

Compensatory Neuroprotective Response of Thioredoxin Reductase Against Oxidative-Nitrosative Stress Induced by Experimental Autoimmune Encephalomyelitis in Rats: Modulation by Theta Burst Stimulation

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Abstract

Cortical theta burst stimulation (TBS) structured as intermittent (iTBS) and continuous (cTBS) could prevent the progression of the experimental autoimmune encephalomyelitis (EAE). The interplay of brain antioxidant defense systems against overproduction of reactive oxygen, nitrogen, and thiol species induced by EAE has not been entirely investigated, just as the effect of iTBS or cTBS on oxidative-nitrogen stress (ONS) in EAE rats. Dark Agouti strain female rats were tested for the effects of EAE and TBS. The rats were randomly divided into the following groups: C - control, EAE - rats immunized for EAE, CFA - rats immunized with Complete Freund's adjuvant; iTBS and cTBS groups, and EAE+iTBS and EAE+cTBS - health and EAE rats exposed to iTBS and cTBS, respectively; EAE+iTBSsh and EAE+cTBSsh - sham stimulated EAE rats with the same noise artifacts of iTBS and cTBS, respectively. Superoxide dismutase activity, levels of superoxide anion ($O_2^{\cdot-}$), lipid peroxidation, glutathione (GSH), nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase (TrxR) activity were analyzed in rat spinal cords homogenates. The severity of EAE clinical coincided with the climax of ONS, based on the increase of superoxide anion and lipid peroxidation; depletion of total thiols, GSH and NADPH; and decrease of SOD activity. The TrxR imposed the most sensitive response against the applied central nervous system (CNS) stressors to rats. We concluded that the TrxR upregulation meritoriously compensates decreased ROS sequestering and GSH systems in EAE. Both iTBS and cTBS modulate the biochemical environment at a distance from the area of stimulation against ONS, accomplish a similar effect on TrxR activity to EAE and healthy rats, and alleviate symptoms of EAE.

Keywords: thioredoxin reductase; oxidative stress, nitrosative stress; theta burst stimulation; experimental autoimmune encephalomyelitis; rats

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an animal model of relapsing-remitting multiple sclerosis (MS). Repeated neuroinflammatory attacks induce demyelination, neuronal damage/loss, reactive

gliosis, the formation of sclerotic plaques, and aggravate remyelination. Those processes are oxidative-nitrosative stress (ONS) associated [1].

The mitochondrial respiratory chain is a primary source of reactive oxygen species (ROS) in physiological conditions [2]. Overproduction of free oxygen and nitrogen radicals, arising mainly from activated glial and T cells, overwhelm the antioxidant capacity in EAE neuroinflammation. The depletion of the endogenous sources of reducing equivalents [nicotinamide adenine dinucleotide phosphate (NAD(P)H) and glutathione (GSH)] by antioxidant enzymes, such as superoxide dismutase (SOD), and glutathione reductase (GR), glutathione peroxidase or others of glutathione or thioredoxin cycles is a recognizable feature in many diseases associated with ONS [3].

Besides GSH system, the antioxidative responses of brain tissue engage other thiol-reducing systems the thioredoxin (Trx) system, which has not clarified entirely [4, 5]. It is known that thioredoxin (Trx) is an endogenous antioxidant that reduces disulfides of proteins. Regeneration of Trx arises from thiol reduction catalyzed by the flavo- and selenoprotein–thioredoxin reductase (TrxR) and cofactor NADPH. Likely to GSH, Trx is likewise an astroglia-derived neuro-protectant [6-8].

The inability of the antioxidant defense system to counteract ROS overproduction in the central nervous system (CNS) is coupled with low adenosine three phosphates production in MS, hence the function of mitochondria is slaughtered [9]. Feedback mechanisms by which glucose-6-phosphate may be utilized in glycolysis to produce energy in the form of ATP and NADH, used to store energy in the form of glycogen, or used by the pentose phosphate pathway compensates energy loss and keep the viability of cells [10]. Oxidative stress hinders oligodendrocytes from performing myelination of axons and also obstructs oligodendrocyte progenitor cells (OPCs) to differentiate into oligodendrocytes [11].

It has been shown that repetitive transcranial stimulation arouses remyelination and fosters the expression of neurotrophic factors that are of significant importance for neuro-regeneration in EAE animals [12]. In the cerebral cortex, it may promote neuronal activities in remote zones of the spinal cord, which is interconnected with the main motor pathway - corticospinal tract. Also, neuronal activity is found essential for OPCs differentiation into oligodendrocytes in that part of CNS [11, 13].

Structured patterns of repetitive transcranial stimulation such as theta burst stimulation (TBS), modulate motor cortical excitability. Intermittent TBS (iTBS) provokes an early stage of long-term potentiation, while

continuous TBS (cTBS) induces an early stage of long-term depression. Cortical neuronal activities (disinhibition/inhibition) associated with neuronal plasticity, support, and orchestrate remyelination of the affected neural tissue in the lumbar spinal cord, where the redox homeostasis plays an extraordinary role [14]. The precise underlying mechanisms of iTBS or cTBS have not revealed yet.

Considering the above data, in the present study, we evaluated if iTBS and cTBS affect redox homeostasis, including the status of thiols and thioredoxin in the spinal cord of EAE in rats.

Materials and methods

Animals and experimental procedure

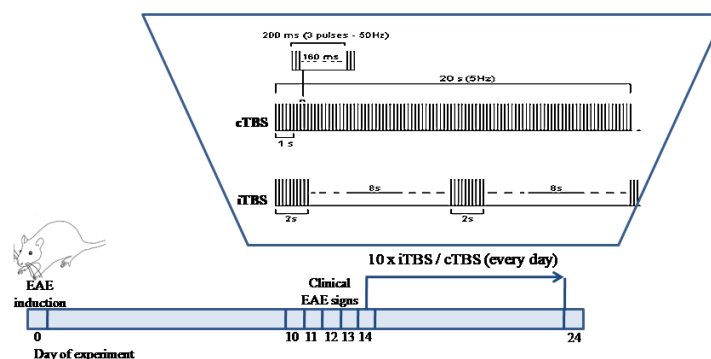
The experiment was conducted in female Dark Agouti (DA) rats, weighed 150-200 g, and aged 10-14 weeks [15]. The animals (6 per cage) were housed in polyethylene cages under standardized housing conditions (ambient temperature of 23 ± 2 °C, a relative humidity of 55 ± 3 %, a light/ dark cycle of 13/11 h). The access to laboratory food and water was *ad libitum*, and for welfare assistance to animals with developed EAE, food, and water were bottom-positioned. Rats were habituated to the ambient and laboratory staff five days before the experimental procedure. The experiment lasted 25 days. The TBS started from 14th post-immunization day (dpi) in EAE rats and lasted ten days. Rats were intraperitoneally anesthetized with sodium pentobarbital and decapitated after 24 hours of the last treatment.

This EAE animal study is a part of the more significant EAE project, approved by the Ethical Community of the Military Medical Academy (Belgrade, Serbia) (license no. 323-07-00622/2017-05) which followed the principles of the governmental policy of Official Gazette Republic of Serbia (No. 14/2009) and Directive 2010/63/EU.

To immunize rats for EAE, we injected subcutaneously 0.1 ml of rat spinal cord homogenate (50% w/v in saline) suspended in Complete Freund's Adjuvant (CFA), containing 1 mg/ml *Mycobacterium tuberculosis* (CFA; Sigma, St. Louis, MO, USA) in the right hind footpad. Rats were previously anesthetized intraperitoneally with sodium pentobarbital 45 mg/kg body weight (Tritay, Germany) [16].

Theta burst stimulation was applied with MagStim Rapid2 device with a 25 mm figure-of-eight coil (The MagStim Company, Whitland, Dyfed, UK). Rats were manually held, and the coil was positioned over bregma in direct physical contact with the animal head [14]. The iTBS block consisted of 600 pulses during

192 s. Twenty trains of 10 bursts with three pulses at a frequency of 50 Hz, repeated at 5 Hz, with 10 s pauses between trains were applied [17, 18]. The cTBS consisted of 600 pulses set in a single train of bursts during 40 seconds, repeated at 5 Hz. The applied intensity corresponded to the 30% of maximal strength of TBS, which was merely under a motor threshold of rats, evaluated as a visible contraction of upper limbs without any other apparent distress (Scheme 1).



Scheme 1. Theta burst stimulation of rats

The rats (n=54) were randomized into two categories, non-EAE, and EAE groups, besides the C-control group. Nine groups (n=6/group) in total. Non-EAE groups: CFA – rats treated with Complete Freund's Adjuvant, iTBS, and cTBS groups – rats subjected to iTBS and cTBS. -EAE groups: EAE-rats with experimental autoimmune encephalomyelitis, EAE+iTBS, and EAE+cTBS groups – EAE rats subjected to iTBS and cTBS, and EAE+iTBSsh and EAE+cTBSsh – EAE rats subjected to iTBS's and cTBS's noise artifact (sham-treated EAE rats).

Rats were monitored daily and clinically scored for EAE neurological signs.

Clinical evaluation

The double-blind approach was applied for the rats' daily observation and scoring of EAE signs, up to 24 dpi. The clinical symptoms and signs were ranged from 0 to 5: 0 – no oddity; 0.5 – fairly lose tail tone and inability to rotate the posterior side of the tail; 1 – depressed tail tonus; 1.5 – moderately unsteady walk and

reduced ability to straight-up or their combination; 2 – weakness of hind limbs; 2.5 – incomplete hind limb paralysis; 3 – complete hind limb paralysis; 3.5 – complete paralysis of hind limbs and weakness of the front limbs; 4 – quadriplegia with breathing effort; and 5 – moribund or death [19].

The following descriptors (presented as Mean \pm STDEV) for the severity of EAE clinical symptoms and monitoring of the iTBS or cTBS effects were considered: incidence (proportion of rats with clinical score \geq 0.5 per group); daily clinical score (rating of clinical symptoms per rat per day); maximal clinical severity score [duration of the most severe clinical symptoms (days)]; EAE onset [the appearance of the early clinical signs (day)]; duration of paralysis [period after the EAE onset, when rats had a score \geq 2.5 (days)]; and mortality rate.

Tissue homogenates and biochemical analyses

Spinal cords were dissected, and lumbar region slices were transferred separately into saline solution (0.9 % w/v). A crude mitochondrial fraction was prepared as follows: aliquots (1 mL) were homogenized (homogenizer-Tehnica Zelezniki Manufacturing, Slovenia) twice using Teflon pestle at 800 rpm (1000 g) for 15 min at 4 °C, and centrifuged at 2500 g for 30 min at 4 °C. The subcellular membranes pellet was solubilized in 1.5 mL of deionized water, by constant mixing with Pasteur pipette, for 1h. The mixture was then centrifuged at 2000 g for 15 min, at 4 °C, and supernatant (a crude mitochondrial fraction) was used for analysis [20].

Nitrite and nitrate concentration ($\text{NO}_2 + \text{NO}_3$) was determined spectrophotometrically at 492 nm, using the colorimetric Griess method [21].

Lipid peroxidation was determined spectrophotometrically by using the thiobarbituric acid reactive species (TBARS) method, as described by Girotti et al. The results are expressed as nmol of malondialdehyde (MDA) per milligram of proteins (nmol MDA /mg protein) [22].

Superoxide anion ($\text{O}_2^{\cdot-}$) was determined spectrophotometrically at 550 nm. The principle of the method was based on the reduction of nitroblue-tetrazolium (Merck, Darmstadt, Germany) in the alkaline, nitrogen saturated medium [23].

Superoxide dismutase (EC 1.15.1.1.; SOD) activity was expressed as the degree of inhibition of epinephrine auto-oxidation by SOD, in the presence of O₂, in alkaline pH. It was measured spectrophotometrically at 480 nm [24].

Total sulfhydryl groups (SH) were measured spectrophotometrically at 412 nm by Elman's method [25].

Total GSH was measured spectrophotometrically at 412 nm, for 6 minutes, by 5,5-dithiobis-2-nitrobenzoic acid (DTNB) - oxidized glutathione (GSSG) recyclable method. The level of produced 5-thio-2-nitrobenzoic acid (TNB) was proportional to the total GSH concentration [26]. The content of total GSH was expressed as nmol/ mg protein.

Thioredoxin reductase activity was measured spectrophotometrically at 412 nm with a commercially available Assay kit (Abcam; ab83463). The principle of the method refers to the reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) into yellow-colored 5-thio-2-nitrobenzoic acid (TNB), catalyzed by thioredoxin reductase that utilizes NADPH. The results are expressed as mU/mg proteins (one unit of thioredoxin reductase activity equals the formation of 1,0 μ mol of TNB per minute at 25°C) [27].

The reduced form of NADPH was determined spectrophotometrically using commercial NADPH Assay Kit at OD450nm (Abcam 65349, UK) [28].

Total proteins were determined by the Lowry method [29].

Statistical analysis

One-way ANOVA and Bonferroni's post hoc multiple tests were used (software GraphPad Prism, version 5.03) for statistical data analysis. The values are presented as means \pm SD. The level of confidence referred to $p < 0.05$.

Results

Clinical observation

The onset of EAE symptoms was established at 10.5 ± 0.23 dpi in all immunized rats (100% incidence). No lethality occurs in any of the experimental groups. The same pattern, addressing the onset and the duration of EAE clinical manifestations was recognized within non- and TBS treated EAE groups. Non-TBS treated groups refer to EAE and sham i/cTBS treated EAE groups, and TBS treated EAE groups refer to i/cTBS

treated EAE groups. Duration of limb paralysis ($p < 0.05$) and severity of EAE symptoms were lower in TBS treated EAE rats than in EAE rats (Table 1).

Table 1. Alleviation of EAE clinical symptoms by iTBS and cTBS in rats

Experimental groups	Maximal clinical severity score		Paralysis		Body mass loss and gained	
	Started (dpi)	Lastest (days)	Started (dpi)	Lastest (days)	Min. weight (%)	Gained weight (%)
NonTBS-EAE groups: EAE EAE+i/cTBSsh	11.8	3.08±0.97	12	~3.5 ~2.5	~70 (14 th dpi)	~96 (20-24 th dpi)
TBS-EAE groups: EAE+i/cTBS	11.5	2.79±0.76	11.8	~1.7 ^a	~81 (15 th dpi)	~90 (20-24 th dpi)

The progress of EAE (development and withdrawal of severe clinical signs) overlapped with body mass loss and its gaining back (Figure 1).

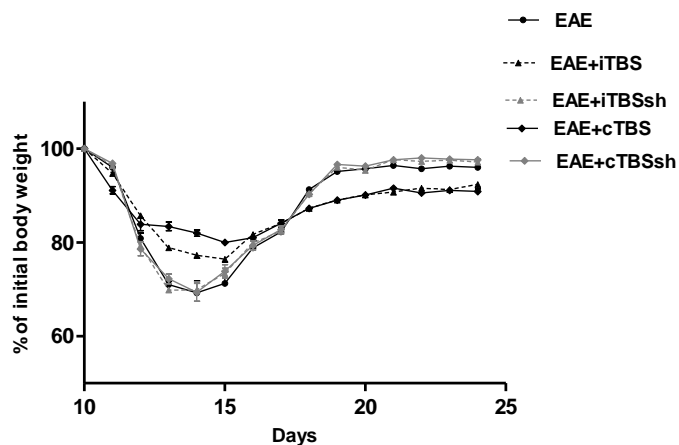


Figure 1. The effect of EAE, i/cTBS and sham i/cTBS on the rat body mass

The average values of body mass changes per group were presented linearly and are expressed as a percentage of initial body weight.

The drop in the body mass and gaining back was more intense in non-TBS treated than in TBS treated EAE rats (reduction for 30 % and 19%; and achieved 96% vs. 90% of the initial weight, respectively) as compared to 10th dpi, when EAE onset happened. The body mass loss coincided with paralysis (Table 1, Figure 1).

Clinical symptoms, including motoric/walking possibilities and developed inabilities, were observed daily and scored in EAE, TBS- and sham-TBS- treated EAE rats. The totals of daily clinical scores were summarized

for days before (0-13th dpi) and during (14-24th dpi) the applied TBS or sham TBS to EAE rats (Figure 2). The summarized clinical scores for 14-24th dpi were significantly lower in cTBS treated EAE rats compared to EAE rats ($p < 0.05$).

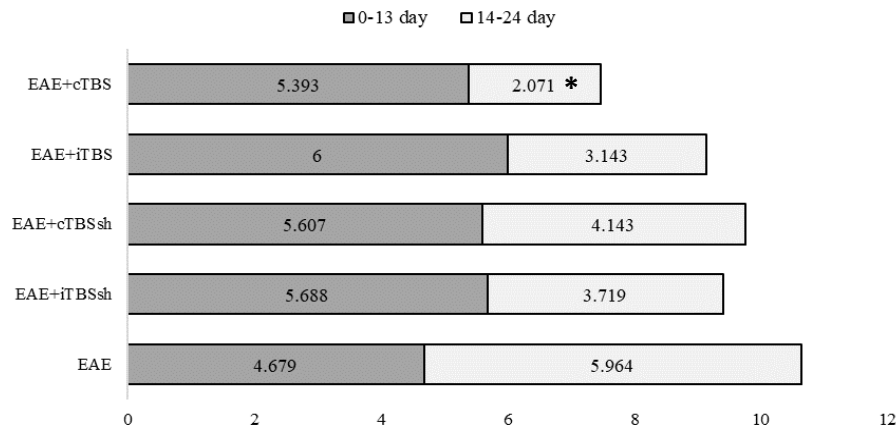


Figure 2. Summarized daily scores of EAE clinical symptoms before and during the TBS or sham TBS to EAE rats

The number of rats per group was 8. The totals of daily EAE clinical symptoms scores were calculated for 0-13th dpi (bottom part of the bars) and during 14-24th dpi (upper part of the bars). Clinical symptoms were numerically evaluated (see Figure 1 capture). The statistical significance compared to the EAE group was considered at ^a $p < 0.05$.

Oxidative stress biochemical analyses

The concentrations of NO_2+NO_3 increased in TBS-untreated groups (EAE and EAE+iTBSsh and EAE+cTBSsh) ($p < 0.05$) compared to both iTBS and cTBS - treated EAE groups, which remained equal to the control values (Fig. 3a).

The concentrations of TBARS increased with progress and peak of the disease (EAE14; $p < 0.01$), but lately it decreased (EAE24; $p < 0.01$). Decreased TBARS values were documented in both TBS-treated EAE groups (EAE+iTBS and EAE+cTBS) and TBS-untreated EAE groups (EAE+iTBSsh and EAE+cTBSsh), compared to EAE14 ($p < 0.01$) (Fig 3b).

In both of TBS-treated (EAE+iTBS, EAE+cTBS) and TBS-untreated EAE groups (EAE+iTBSsh, EAE+cTBSsh), production of O_2^- decreased, compared to the peak of the disease (EAE14; $p<0.01$). In this term, O_2^- elevated, compared to controls ($p<0.001$). Remarkable data was depleted production of O_2^- in healthy animals treated with iTBS ($p<0.05$) and cTBS ($p<0.01$), compared to controls (Fig. 3c).

A significant drop of SOD activity was measured on the 10th day in EAE rats (EAE10; $p<0.001$) compared to the controls, while afterward, it started to rise (EAE14/24, $p<0.01$; compared to EAE10). The activity of SOD also decreased in CFA treated animals ($p<0.001$), cTBS-treated EAE group (EAE+cTBS; $p<0.001$) and in TBS-nontreated rats (EAE+iTBSsh and EAE+cTBSsh; $p<0.001$). Increased SOD activity occurred in EAE+iTBS rats, compared to all EAE groups (EAE10, $p<0.001$; EAE14, $p<0.01$; EAE24, $p<0.05$) (Fig. 3d).

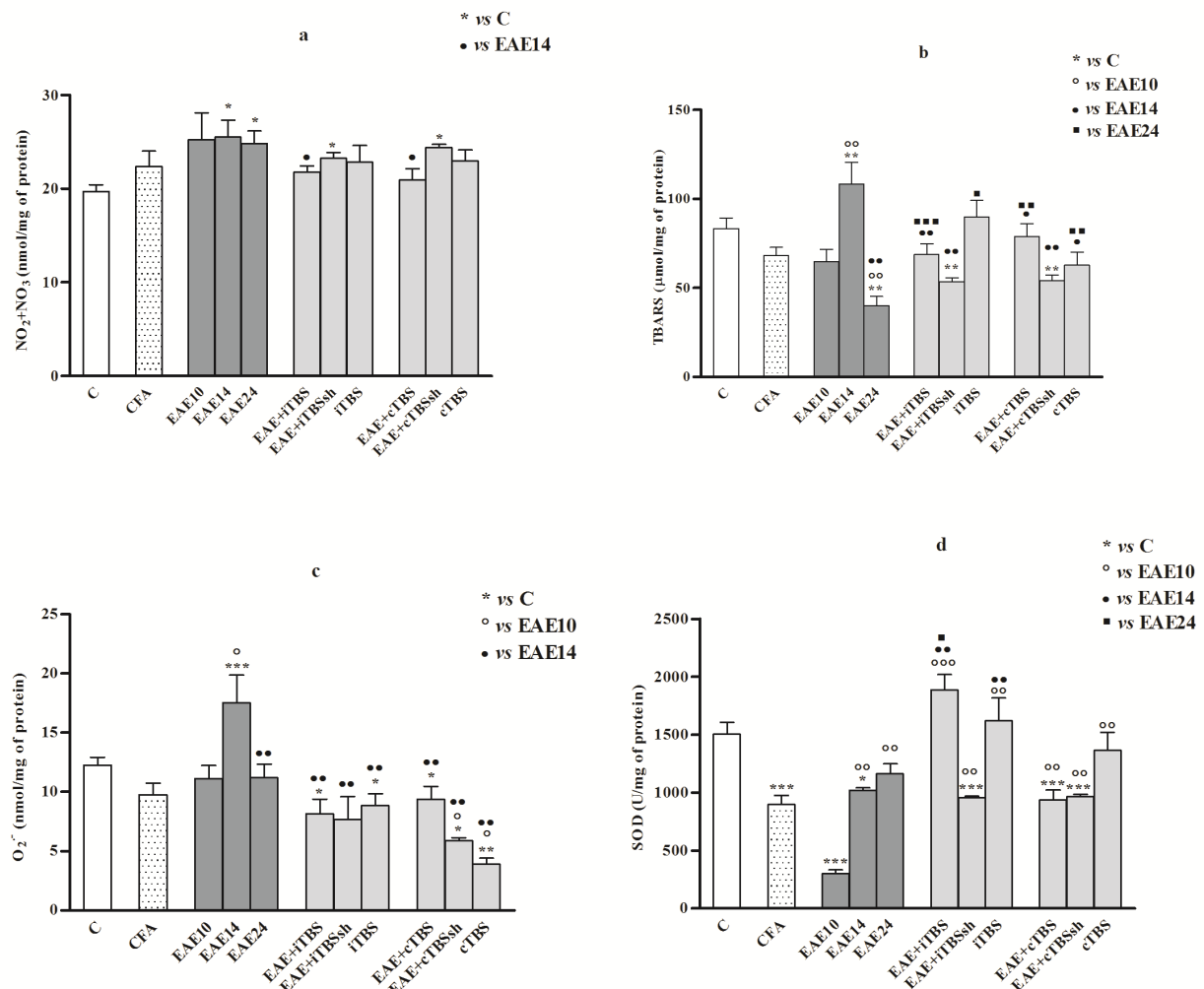


Figure 3a-d. Reactive oxygen and nitrogen species biochemistry and lipid peroxidation status in the rat spinal cord

*Oxidative/nitrosative stress parameters in the spinal cord at 10, 14 and 24 dpi in EAE rats, and after iTBS/cTBS: (a) nitrite and nitrate (NO_2+NO_3 ; nmol/mg proteins); (b) lipid peroxidation (TBARS; $\mu\text{mol/mg}$ proteins - corresponds to malondialdehyde (MDA)); (c) superoxide anion radical (O_2^- ; nmol/min/mg proteins); (d) superoxide dismutase (SOD; U/mg proteins). Values are presented as means \pm SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ statistically significant difference compared to control, $^{\circ}p<0.05$, $^{\circ\circ}p<0.01$, $^{\circ\circ\circ}p<0.001$ compared to EAE10, $^{\bullet}p<0.05$, $^{\bullet\bullet}p<0.01$, $^{\bullet\bullet\bullet}p<0.001$ compared to EAE14, $^{\text{I}}p<0.05$, $^{\text{II}}p<0.01$, $^{\text{III}}p<0.001$ compared to EAE24* The concentration of thiols (SH-containing compounds) declined with the disease progress (EAE14, $p<0.05$: compared to the controls). Elevated values of thiols were measured in both iTBS and cTBS treated EAE compared to all EAE groups (EAE10/14/24 groups, $p<0.05$) (Fig. 4a).

The GSH decreased in EAE10 and EAE14 groups compared to the controls ($p<0.05$). The renewal of GSH content was noticed on the 24th day (EAE24). The cTBS increased GSH in EAE rats (EAE+cTBS), compared to EAE24 ($p<0.05$). Interestingly, iTBS increased GSH in healthy animals ($p<0.001$) (Fig 4b).

The activity of TrxR increased in all experimental groups [CFA ($p<0.05$); EAE10,14,24 ($p<0.01$); EAE+i/cTBS ($p<0.01$); EAE+i/cTBSsh ($p<0.01$); i/cTBS ($p<0.01$)] compared to controls. The activity of TrxR decreased in TBS-untreated groups [(EAE+iTBSsh ($p<0.05$) and EAE+cTBSsh ($p<0.05$))] as well as in EAE+cTBS rats ($p<0.05$), compared to EAE24 group (Fig. 4c).

The reserves of NADPH in the spinal cord of EAE 10/14/24, CFA group, and both sham TBS-treated EAE rats were depleted, compared to the controls (all $p<0.05$). The iTBS and cTBS increased NADPH concentration back to control levels in treated EAE rats. Additionally, NADPH in EAE+iTBS rats was higher from EAE10/14/24 groups ($p<0.05$) (Fig 4d).

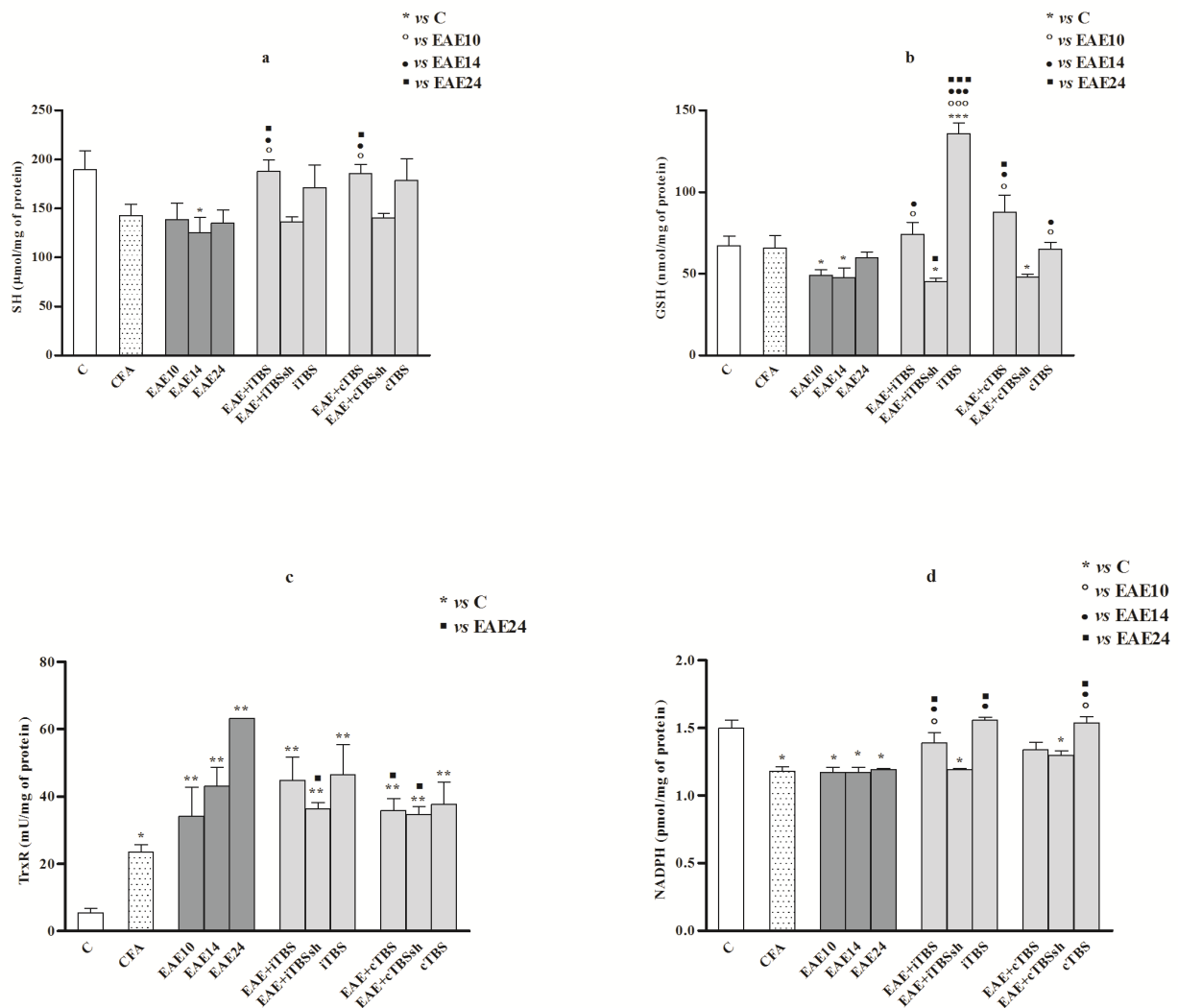


Figure 4 a-d. Thiol-associated biochemistry and NADPH status in the rat spinal cord

Parameters of thiol species-associated biochemistry in the spinal cord at 10, 14 and 24 dpi in EAE rats, and after iTBS/cTBS: (a) total sulfhydryl groups (SH; $\mu\text{mol/mg}$ proteins); (b) Glutathione (GSH; nmol/mg proteins); (c) Thioredoxin reductase (TrxRs, mU/mg proteins); (d) Nicotinamide adenine dinucleotide phosphate-reduced form (NADPH) (pmol NADPH/mg proteins). Values are presented as means \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ statistically significant difference compared to control, ° $p < 0.05$, °° $p < 0.01$, °°° $p < 0.001$ compared to EAE10, • $p < 0.05$, •• $p < 0.01$, ••• $p < 0.001$ compared to EAE14, ' $p < 0.05$, '' $p < 0.01$, ''' $p < 0.001$ compared to EAE24.

Discussion

Subtle pathological changes occur in the brain or spinal cord of rats immunized for EAE before the onset of EAE signs. Astrogliosis has been reported as an underlying mechanism of EAE [12]. Gliosis is usually established after two to three weeks of a CNS injury in rats, which coincided with our results that the peak of the EAE clinical score was achieved at 14dpi and overlapped with the highpoint of ONS measured in the spinal cord of treated rats ([30]). Obtained results confirm that EAE pathology is closely associated with OS and NS [31].

Herein, we showed that TBS of EAE rats mitigated the ONS in the spinal cord and improved clinical symptoms of EAE. Contrary to the report that modulation of cortical excitability by cTBS and iTBS have been reported to occur in opposite directions in EAE, we obtained almost similar outcomes by both iTBS and cTBS, with somewhat more beneficial effects accomplished by iTBS. Underlying mechanisms of TBS have not been fully elucidated. Theta burst stimulation raises neuronal activity, adult neurogenesis, and remyelination by affecting astrocytes and microglia/macrophages. The effect was realized, both at the site of stimulation and in the zone of the spinal cord [32, 33].

The NOx levels were the highest in EAE rats, while lately, applied TBS reduced it slightly (Figure 3a). Increased metabolism of nitric oxides is the hallmark of glutamate turnover, i.e., excitotoxicity in EAE. Glutamate-mediated excitotoxicity occurs via ionotropic glutamate receptors activation and increased calcium (Ca^{2+}) influx. Cells Ca^{2+} overload affects mitochondrial respiratory chain and ATP production, which is an early occurrence in EAE lesions. Also, macrophages and microglia influence metabolic and regenerative processes, stripping myelin and inducing matrix metalloproteinases. The localization of mitochondria within axons is essential for maintaining axonal integrity, demyelination, and remyelination [34]. Release of ROS from macrophages occurs during phagocytosis ("oxidative burst") of engulfed debris and toxic products from the surrounding environment [35]. Oxidatively damaged neighbor health tissue, though not affected by inflammation can facilitate the infiltration of immune cells, what is the scenario recognized in an early stage of EAE [36]. Assumingly, that could be the explanation of the simultaneous occurring of EAE symptoms severity and climax of ONS.

Antioxidant enzyme SOD converts $\text{O}_2^{\cdot-}$ into molecular oxygen and hydrogen peroxide, by utilizing NAD(P)H. Its deficient activity on 10dpi was followed with reduced elimination of $\text{O}_2^{\cdot-}$, which resulted in its peak on

14dpi, which coincided with the severity of lipid peroxidation (expressed as TBARS) and the most severe clinical signs in EAE rats. However, the return of SOD to normal on 14th dpi resulted in the consequent decrease of O_2^- and TBARS to control values (Figure 3b,c), according to the study of Thimm et al. (Fig. 3c, d) [37].

Reduced SOD activity along with NADPH in CFA, EAE, and sham-TBS treated EAE rats (Fig. 3d) indicate that both nonspecific immuno-stimulation (CFA-mediated) and specific (immunization for EAE) deteriorated redox homeostasis in the direction of OS and NS [38]. The distinctive impact of iTBS and cTBS on SOD activity was documented in EAE treated rats. Its activity increased under the iTBS, while cTBS did not impose any effect. The upregulation of antioxidative enzymes is mediated by the nuclear factor (erythroid-derived-2)-like 2 factor (Nrf2) pathway in nervous tissue. The Nrf2 path is extinguished in EAE; thus, we speculated that iTBS could upregulate it and contributing to the enabling of SOD [39]. Mitochondrial function is particularly compromised by the excess of glutamate and pro-inflammatory mediators (proteases, inflammatory cytokines, etc.). Reactive species oxidatively damage neuronal and glial cells, including oligodendrocyte progenitor cells, as well as recruit and activate T cells at the site of inflammation. That could be a reason for matrix metalloproteinases production and the blood-brain barrier disrupter.

The occurrences mentioned above coincided with a decrease of GSH, total SH pool, and NAD(P)H (Figure 4 a,b,d). The changes in total SH-containing compounds and GSH were similar (Fig. 4a). It is well-known that -SH groups of cysteine of GSH is responsible for its antioxidant and other physiological roles. As a strong nucleophile, SH-groups are prone to oxidation what was probably the reason for the reduced content of total SH-containing compounds and GSH documented in EAE and EAE+iTBSsh or EAE+cTBSsh groups, compared to controls [40]. Thiols reduce an oxidative attack and preserves surrounding biomolecules from oxidative damage. The functional impairment of SH-groups containing molecules could arise from metabolic disorders but also long-lasting overstimulation of neurons in EAE. In excitotoxicity, an overload of the glutamate-cysteine transporters blocks cysteine transport, affecting cysteine, which is the source of SH-group in GSH, and other thiol-proteins [41]. The GSH cycle encompasses several antioxidant enzymes that utilize GSH or NADPH as donors for reducing equivalents. The main representatives are glutathione reductase (GR) [catalyzes NAD(P)H-dependent reduction of oxidized glutathione (GSSG) back into GSH], glutathione

peroxidase (catalyzes GSH-dependent reduction of lipid hydroperoxides or H_2O_2 , into alcohol or water, respectively), glutathione-S-transferase utilizes GSH in thiolation of compounds, etc. [3].

Nevertheless, if applied to EAE or health rats, both iTBS and cTBS, returned the -SH and GSH levels to the control values. Mitochondrial GSH is recognized to be a critical factor in antioxidant protection. Thus impairment of mitochondrial physiology may considerably ruin the GSH antioxidant capacity [42]. The antioxidant effect of thiols may be the primary underlying mechanism of ROS sequestration during reactive astrogliosis treated with TBS, particularly by iTBS that arrives more effective than cTBS in preserving/restoring of the GSH reserves (documented in health rats) (Fig. 4b).

Unlikely to the GSH antioxidant role, little is known about the physiological significance of the Trx system in this capacity [5, 43-45]. To our knowledge, the present study is the first in the literature to address the role of the Trx/TrxR system relative to the GSH system in the response of the brain to induce EAE in rats.

Down-regulated antioxidant enzymes, next to impaired mitochondrial GSH pool in EAE rats (Figure 4b), imply that GSH may not be effective enough in coping with the ONS evoked by EAE [42]. Contrary to the reduced ROS sequestering system (herein represented by SOD) and GSH system, we obtained a remarkable increase of TrxR in rats immunized for EAE (specific immunization), CFS (nonspecific vaccination), and TBS-treated health rats (Fig. 4c). The decreased level of total thiols (Figure 4a) correlates with increased TrxR activity. By that, we confirmed the exceptional protection role of TrxR against brain oxidant injury in rats and its outstanding sensitivity to the applied CNS stressors, according to the Ruszkiewicz J. and Jan Albrecht J study [42]. Herein we interpret the increased TrxR activity as a neuroprotective response to ONS in rats against all the applied treatments. We showed that upregulation TrxR activity in EAE might compensate reduced GSH-cycle antioxidant role and SOD activity, thus significantly contributes to brain antioxidant response to ONS in rats (Figure 4c). No statistical significance of TrxR activity between EAE or healthy rats subjected to TBS was obtained. The underlying mechanisms of TrxR response in EAE and EAE exposed to TBS are challenging to explain, due to uncertain available evidence of the Trx/TrxR role in different neurological diseases. Thioredoxin reductase imposes additional activities, besides the reduction of Trxs. It recycles ascorbate and imposes another biological role in cell growth and transformation. However, increased brain Trx/TrxR role was seen in hyperoxia in newborn rats and ischemia induced by transient middle cerebral artery occlusion in adult mice, and in Alzheimer's disease-affected brain [46-48]. The underlying

neuroprotective role of the Trx/TrxR system may derive from its vigorous release from an astroglia-derived cell line exposed to hydrogen peroxide. Accordingly, the extreme upregulation of TrxR in EAE rats may be explained by the ONS-associated astrogliosis [12, 49, 50].

The decrease of NADPH in EAE rats was expected, due to its cofactor role in numerous enzymatic oxidoreductive reactions, including GSH turnover by GR; reduction of Trx, and other SH-compounds, prone to oxidation (Fig. 4d) [31]. The obtained result confirmed the increased utilization of NADPH during OS and NS in EAE rats. As a cofactor in lipid and cholesterol synthesis and fatty acid chain elongation, NAD(P)H is vital for neuronal tissue maintenance [51]. The consumption of GSH and NAD(P)H in enzymatic reactions of FRs' sequestrations entails the involvement of the pentose phosphate pathway [52]. The recovery of NADPH and total thiols and glutathione under the iTBS or cTBS promoters the better metabolic achievement in EAE. No statistical significance of TrxR activity between EAE or healthy rats subjected to TBS was obtained.

The maintenance of redox homeostasis and energy is a predominant target for EAE treatment. The peak of EAE clinical symptoms positively correlated with the climax of ONS, including depletion of total thiols and GSH, fall of SOD, and the rise of TrxR activity and consumption of NADPH. We confirmed that the Trx system efficiently compensates decreased ROS sequestering and GSH systems in EAE. No differences in TBS effect on TrxR activity were obtained between EAE and healthy rats.

Both iTBS and cTBS modulate the biochemical environment against spinal cord ONS and alleviate symptoms of EAE. Both TBSs shifted neuronal antioxidative defense towards a more reductive state to improve physiological resilience to ONS by modulating the biochemical environment in EAE at a distance from the area of stimulation.

Acknowledgments: The authors acknowledged the University of Defense (grant number MFVMA/01/18-20) and the Ministry of Education and Science Republic of Serbia (Grant No: III 41018) for supporting this study, and technical staff from the Center of Veterinary Services of Military Health Department. This research received no external funding.

List of abbreviations

CFA - Complete Freund's Adjuvant

CNS - central nervous system

cTBS - continuous theta burst stimulation

cTBSsh - sham stimulated EAE rats, exposed to the sound artifacts of cTBS

dpi - postimmunization days

EAE - experimental autoimmune encephalomyelitis

iTBS - intermittent theta burst stimulation

iTBSsh - sham stimulated EAE rats, exposed to the sound artifacts of iTBS

MS - multiple sclerosis

TBS - theta burst stimulation

Declarations

Ethics approval: The Ethical Community of the Military Medical Academy (Belgrade, Serbia): license no. 323-07-00622/2017-05, following the principles of the governmental policy of Official Gazette Republic of Serbia (No. 14/2009) and Directive 2010/63/EU.

Availability of data and materials: The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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

Funding: The University of Defense (Grant No: MFVMA/01/18-20) and the Ministry of Education and Science Republic of Serbia (Grant No: III 41018) supported this study. This research received no external funding.

Authors' Contributions: IS – Investigation, Methodology, Supervision, Writing-original draft; MN - Conceptualization, Investigation, Supervision, Writing-original draft; BM – Methodology, Resources, MM – Methodology, Resources; IS – Conceptualization, Project administration, Visualization; TI – Conceptualization, Project administration, visualization; MV – Methodology, Resources; MDj-corresponding author – Conceptualization, Investigation, Visualization Writing – review and editing.

All authors approved the submitted manuscript.

All authors agreed to be both personally accountable for the author's contributions and ensure the accuracy or integrity of any part of the work.

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