production

ATP production was measured by bioluminescence method as previously described [25]. Briefly, an ATP Bioluminescence Assay Kit CLS II with 5 mM malate, 5 mM pyruvate, 10 mM succinate, 5 mM glutamate and 1 mM ADP as substrates was used to determine ATP formation fluorometrically using FluoroMax3 (Jobin Yvon, Edison, New Jersey, USA). The reaction was initiated by the addition of luciferase reagent and luminescence was measured at 532 nm.

Statistical analysis

Normal distribution of the data was checked using the Shapiro-Wilk test. To compare the differences between the groups regarding parameters of mitochondrial function, markers of OS, and gene expression, ANOVA with *post-hoc* Bonferroni test was used. To compare clinical scores, as well as inflammatory scores between the ET and CG1 groups, unpaired t-test (for data with normal distribution) and Mann-Whitney test (for data without normal distribution) were used. The results for the variables' data are expressed as mean and standard deviation (SD) or mean and standard error of the mean (SEM). The differences were considered statistically significant when $P < 0.05$. Statistical analyses and data visualization were performed using GraphPad Prism, version 8.0.0 for Windows, GraphPad Software (San Diego, California, USA).

Results

Edaravone ameliorated EAE severity

Treatment with edaravone ameliorated clinical scores over the course of the experiment (Figure 1). There was a significant difference between the clinical scores in the ET and CG1 group at the peak of the disease, as well as in the cumulative disease index (defined as total disease score

over experiment and calculated as a mean of daily scores of all mice in the group during the whole experiment). There was no significant difference in the clinical scores on the last day of the experiment (28th day after EAE induction).

Edaravone suppressed inflammation in the SC

Hematoxylin-eosin staining was used to determine the extent of infiltration of mononuclear cells and perivascular cuffing in the SC (Figure 2). In accordance with the clinical results, the CG1 group exerted massive infiltration of mononuclear cells into the SC with several foci of inflammation, whereas ET group showed significantly fewer infiltrating cells and perivascular cuffing, as verified by the inflammatory scores ($p < 0.05$).

Edaravone attenuated OS in the SC and the brain

In the homogenates of the SCs, there was a significant difference between the ET and CG1 group, but no differences were observed for the level of TBARS. Conversely, in the brain homogenates, there was a significantly lower TBARS level in the ET group compared to the CG1 group, whereas no difference was observed for the CD level (Figure 3).

Edaravone changes genes expression in the SCs

Gene expression of Nrf2, HO-1, PGC1-α and iNOS was determined in the brains and the SCs of mice at the peak of the disease (6th day after the symptoms onset) and at the end of the experiment (28th day after EAE induction). There were no significant differences in the brain samples for any gene neither at the peak of the disease, nor at the end of the experiment (data not shown). In the SCs, mRNA for HO-1 was significantly higher in the ET group compared to

mRNA of HO-1 in the CG2 group at the peak of the disease (Figure 4B). There were no significant differences in other genes expression between the ET, CG1 and CG2 groups (Figure 4A, C and D). At the end of the experiment, mice in the ET group expressed higher levels of mRNA of Nrf2 and HO-1 compared to the mice in the CG2 group (Figure 5A and B). Additionally, there was a difference in the PGC1-α mRNA expression between the CG1 and CG2 groups (Figure 5D). No differences were found in the iNOS mRNA expression between the groups neither at the peak of the disease, nor at the end of the experiment.

Edaravone has a profound effect on the mitochondrial functions in the brain and the SC

Activity of respiratory chain complexes

No changes in the ETC complex I activity were detected in the SC and brain in the ET and CG1 groups compared with the CG2 group (Figure 6C and D). However, edaravone treatment was associated with an increase in the activity of ETC complex II+III (Figure 6E) in the SC. Similar observations were noted in the brain (Figure 6F). Additionally, edaravone treatment caused inhibition of the ETC complex IV in the brain and SC, as activity of ETC complex IV was lower in the ET group compared to both CG1 and CG2 groups (Figure 6G and H).

Mitochondrial respiration

Complex I-linked respiration was also not altered neither by EAE, nor by edaravone treatment (Figure 6I and J). Complex II-linked respiration in the SC was increased in the ET group compared to the CG2 group (Figure 6K). In the brain, respiration linked to the ETC complex II was significantly decreased in the ET group when compared to the CG1 group (Figure 6L).

ATP production

Level of ATP production in the SC was significantly reduced in the CG1 group compared to the CG2 group (Figure 6A), but this was not observed in the ET group. This indicates that treatment with edaravone preserves ATP production in the mitochondria in the SC. This effect was not seen in the brain (Figure 6B).

Discussion

This study has shown that edaravone ameliorates disease severity in the EAE model, attenuates inflammation in the SC, reduces OS and causes profound effects on the mitochondrial function in the brain and SC of the EAE mice. Additionally, edaravone was found to increase gene expression ofNrf2 and HO-1. These findings provide additional evidence of edaravone potential for treatment in MS and expand our knowledge of the mechanisms of action of edaravone in the EAE model.

Edaravone is a potent antioxidant that is currently approved for the treatment of ALS and the management of neurological symptoms associated with acute ischemic stroke. In this study, edaravone treatment decreased levels of OS markers TBARS and CD in the homogenates of the brains and SCs, respectively. In addition to its anti-oxidative properties, edaravone has a role in attenuation of inflammation in the CNS, as well as in apoptotic cell death prevention, therefore it fits to the class of multi-target compounds [26]. We have shown that edaravone reduces clinical scores in the EAE model, and ameliorates inflammatory cellular infiltration into the SC of the EAE mice. These results are in accordance with the findings of an earlier study by [13].

The antioxidative properties of edaravone have been mainly attributed to its ability to scavenge ROS. However, there are a growing number of studies demonstrating the ability of edaravone

to act as an Nrf2/HO-1 pathway inducer. Edaravone was shown to increase Nrf2 and HO-1 mRNA expression in various animal models, including vascular dementia [11], cerebral infarction [10], traumatic brain injury [12]. We have examined the expression of Nrf2/HO-1 pathway at the peak of the disease (5 days of edaravone treatment) and at the end of experiment (14 days of edaravone treatment), as the level of gene expression is time-dependent. At the peak of the disease, there were no significant changes between the groups regarding Nrf2, whereas expression of HO-1 mRNA was markedly augmented in the ET group in comparison to the CG2 group. At the end of the experiment, i.e., after 14 days of edaravone treatment, the mRNA expression of both Nrf2 and HO-1 was significantly increased in the ET group compared to the CG1 group.

Mitochondrial dysfunction is a critical event in the pathophysiology of MS which leads to impaired oxidative phosphorylation and consequent energy failure [4,8]. In addition to its antioxidant and anti-inflammatory properties, Nrf2 plays a role in maintaining mitochondrial homeostasis. There is an emerging body of evidence of the existence of the regulatory loop involving Nrf2 and PGC-1α [7]. PGC-1α, a transcriptional coactivator, is a major regulator of mitochondrial biogenesis and energy metabolism, which seems to be decreased in MS probably due to prolonged microglia activation [27]. PGC-1α downregulation has been linked to an augmented level of mitochondria - derived ROS, whereas its upregulation was associated with protection of neural cells against OS [28]. In this study, the level of mRNA expression of PGC-1α was lower in the ET and CG1 groups compared to the CG2, however, a significant difference was observed only between the CG1 and CG2 groups.

Reduced intracellular ATP level is an important indicator of mitochondrial dysfunction. Importantly, edaravone treatment preserved ATP level in the SCs of the EAE mice. Conversely, ATP production was significantly reduced in the CG1 group compared to the CG2 group. Previously, edaravone protected against hyperosmolarity-induced OS in primary human

corneal epithelial cells by increasing the levels of ATP and mitochondrial membrane potential (MMP) [29]. Similarly, edaravone treatment improved kidney function in rats with ischemia-reperfusion injury by increasing ATP levels and MMP [30]. In this study, edaravone administration induced significant changes in the ETC complexes activity in the brain and the SCs of the mice. ETC complex II/III activity was increased in the ET group, whereas ETC complex IV activity was reduced in the ET group compared to the CG1 and CG2 groups. In accordance with this finding, complex II-linked respiration in the SC was also increased in the ET group. During complex I-linked respiration, ROS are produced from complexes I and III, whereas complex II-linked respiration leads to ROS production to some extent by complex III, but also through reverse electron flow to complex I. We can presume that reduced ROS production occurs when ETC complex II/III activity is increased and enhanced by edaravone. The reduction in the ETC complex IV activity might represent a compensatory mechanism of increased complex II/III activity. In a recent study, edaravone treatment completely restored activity of ETC complexes I – IV in the muscles of transgenic mice with impaired oxidative phosphorylation [31]. These effects were observed after a month of treatment with edaravone, whereas in this study ETC complexes activity were determined at the peak of the disease (5 days after edaravone treatment). Therefore, it is reasonable to assume that a longer treatment with edaravone is needed for a complete restoration of ETC complexes activity and this should be investigated in the future studies.

In the previous EAE study [13], edaravone reduced iNOS mRNA expression in the SC of the EAE mice. iNOS is responsible for the synthesis of NO, an important inflammatory player in the pathophysiology of MS. In our study, the iNOS mRNA expression in the SC in the CG1 group was higher compared to the CG2 and ET groups, however, these differences were not statistically significant. Discrepancies between the results are probably due to the fact that iNOS

mRNA expression in the previous study was examined specifically in the microglia in the SC, whereas in this study it was assessed in the whole SC samples.

A limitation of the current study is that the brain tissue was not sufficiently explored. This was due to the fact that in the EAE model, inflammation is mainly limited to the SC, whereas the brain stem, the cerebellum and the forebrain are affected to a lesser extent [32]. Moreover, inflammation and demyelination are confined only to the specific regions of hippocampus, striatum, cerebellum, corpus callosum, and the cerebral cortex [33]. This could actually be a reason why we found no significant differences in the levels of mRNA expression of Nrf2, HO-1, PGC1-α and iNOS, as these analyses were performed in the whole brain samples, but not in the specific regions of the brain.

Conclusion

Edaravone treatment attenuates disease severity in the EAE model by reducing inflammation in the SC, diminishing OS, and improving mitochondrial function in the CNS of the EAE mice, and increases expression of Nrf2 and HO-1. Findings in this study provide additional evidence of edaravone potential for treatment in MS and expand our knowledge of the mechanisms of action of edaravone in the EAE model.

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Conflicts of Interest: The authors declare no conflict of interest.

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455 **Figures**

456 Figure 1. Clinical scores in the ET and CG1 group during the experiment. Results are
457 presented as mean ± SEM (n = 28 samples per group)

458 Figure 2. Morphology of the spinal cord in animals with EAE treated with edaravone. A.
459 Image of the spinal cord from an animal with EAE (CG1 group). Widespread infiltration
460 extending from perivascular infiltrate into the surrounding white matter is visible (asterisks).
461 In addition, numerous abnormally enlarged axons can be seen (arrowheads). Scale
462 bar=200μm. B. High magnification view of white matter of the spinal cord shown in A. Two
463 inflammatory foci are visible (asterisks) together with multiple swollen axons (arrowheads).
464 Scale bar=50μm. C. Image of the spinal cord from an animal with EAE treated with
465 edaravone (ET group). Isolated inflammatory focus is visible (asterisk). Swollen axons are
466 rare. Scale bar=100μm. D. High magnification view of the white matter of the spinal cord
467 shown in C. An inflammatory focus is visible (asterisk) as well as some swollen axons
468 (arrowheads). Scale bar=50μm. E. Spinal cord from the control healthy animal (CG2 group).
469 No inflammatory foci can be detected. Scale bar=200μm. F. Image of white matter of the
470 spinal cord shown in E. Structure of the spinal cord is well-preserved, and no inflammatory
471 infiltration is present. Scale bar=50μm. Hematoxylin-eosin staining. Wm=white matter,
472 gm=gray matter. G. Inflammatory scores in the ET and CG1 groups. Results are presented as
473 mean ± SD (n = 4 samples per group), * $p < 0.05$

474 Figure 3. Markers of oxidative stress in the brain and the spinal cord (SC) in the group treated
475 with edaravone (ET), control group with EAE (CG1) and control group consisting of healthy
476 mice (CG2). A. Level of Thiobarbituric Acid Reactive Substances (TBARS) in the brain B.
477 Level of TBARS in the SC C. Level of conjugated dienes (CD) in the brain D. Level of CD in
478 the SC. Results are presented as mean ± SD (n = 12 samples per group). * $p < 0.05$

Figure 4. Gene expression in the spinal cord (SC) in the group treated with edaravone (ET), control group with EAE (CG1) and control group consisting of healthy mice (CG2) at the peak of the EAE. A. Relative gene expression of nuclear factor erythroid 2-related factor 2 (Nrf2) B. Relative gene expression of heme oxygenase (HO-1) C. Relative gene expression of induced NO synthase (iNOS) D. Relative gene expression of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1-α). Results are presented as mean ± SD (n = 12 samples per group). * $p < 0.05$

Figure 5. Gene expression in the spinal cord (SC) in the group treated with edaravone (ET), control group with EAE (CG1) and control group consisting of healthy mice (CG2) at the 28th day after EAE induction. A. Relative gene expression of nuclear factor erythroid 2-related factor 2 (Nrf2) B. Relative gene expression of heme oxygenase (HO-1) C. Relative gene expression of induced NO synthase (iNOS) D. Relative gene expression of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1-α). Results are presented as mean ± SD (n = 6 samples per group). * $p < 0.05$

Figure 6. Mitochondrial functions in the spinal cord (SC) and the brain in the group treated with edaravone (ET), control group with EAE (CG1) and control group consisting of healthy mice (CG2) at the peak of the EAE. A. Activity of mitochondrial complex I in the SC B. Activity of the complex I in the brain. C. Activity of the complex II+III in the SC D. Activity of the complex II+III in the brain E. Activity of the complex IV in the SC F. Activity of the complex IV in the brain. G. Complex I-linked respiration in the SC H. Complex I-linked respiration in the brain I. Complex II-linked respiration in the SC J. Complex II-linked respiration in the brain. K. ATP production in the spinal cord (SC) L. ATP production in the brain. Results are presented as mean ± SD (n = 3 samples per group). * $p < 0.05$

Fig. 1

Clinical scores

Group	Maximal score	Score on the last day	CDI
ET	2.7 ± 0.8*	3.0 ± 1.3	2.4 ± 0.6*
CG1	3.4 ± 0.6	3.2 ± 1.2	2.8 ± 0.7

Abbreviations: ET – edaravone; CG1 = control group receiving vehicle; CDI = cumulative disease index (defined as total disease score over experiment).
* p<0.05 between ET and CG1

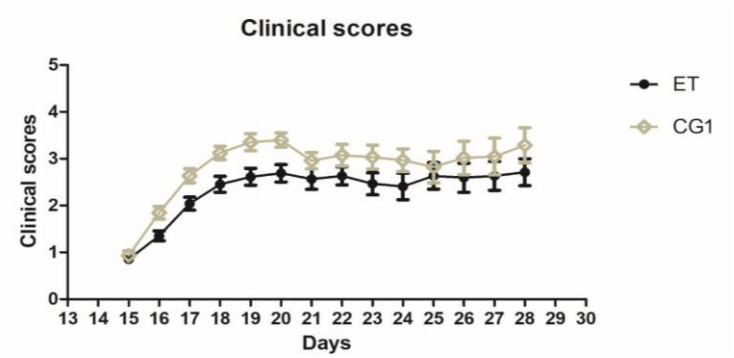
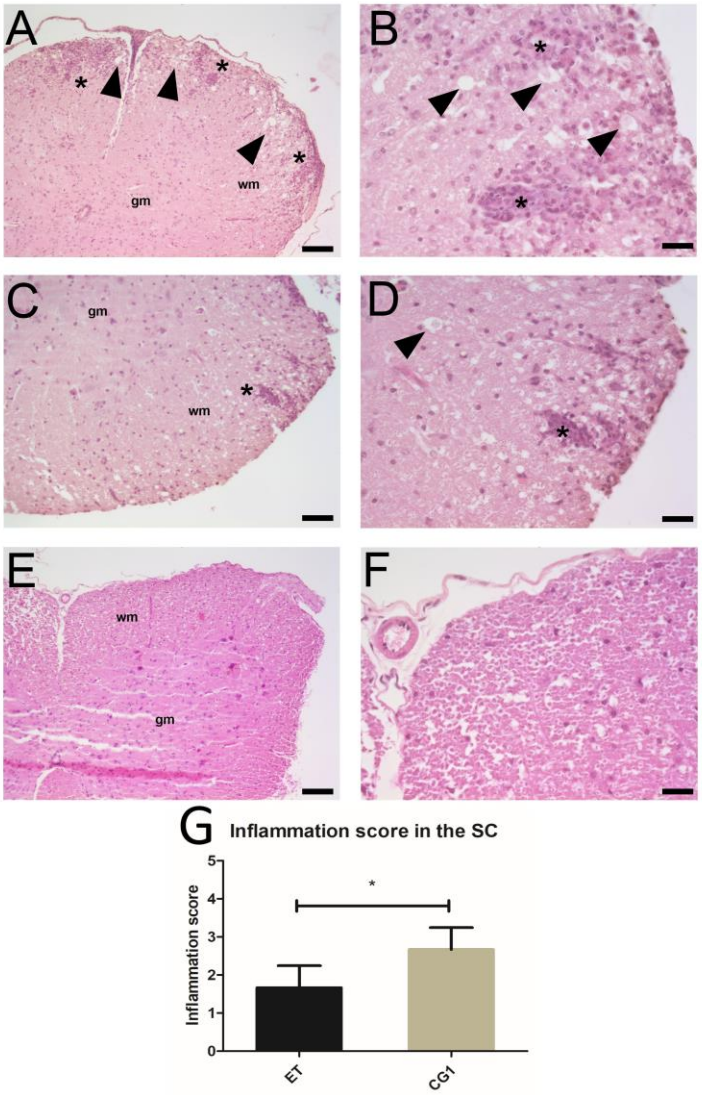
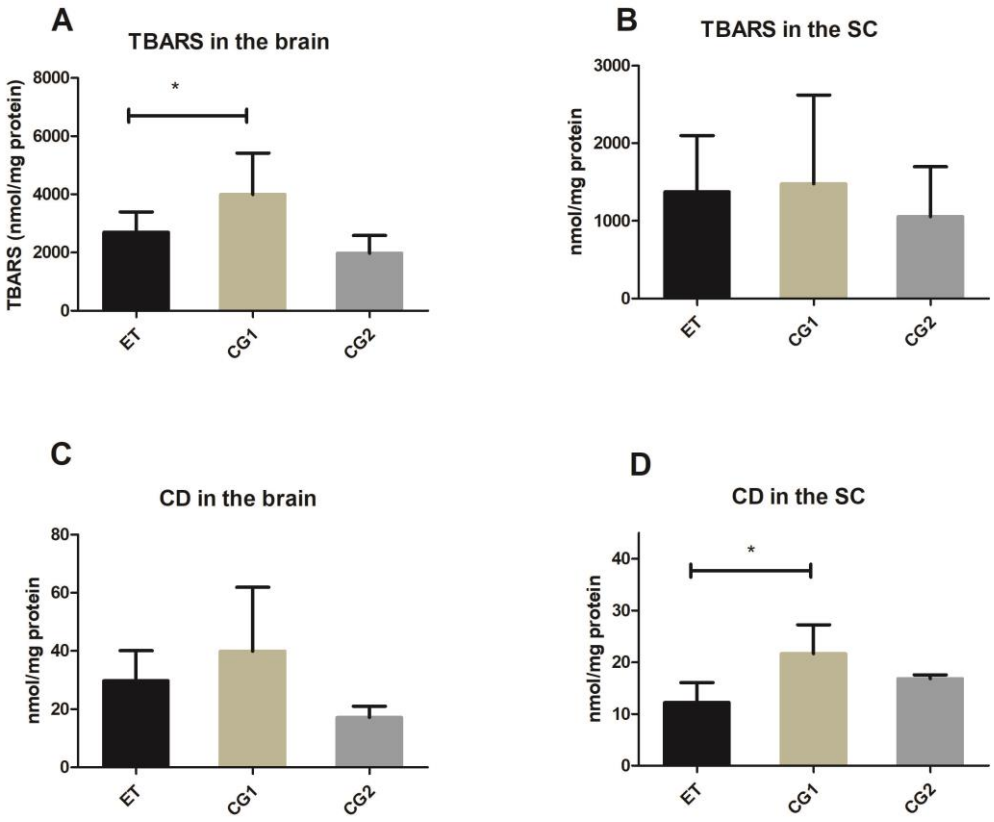


Fig. 2



509 Fig. 3



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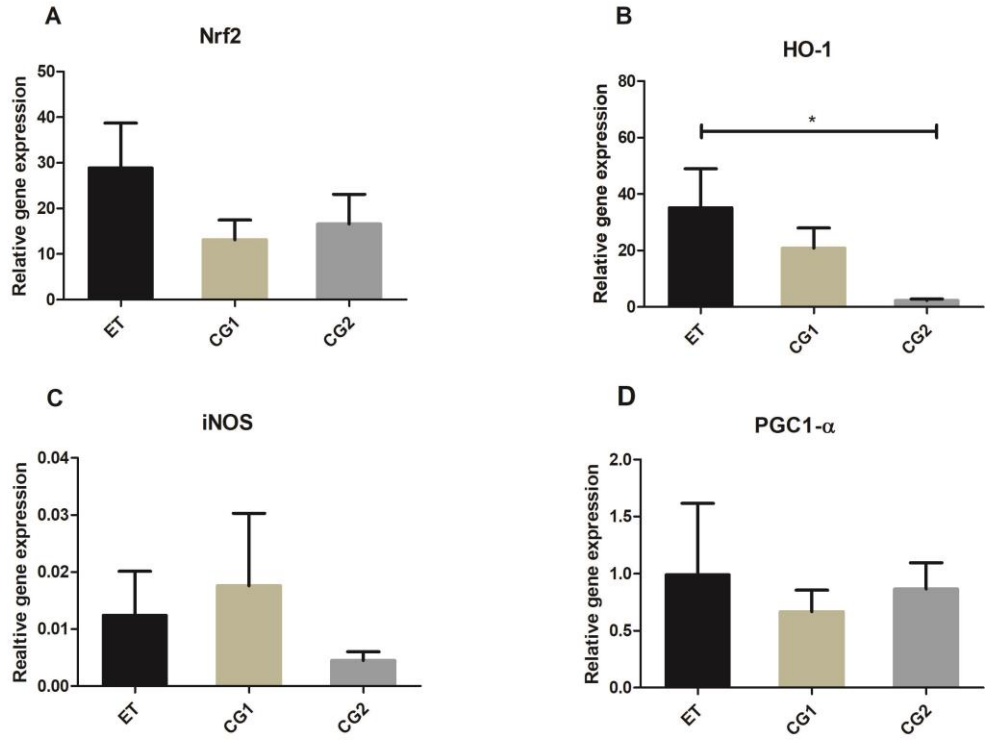
512 Fig. 4



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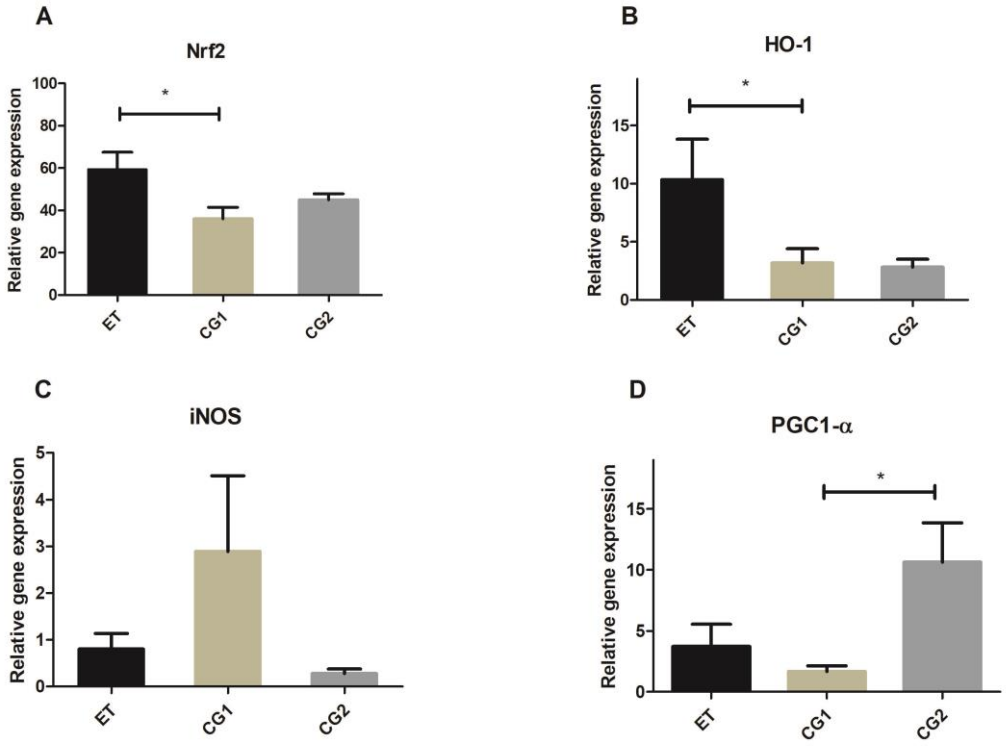
515 Fig. 5



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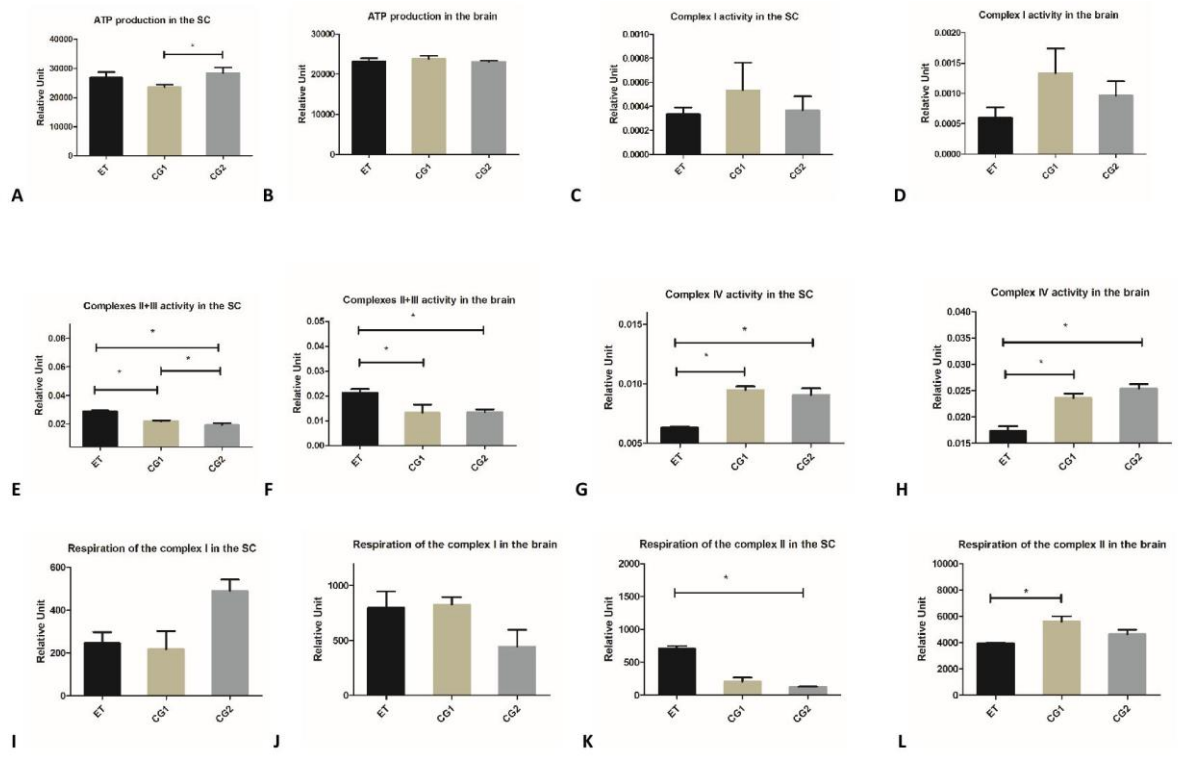
518 Fig. 6



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