

*Original article* ■

# Benefit Agmatine Effects in Experimental Multiple Sclerosis. CNS Nitrosative and Oxidative Stress Suppression

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## SUMMARY

The aim of this study was to investigate the exogenous agmatine influence on nitrosative and oxidative stress parameters in acute phase of multiple sclerosis (MS) experimental model, experimental autoimmune encephalomyelitis (EAE).

EAE was induced by subcutaneous injection of myelin basic protein (50 µg per animal). Sprague-Dawley rats were divided into five groups: I group - (CG), treated by PBS (i.p.), II group - (EAE), III group - (CFA), treated with Complete Freund's adjuvant (0.2 ml subcutaneously), IV group - (EAE+AGM), treated by agmatine (75 mg/kg bw i.p.) upon EAE induction and V group - (AGM), received only agmatine in the same dose. The animals were treated every day during experiment – from day 0 to 15, and clinically scored every day. They were sacrificed on day 16 from MBP application. NO<sub>2</sub>+NO<sub>3</sub>, S-nitrosothiols (RSNO), malondialdehyde (MDA) and reduced glutathione (GSH) concentrations and superoxide dismutase (SOD) activity were determined in rat whole encephalic mass (WEM) and cerebellum homogenates.

Agmatine exerted strong protective effects on EAE clinical symptoms (p<0.05). In EAE brain homogenates, NO<sub>2</sub>+NO<sub>3</sub>, RSNO and MDA concentrations were increased compared to CG values. Agmatine treatment diminished NO<sub>2</sub>+NO<sub>3</sub>, RSNO and MDA levels in EAE animals (p<0.05). In EAE rats, GSH level and SOD activity were decreased compared to CG values, but agmatine treatment increased both parameters compared to EAE untreated animals (p<0.05). Immunohistochemical staining supported the clinical and biochemical findings in all groups.

The CNS changes in EAE are successfully suppressed by agmatine application, which could be the the new aspect of the neuroprotective effects of agmatine.

**Key words:** EAE, nitric oxide, S-nitrosothiols, malondialdehyde, glutathione, superoxide dismutase

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## INTRODUCTION

Agmatine, a biogenic amine, synthesized by decarboxylation of L-arginine (1), has been described as neuromodulator (2) and proposed to be neuroprotective (3). It is synthesized and stored in synaptic vesicles and released upon neuronal membrane depolarization (4). Agmatine acts as an agonist for imidazoline and  $\alpha_2$  -adrenergic receptors (5) and an antagonist for N-methyl-D-aspartate (NMDA) receptors (6). Several experimental studies demonstrated that agmatine reduced neuronal damage in various injuries of CNS, such as ischemia (7) or toxic damage (8). The proposed molecular targets of agmatine neuroprotective effects are multiple, including blockade of voltage-dependent  $Ca^{++}$  channels, signalling molecules of cAMP pathway and inducible nitric oxide synthase inhibition.

Experimental autoimmune encephalomyelitis (EAE), the experimental model of multiple sclerosis (MS), is inflammatory demyelinating CNS disease with clinically/pathological and immunologic similarities to MS (9, 10). Early clinical appearance of EAE clinical signs is mediated by autoaggressive T lymphocytes specific for the certain myelin structures (11), as well as macrophages and leukocytes from peripheral blood, infiltrating nerve tissue and inducing the cascade of processes, such as neuroinflammation, demyelination, axon damage and oligodendrocyte loss (12). Besides, infiltrating leukocytes produce enormous quantities of reactive oxygen and nitrogen species which induce further damage of the nerve tissue. In the conditions when the production of free radicals overcome the scavenging capacity of cell antioxidants, oxidative stress is present. The increased superoxide production favors its reaction with nitric oxide and the formation of toxic peroxynitrite, which is considered as the key mediator of cytotoxicity in this type of CNS damage, inducing mitochondria dysfunction, lipid peroxidation, protein nitration, ion channel disability and electrolyte dysbalance (13).

Nitric oxide is synthesized from L-arginine in the reaction catalyzed by nitric oxide synthases (NOS) family: neuronal (nNOS, NOS1), endothelial (eNOS, NOS3) and inducible (iNOS, NOS2) (14). The first two are constitutive,  $Ca^{++}$ -calmoduline-dependent and they produce short-life NO molecules in response to physiological stimuli. iNOS activity, induced by bacterial endotoxins and inflammatory cytokines, results in the production of long-life NO during long period. Beside cytotoxic activity, NO increases CNS cell susceptibility to other cytotoxic agents influence (13).

It has been known that thiols are the main targets of NO, which is converted into highly reactive nitrosonium ion ( $NO^+$ ). This ion reacts with protein thiol groups, forming S-nitrosothiols (RSNO), which can transfer  $NO^+$  group to other sulfhydryl groups of target proteins, forming more stable covalent bonds (15). Formed RSNO are considered as natural depo and the transport forms of NO, controlling its cell flux and availability to other

metabolic pathways. The biological importance of protein S-nitrosylation is pointed out as an important regulatory system in physiological and pathophysiological conditions (16).

The literature data also emphasize the importance of oxidative/nitrosative stress in MS pathogenesis, reporting the correlation between this disease development and diminished or primary lack of CNS antioxidant capacity, at first superoxide dismutase (SOD) activity. SOD catalyses dismutation of superoxide which is produced in excess in neuroinflammation (17). Besides, tripeptide glutathione is the most effective free radical scavenger in mammalian cells, present in oxidized (GSSG) and reduced (GSH) forms. In GSH, cysteine SH groups are hydrogen donors to other unstable compounds, such as oxygen free radicals. GSH is the key substrate of antioxidant enzymes which detoxify hydrogen peroxide and lipid peroxides. The decrease of GSH leads to an overproduction of reactive oxygen species (ROS) and oxidative stress.

Considering contradictory literature data about L-arginine and NO importance in neuroinflammation, as well as the molecular basis of agmatine effects, the aim of this study was the investigation of exogenous agmatine influence on NO production, RSNO concentration and oxidative stress parameters in acute phase of MS experimental model.

## MATERIAL AND METHODS

### *Animals*

The experimental model of EAE, described in literature, was used in the study (18, 19). Female Sprague Dawley rats, 3 months old, weighing  $300 \pm 20$  g, were housed in the Biomedical Research Centre animal care facility of the Faculty of Medicine in Niš throughout the experiment under a 12:12 h light-dark cycle. The rats were kept in plastic cages and fed on a standard diet and water ad libitum.

The experimental protocol was reviewed and approved by the Faculty Ethical Committee. All animals, included in this experiment, received human care in the strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication 80-23, revised 1985).

### *EAE induction*

Experimental autoimmune encephalomyelitis was induced by the subcutaneous injection of myelin basic protein, bovine type in dose 50  $\mu$ g, dissolved in phosphate buffered saline (PBS) emulsified in equal volume of the complete Freund's adjuvant (CFA), on days 0 and 7 in the hind foot pad of the animals under anesthesia. Two intraperitoneal (i.p.) injections of 200 ng Pertussis toxin were given on days 0 and 1. Each of 49 animals was assigned randomly to five groups, each consisting of 9 animals: control group (CG) - animals treated with PBS

in the dose 0.3 ml/i.p./daily; EAE - animals treated with PBS in the dose 0.3 ml/i.p./daily after EAE induction; CFA - animals treated with CFA in the dose 0.3 ml/i.p./daily; EAE + AGM - animals treated with AGM (i.p.) in the dose 75 mg/kg body weight/daily after EAE induction, and AGM - animals treated with AGM (i.p.) in the dose 75 mg/kg body weight/daily. The animals were treated every day from day 0 to 15 of the experiment.

All animals were scored for the clinical signs of EAE daily. EAE clinical expression was assessed as 1=healthy; 2=loss of tail tone; 3=hindlimb weakness; 4=hindlimb paralysis; 5=hindlimb paralysis plus forelimb weakness; 6=moribund or dead. The animals were sacrificed 15 days after EAE induction under Ketalar anesthesia with decapitation.

The brains were dissected from two animals from control and each EAE group, rapidly removed and quickly frozen in tissue freezing medium (Leica, Nussloch, Germany) and cryopreserved at  $-80^{\circ}\text{C}$  for the later immunohistochemistry analyses. From the others animals (7 animals per group), the removed brain tissue was washed in PBS and 10% homogenates of the whole encephalic mass - WEM (consisted of the removed brain tissue without cerebellum and brainstem) and cerebellum tissue were homogenated in PBS on ice with teflon pounder and stored at  $-20^{\circ}\text{C}$  for biochemical analyses.

### *Immunohistochemistry*

Eight  $\mu\text{m}$  thick frozen sagittal sections of subcortical paraventricular forebrain regions were mounted on poly-L lysine coated slides and allowed to air dry. Criostat sections were fixed in acetone. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide containing sodium azide (Dako Cytomation) for 15 minutes. Subsequently, the slides were incubated for 60 minutes at room temperature with one of the following primary antibodies: a mouse monoclonal anti-iNOS (dilution 1:25, Santa Cruz Biotechnology, Santa Cruz, CA, USA); a mouse monoclonal ED1, anti-CD68 antibody that recognizes a lysosomal membrane-related antigen on macrophages/microglia (dilution 1:10, Abcam, Cambridge, UK); a mouse monoclonal OX42, anti-CD11b/c equivalent antibody that reacts with a common epitope shared by CD11b/c which was expressed on monocytes/macrophages and microglia (dilution 1:100, Abcam, Cambridge, UK); a mouse monoclonal antibody directed against GFAP (dilution 1:1000, Abcam, Cambridge, UK), a specific marker of astrocytes; a rabbit polyclonal anti-EAAT1, antibody that reacted with glutamate-aspartate transporter in rat CNS samples (dilution 1:200, Abcam, Cambridge, UK). After washing in TRIS-buffered saline, immunoperoxidase staining was performed by an EnVision antibody complex method using the DakoCytomation EnVision+System-HRP kit (Dako, Glostrup, Denmark). Staining, developed using a liquid 3,3'-diaminobenzidine (DAB) substrate kit, resulted in a brown-colored precipitate at the antigen site. In negative controls, slides were incubated with PBS, in the absence of primary antibody.

Slides were counterstained with hematoxylin, dehydrated through alcohols and xylene and mounted in Kaiser gel (Merck).

In the case of iNOS and EAAT1, the slides were evaluated for staining intensity (negative, weak - pale light brown precipitate, intermediate - golden cytoplasmic stain, or strong - dark brown precipitate) and staining distribution (homogeneous (>50% positive cells), heterogeneous (>5%, but less than 50%), or only within single cells (<5%) (20, 21). In immunohistochemical analysis of ED1, OX42 and GFAP, we have evaluated the sections by counting the number of positive cells in 5 random non-overlapping high power (40x) fields from each section, and then calculated the average value for each section (22, 23). ImageJ software, developed by National Institutes of Health, was used to assess the immunoreactivity of each analyzed field. The investigator who analyzed the slides was blinded to the fact from which experimental group the sections were obtained.

In the case of homogeneous or heterogeneous positivity, we have considered the immunoreactivity strong (+++) if the staining signal was strong, or intermediate (++) if the staining signal was of intermediate strength. Weak immunoreactivity (+) was present in the case of staining of any intensity found in <5% of investigated area, or if staining intensity was weak at any distribution.

### **Biochemical analyses**

#### *Nitrate and nitrite concentration*

After deproteinization, the production of NO $^{\cdot}$  was evaluated by measuring nitrite and nitrate concentrations. Nitrites were assayed directly spectrophotometrically at 543 nm, using the colorimetric method of Griess (Griess reagent: 1.5% sulfanilamide in 1 M HCl plus 0.15% N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water). However, nitrates were previously transformed into nitrites by cadmium reduction (24).

#### *S-nitrosothiol concentration*

S-nitrosothiols were determined spectrophotometrically by Saville-Griess method of (25) with slight modifications (26). The method is based on the reaction of the released nitrosonium ion with naphthylendiamine. The samples were previously added by NH $_4$ -sulfamate to chelate nitrites. Heterolytic degradation of S-NO bond and nitrosonium ion release was induced by mercuri ions from HgCl $_2$ . GSH (0,15-10  $\mu\text{M}$  in 1NHCl) was used as standard.

#### *MDA concentration*

In 10% homogenates, lipid peroxidation intensity was determined by measuring MDA concentration, using spectrophotometric method of Ohkawa et al. (27), based on thiobarbituric acid (TBA)-reacting products, and expressed as nmol/mg prot. Chromogen absorption was measured at 532 nm.

### Reduced glutathione concentration

Reduced glutathione (GSH) concentration was performed immediately after homogenization in 0.02M EDTA solution, using Ellman's reagent (5,5' ditiobis-2-nitrobenzoic acid - DTNB) (28). Reduced glutathione concentration was expressed as nmol/mg of tissue weight.

### SOD activity

SOD activity was determined by the spectrophotometric method of Minami and Yoshikawa (29), based on formazan colored product formation. In the reaction with NBT (nitroblue tetrazolium), superoxide anion ( $O_2^-$ ), produced by pyrogallol autooxidation, forms colored product. SOD, as  $O_2^-$  scavenger, inhibits this reaction. The enzyme activity was expressed as U/mg prot., while one unit represented 50% inhibition.

### Protein concentration

Protein content was measured according to the Lowry procedure, using bovine serum albumin as standard (30).

### Chemicals

Chemicals were purchased from Sigma (St. Louis, MO, USA). All used chemicals were of analytical grade. All drug solutions were prepared on the day of the experiment.

### Statistical analysis

The examined biomarkers values are presented as mean values  $\pm$ SD. The Gaussian distribution of the data (verified by Kolmogorov-Smirnov test) and the number of the groups for comparison (more than two) allowed us to make comparisons using ANOVA followed by Dunnett's test, also, using linear regression analysis for the correlation between examined biomarkers and EAE score. The statistical significance of differences in immunohistochemical findings between groups was tested using the Student t-test. The statistical significance was determined by p values  $<0.05$ . All the statistical calculations were performed using SPSS software version 13.

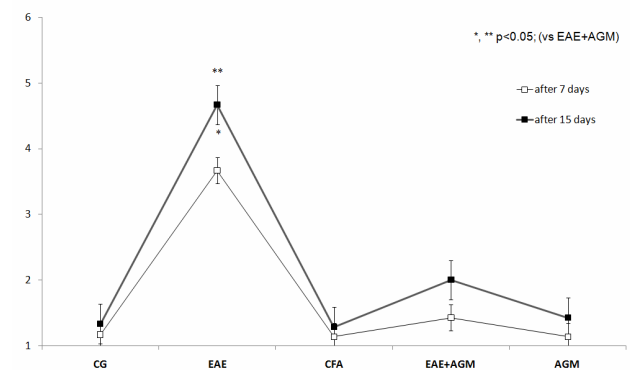
## RESULTS

During the disease development, agmatine expressed strong protective effect on EAE clinical symptoms, reducing significantly the clinical score in rats with EAE compared to EAE group ( $p < 0.01$ ; Figure 1).

The obtained expression of examined biomarkers in EAE affected forebrain paraventricular regions was assessed firstly compared to the samples from healthy animals (control group), where we observed the absence of these biomarkers expression (Figure 2, sections a, d, g, j and m). In healthy animals, iNOS and EAAT1 were not expressed, as well as the presence of GFAP+ astrocytes or activated macrophages/microglia cells. The ex-

pression of iNOS in investigated samples of EAE animals was confined to cytoplasm of individual cells or cells distributed in small clusters (Figure 2, sections a-c). We found that the number and distribution of iNOS-immunoreactive cells was closely associated with the severity of EAE in that group. Inducible NOS showed higher positivity areas with strong cytoplasmic staining in larger number of cells (+++) in EAE group compared to rat forebrain samples from EAE+AGM (+) group. The number and density of ED1 (Figure 2, sections g-i) and OX42 (Figure 2, section j-l) positive cells (monocytes, macrophages and microglia) was markedly increased in EAE group (+++), as well as the number of GFAP (Figure 2, section d-f) positive astrocytes (+++), compared to agmatine-treated EAE samples (+) ( $p < 0.05$ ). Staining for excitatory amino acid transporter EAAT1 (Figure 2, section m-o) resulted in diffuse immunoreactivity, with the strongest signal in EAE group (+++), compared to those in AGM-treated animals ( $p < 0.05$ ).

Figures 3 and 4 show nitrite and nitrate ( $NO_2 + NO_3$ ) and RSNO concentrations in WEM and cerebellum.  $NO_2 + NO_3$  and RSNO concentrations in WEM and cerebellum of rats with EAE are significantly increased ( $p < 0.05$ ) compared to the control values, while agmatine application to EAE animals significantly decreases both parameter concentrations in both examined homogenates ( $p < 0.05$ ). Agmatine application to healthy animals did not cause significant changes of these parameters in WEM and cerebellum of treated rats compared to the control group animals, as well as CFA treatment ( $p > 0.05$ ).

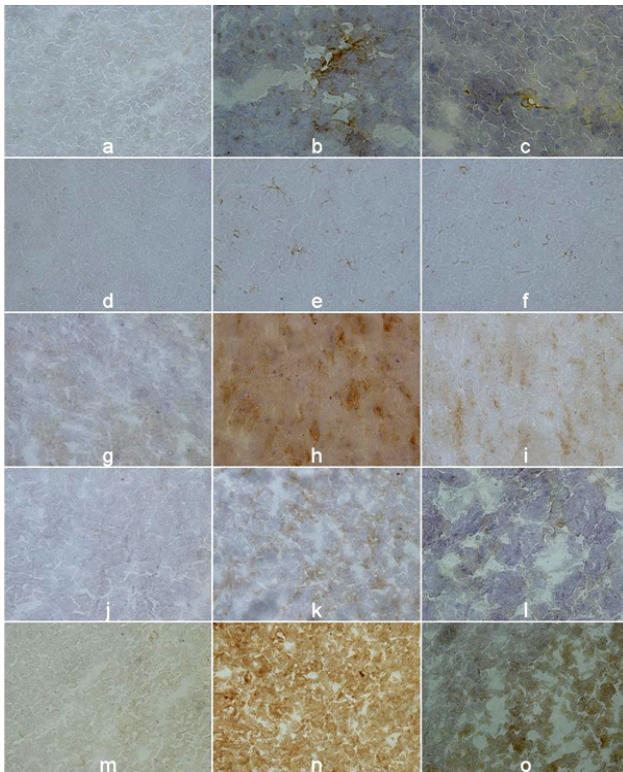


**Figure 1.** The clinical score of neurological disorder (1-6) of rats during the EAE course.

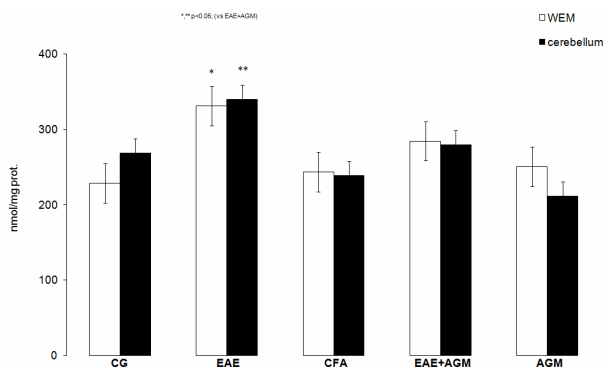
1-healthy rats, 2-loss of tail tone, 3-hindlimb weakness, 4-hindlimb paralysis, 5-hindlimb paralysis plus forelimb weakness, 6-moribund or dead.

CG-control group, EAE-rats with experimental autoimmune encephalomyelitis, CFA-rats treated with Complete Freund's adjuvant, EAE+AGM-agmatine treated EAE rats, AGM-rats treated with agmatine alone.

\*\*\* $p < 0.01$  (ANOVA, Dunnett's test)

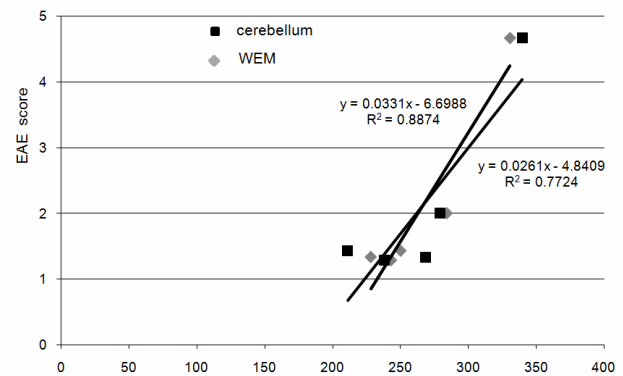


**Figure 2.** The representative immunohistochemical staining of sagittal subcortical paraventricular forebrain sections in control group (photomicrographs in the first column - sections a, d, g, j, m), EAE group (b, e, h, k, n) and EAE+AGM group (c, f, i, l, o). Serial sections were stained for iNOS (a-c); ED1, anti-CD68 antibody that recognizes a lysosomal membrane-related antigen on macrophages/microglia (d-f); OX42, that reacts with a common epitope shared by CD11b/c, expressed in macrophages/microglia (g-i); Glial fibrillary acidic protein (GFAP), astrocyte marker (j-l) and EAAT1, glutamate-aspartate transporter (m-o). Note the increased expression of inducible NOS and EAAT1 (+++), as well as higher density of activated microglia/macrophages and GFAP+ astrocytes in sections from EAE group compared to EAE animals treated by AGM. The number of tested rats was two for each experimental group. Original magnification x400. The data were statistically compared between groups by Student t-test ( $p < 0.05$ ).



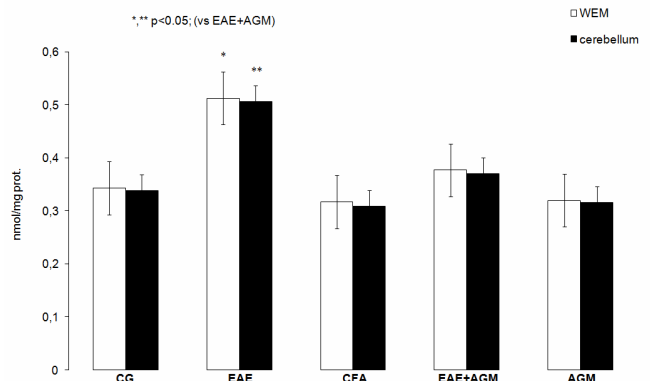
**Figure 3.**  $\text{NO}_2$  and  $\text{NO}_3$  concentration (nmol/mg prot.) in the rat WEM and cerebellum.

CG-control group, EAE-rats with experimental autoimmune encephalomyelitis, CFA-rats treated with Complete Freund's adjuvant, EAE+AGM - EAE-rats treated with agmatine, AGM-rats treated with agmatine alone. Bars in the graph represent mean  $\pm$ SEM from 7 animals for each group. AGM treatment decreases  $\text{NO}_2$  and  $\text{NO}_3$  level in the WEM and cerebellum homogenates.  $^{***}p < 0.05$ , (ANOVA, Dunnett's test).



**Figure 3A.**  $\text{NO}_2$  and  $\text{NO}_3$  concentration (nmol/mg prot.) and EAE score correlation.

EAE score: healthy=1; loss of tail tone=2; hindlimb weakness=3; hindlimb paralysis=4; hindlimb paralysis plus forelimb weakness=5; moribund or dead=6. Parallel to  $\text{NO}_2$  and  $\text{NO}_3$  concentration decrease in both examined tissues (WEM and cerebellum), EAE clinical expression decreases (better clinical condition),  $R^2 = 0.88$ ,  $R^2 = 0.77$  for WEM and cerebellum, respectively. There is a positive linear relationship between these variables ( $p < 0.01$ ).

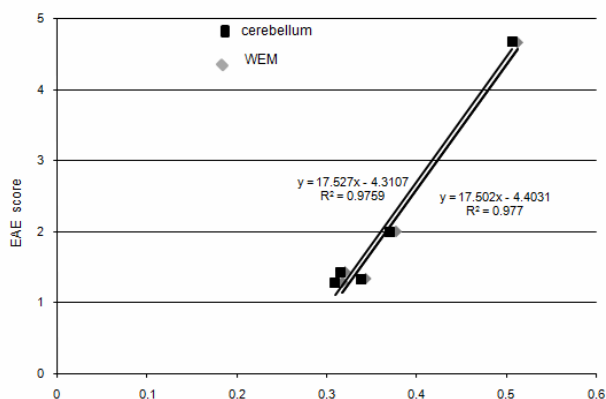


**Figure 4.** RSNO concentration (nmol/mg prot.) in rat WEM and cerebellum.

CG-control group, EAE-rats with experimental autoimmune encephalomyelitis, CFA-rats treated with Complete Freund's adjuvant, EAE+AGM - agmatine treated EAE rats, AGM-rats treated with agmatine alone. Bars in the graph represent mean  $\pm$ SEM from 7 animals for each group. AGM treatment decreases RSNO concentration in the WEM and cerebellum.

$^{***}p < 0.05$ , (ANOVA, Dunnett's test).





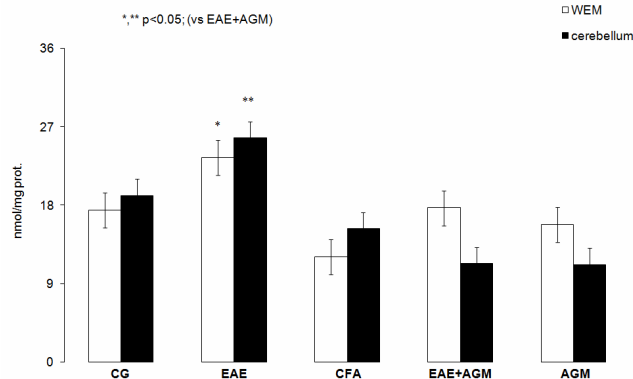
**Figure 4A.** RSNO concentration (nmol/mg prot.) and EAE score correlation. EAE score: healthy=1; loss of tail tone=2; hindlimb weakness=3; hindlimb paralysis=4; hindlimb paralysis plus forelimb weakness=5; moribund or dead=6. Parallel to RSNO concentration decrease in both examined tissues (WEM and cerebellum), EAE clinical expression decreases (better clinical condition),  $R^2=0.97$ ,  $R^2=0.97$  for WEM and cerebellum, respectively. There is a positive linear relationship between these variables ( $p<0.01$ ).

In EAE rats WEM and cerebellum, MDA concentrations showed a significant increase ( $p<0.05$ ) related to the control values and the group of animals treated by CFA (Figure 5). Agmatine treatment decreased significantly MDA concentration in both homogenates of EAE rats ( $p<0.05$ ), but it did not induce significant changes in healthy treated rats in comparison to the control and CFA groups ( $p>0.05$ ).

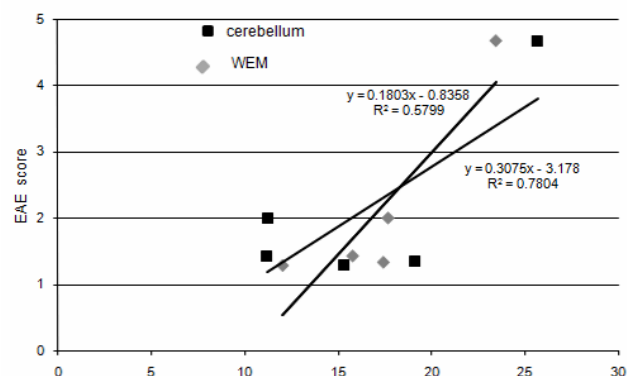
The application of agmatine to EAE animals significantly ( $p<0.05$ ) increased WEM and cerebellum GSH content, which had been significantly diminished compared to the values in the control and CFA treated groups (Figure 6). Agmatine alone did not change the GSH level in examined homogenates of healthy rats ( $p>0.05$ ).

In both types of homogenates, SOD activity in EAE animals was significantly reduced ( $p<0.05$ ) compared to the values in control group animals (Figure 7). Both agmatine and CFA did not lead to the significant increase of this enzyme activity in healthy animals related to the control values ( $p>0.05$ ), while, in EAE rats, agmatine treatment induced the significant increase of SOD activity ( $p<0.05$ ).

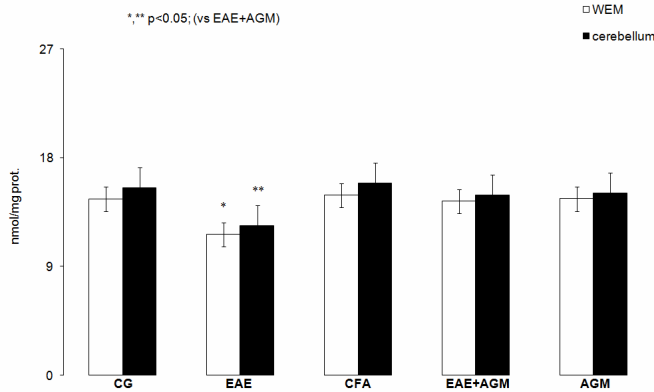
The performed linear regression analysis in all compared cases showed the high ratio of positive or negative correlation between the changes in all examined biomarkers concentrations (nitrite and nitrite, RSNO, MDA, GSH, SOD) in WEM and cerebellum of both EAE groups - AGM-treated and untreated (Figure 3A, 4A, 5A, 6A, 7A -  $p<0.01$ ).



**Figure 5.** MDA concentration (nmol/mg prot.) in the rat WEM and cerebellum. CG-control group, EAE-rats with experimental autoimmune encephalomyelitis, CFA-rats treated with Complete Freund's adjuvant, EAE+AGM - agmatine treated EAE rats, AGM-rats treated with agmatine alone. Bars in the graph represent mean  $\pm$ SEM from 7 animals for each group. AGM treatment decreases MDA concentration in the WEM and cerebellum. \*\*\* $p<0.05$ , (ANOVA, Dunnett`s test).



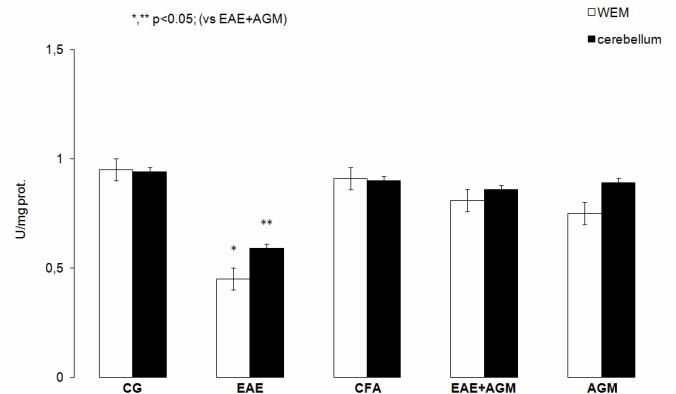
**Figure 5A.** MDA concentration (nmol/mg prot.) and EAE score correlation. EAE score: healthy=1; loss of tail tone=2; hindlimb weakness=3; hindlimb paralysis=4; hindlimb paralysis plus forelimb weakness=5; moribund or dead=6. Parallel to decrease in MDA concentration in both examined tissues (WEM and cerebellum), EAE clinical expression decreases (better clinical condition),  $R^2=0.58$ ,  $R^2=0.78$  for WEM and cerebellum, respectively. There is a positive linear relationship between these variables ( $p<0.01$ ).



**Figure 6.** GSH concentration (nmol/mg prot.) in the rat WEM and cerebellum.

CG-control group, EAE-rats with experimental autoimmune encephalomyelitis, CFA-rats treated with Complete Freund's adjuvant, EAE+AGM – agmatine treated EAE rats, AGM-rats treated with agmatine alone. Bars in the graph represent mean  $\pm$ SEM from 7 animals for each group. AGM treatment increases GSH concentration in the WEM and cerebellum.

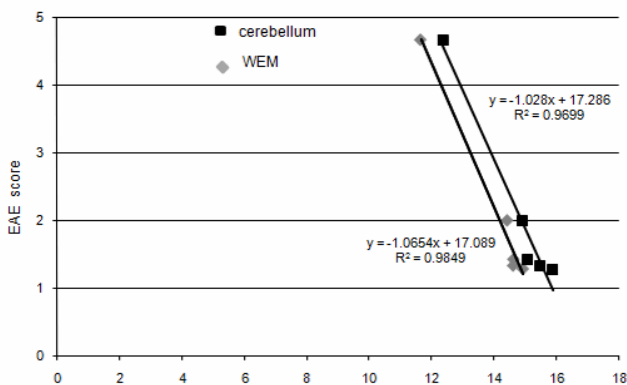
\*\*\* $p < 0.05$ , (ANOVA, Dunnett's test).



**Figure 7.** SOD activity (U/mg prot.) in the rat WEM and cerebellum.

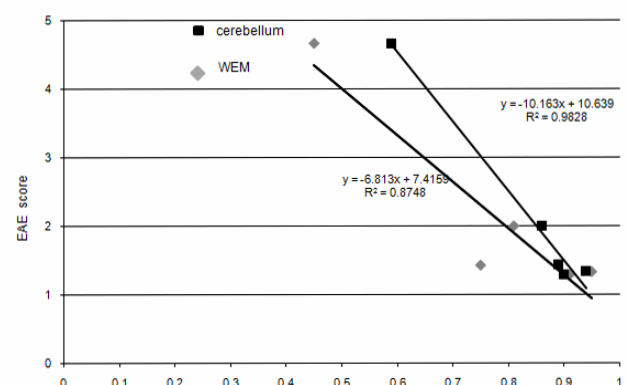
CG-control group, EAE-rats with experimental autoimmune encephalomyelitis, CFA-rats treated with Complete Freund's adjuvant, EAE+AGM – agmatine treated EAE rats, AGM-rats treated with agmatine alone. Bars in the graph represent mean  $\pm$ SEM from 7 animals for each group. AGM treatment increases GSH concentration in the WEM and cerebellum.

\*\*\* $p < 0.05$ , (ANOVA, Dunnett's test).



**Figure 6A.** GSH concentration (nmol/mg prot.) and EAE score correlation.

EAE score: healthy=1; loss of tail tone=2; hindlimb weakness=3; hindlimb paralysis=4; hindlimb paralysis plus forelimb weakness=5; moribund or dead=6. Parallel to increase in GSH concentration in both examined tissues (WEM and cerebellum), EAE clinical expression decreases (better clinical condition),  $R^2=0.98$ ,  $R^2=0.97$  for WEM and cerebellum, respectively. There is a negative linear relationship between these variables ( $p < 0.01$ ).



**Figure 7A.** NO<sub>2</sub> and NO<sub>3</sub> concentration (nmol/mg prot.) and EAE score correlation.

EAE score: healthy=1; loss of tail tone=2; hindlimb weakness=3; hindlimb paralysis=4; hindlimb paralysis plus forelimb weakness=5; moribund or dead=6. Parallel to increase in SOD activity in both examined tissues (WEM and cerebellum), EAE clinical expression decreases (better clinical condition),  $R^2=0.87$ ,  $R^2=0.98$  for WEM and cerebellum, respectively. There is a negative linear relationship between these variables ( $p < 0.01$ ).

## DISCUSSION

Together with the cognition of NO critical role in inflammation, the attention of investigators has been directed to its involvement in the mechanisms of the appearance and the development of the symptoms of MS and its experimental model - EAE (31, 32). The literature data document increase of NO oxidation products in demyelinating lesions in EAE animal brains, as well as in CSF of MS patients (33, 34). The recent studies have reported that redox signalization dysregulation and the consequent oxidative stress change thiol-disulfide status of key proteins, which regulate balance between cell death and survival (35).

It has been known that in neuroinflammatory reaction there is an increased expression of mRNA for pro-inflammatory cytokines, such as TNF- $\alpha$  i INF- $\gamma$ , the important inducers of iNOS. Besides, Fabian and Rea (36) reported that activated glial cells, also proved in our experiment (Figure 2), secreted NO and its reactive metabolites, which was an explanation for the expected increase of NO production in EAE rats brains (Figure 3). Meanwhile, the role of NO in the pathogenesis of EAE is complex, which is supported by the results of the experiments with NOS inhibitors, which, sometimes, worsen the disease symptoms. Reactive nitrogen species (N<sub>2</sub>O<sub>3</sub>, N<sub>2</sub>O<sub>4</sub> and peroxyxynitrite), may induce oxidative and nitrosative stress, resulting in myelin, i.e. oligodendrocyte, destruction. It has been proved that the activation of microglia directly leads to cytotoxicity to oligodendrocytes and surrounding neurons, potentiating lipid peroxidation process, which is documented by MDA concentration increase in EAE animals not treated with agmatine (Figure 5) in our study.

In addition, the possible explanation of worse clinical presentation in EAE rats, reflected in tri- and quadriplegia, is the existence of direct correlation between high NO concentrations and the block in neuronal action potential transduction, taking in account that NO donors induced reversible conductance block in normal and demyelinated axons in CNS (37). This can be the consequence of NO direct influence on glutamatergic transmission, considering the fact that nitrosylation of NMDA receptors leads to their inactivation (38).

Although agmatine influence on NO production and its biological effects are not clear, it is thought that one of its important functions is iNOS inhibition with the consequent reduction in NO synthesis, which is also supported by the results of our investigation (Figure 2, Figure 3). In cell culture, iNOS inhibitory action of agmatine has been reported (39), while experiments with purified enzyme showed that agmatine is neither substrate, nor NOS inhibitor. On the contrary, the results of Satriano et al. (40) proved inhibitory effects of agmatine aldehyde on iNOS *in vivo*.

In addition, the maintaining of high NO concentrations in neurons is provided by permanent calcium influx through Ca<sup>++</sup>-voltage channels. Agmatine blocks

NMDA receptors, highly expressed in EAE (Figure 2), Ca<sup>++</sup>-voltage channels and glutamate release, which is also a possible explanation for its neuroprotective effects. In our study, agmatine reduced EAE clinical symptoms (Figure 1), in association with the decrease of NO level in brains of treated animals. These results correlate with the findings of Regunathan and Piletz (41), who reported agmatine-induced decrease of iNOS protein level, providing the molecular basis of its neuroprotective and anti-inflammatory effects, which was also supported by the results of our laboratory from the recent experiments on iNOS -/- knockout mice (unpublished data).

As it was mentioned before, the majority of NO physiologic and pathophysiologic effects are mediated by peptide and protein S-nitrosylation, leading to RSNO formation. Foster et al. (42) suggested that dysregulation of RSNO homeostasis contributes to the pathogenesis of different diseases, including neuroinflammatory (43) and neurodegenerative diseases. RSNO concentration increase is documented in our experiment (Figure 4), which is in correlation with the results of Bizzozero et al. (44), who showed the increase of S-nitrosylated proteins in white matter of MS patients. RSNO increase favors further nitrosylation of protein thiol groups, promoting damaging effects of nitrosative stress in CNS. The "burst" of RSNO production, evident in neuroinflammation, is considered to be the key point in cell fate determination, i.e. the increase of neuronal susceptibility to NO effects (45).

In addition, S-nitrosoglutathione (GSNO), endogenous signal molecule, formation reduces CNS GSH pool, which is the switch of redox-dependent changes in cell signalization, leading to the modification of key intracellular enzymes and disturbing mitochondrial respiratory chain activity (46). Reduction of intracellular GSH pool in oligodendrocytes is considered as contributing factor to oxidative damage and neuronal death (47). This is supported by the results of Calabrese et al. (48), who reported diminished levels of GSH and SH groups in CNS of MS patients. The GSH level decrease, also proved in our investigation (Figure 6), is suggested by Chi et al. (49) to cause dysfunction of mitochondria, facilitating apoptosis inducing factor (AIF) translocation, cytochrome c release and caspase-3 activation, which induce apoptosis of neurons in both *in vitro* i *in vivo* conditions. Therefore, considering high NO concentrations, GSNO formation could be one of the mechanisms leading to GSH decrease in EAE and the explanation of the results in this study.

Meanwhile, in the conditions of excessive ROS production, GSH decrease can also induce the damage of blood-brain barrier endothelial cells, contributing to further CNS infiltration by macrophages and T-lymphocytes. These cells, as well as reactive microglial cells, had been documented to be present in demyelinating lesions (50), which was also supported by our results (Figure 2), leading to further oxidative burst and damaging myelin sheath and phagocytosing myelin particles (51),



which could also be an explanation for worse EAE clinical presentation in animals with lower GSH levels (Figure 1, Figure 6).

In oxidative stress, mitochondria are the main sources of intracellular superoxide which is converted to H<sub>2</sub>O<sub>2</sub> by SOD. The literature data about SOD alterations in MS are controversial. Qi et al. (52) reported increased SOD expression in demyelinating lesions in EAE, while others documented this enzyme decrease in plasma and erythrocytes of MS patients, which was also supported by our results (Figure 7), indicating the reduction of antioxidant capacity in neuroinflammation. The protective effects of exogenous agmatine on SOD activity could be indirectly related to its antioxidant and anti-nitrosative potential.

In the light of earlier investigations, our results support the hypothesis that nitrosative and oxidative stress, induced by neuroinflammation, are important pathogenetic factors of CNS morphological and functional

damage in the acute phase of demyelination. Through S-nitrosylation of different key proteins, important for neuronal survival, NO and free radical overproduction by activated microglia, as well as diminished antioxidant pool of nerve tissue, contribute to the pathogenetic mechanisms of early demyelination.

The obtained results point to the benefit effects of L-arginine bioactive metabolite - agmatine in MS experimental model, but the precise mechanisms of agmatine action remains to be elucidated.

### Acknowledgments

This work was supported by a grant from the scientific project number 41018 financed by the Ministry of Education and Science, Republic of Serbia. No conflict of interest exists for any of the authors listed in the article.

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## PROTEKTIVNI EFEKTI AGMATINA U EKSPERIMENTALNOJ MULTIPLOJ SKLEROZI. SUPRESIJA NITROZATIVNOG I OKSIDATIVNOG STRESA U CNS-u

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### Sažetak

Cilj ovog rada bio je ispitivanje uticaja egzogeno aplikovanog agmatina na parametre nitrozativnog i oksidativnog stresa u velikom mozgu i cerebelumu pacova u akutnoj fazi eksperimentalnog autoimunog encefalomijelitisa (EAE), eksperimentalnog modela MS.

EAE je izazivan subkutanom injekcijom mijelin bazičnog proteina (50 µg po životinji). Sprague-Dawley pacovi bili su podjeljeni u pet grupa: I grupa – (KG) tretirana PBS-om (i.p.), II grupa – (EAE), III grupa – (CFA) - tretirana kompletnim Freundovim adjuvansom (0,2 ml subkutano), IV grupa – (EAE+AGM) - tretirana samo agmatinom (75 mg/kg bw i.p.) nakon indukcije EAE i V grupa – (AGM) - dobijala samo agmatin u istoj dozi. Životinje su tretirane svakog dana eksperimenta, od 0 do 15. dana i klinički skorovane svakog dana. One su žrtvovane 16. dana nakon aplikacije MBP. Koncentracije NO<sub>2</sub>+NO<sub>3</sub>, S-nitrosotiola (RSNO), malondialdehida (MDA) i redukovano glutationa (GSH) i aktivnost superoksid dizmutaze (SOD) određivani su u homogenatima celokupne mase velikog mozga (whole encephalitic mass -WEM) i malog mozga.

Agmatin ispoljava snažne protektivne efekte u odnosu na kliničke simptome EAE (p<0,05). U EAE homogenatima mozga, koncentracije NO<sub>2</sub>+NO<sub>3</sub>, RSNO i MDA su povećane u odnosu na kontrolne vrednosti. Tretman agmatinom smanjuje nivoe NO<sub>2</sub>+NO<sub>3</sub>, RSNO i MDA u EAE životinja (p<0,05). Kod EAE pacova, nivo GSH i aktivnost SOD smanjeni su u poređenju sa kontrolnim vrednostima, dok tretman ovih životinja agmatinom povećava oba parametra u odnosu na netretirane EAE životinje (p<0,05). Imunohistohemijske analize podržavaju kliničke i biohemijske nalaze u ovim grupama.

Aplikacija agmatina EAE životinjama uspešno suprimira promene u CNS-u, što može predstavljati novi aspekt neuroprotektivnih efekata agmatina.

**Ključne reči:** EAE, azot monoksid, S-nitrozotoli, malondialdehid, glutation, superoksid dizmutaza

