ORIGINAL ARTICLE

UDC: 616.832-004-08-092.9
DOI: https://doi.org/10.2298/VSP190707145S

Therapeutic potential of agmatine in the experimental autoimmune encephalomyelitis

Terapijski potencijal agmantina u eksperimentalnom autoimunskom encefalomijelitisu

Ivana Stevanović*†, Milica Ninković*†, Jelena Stanojević*†, Bojana Mančić*†, Ivana Stojanović‡

***University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia; †Military Medical Academy, Institute for Medical Research, Belgrade, Serbia; ‡University of Niš, Faculty of Medicine, Institute for Biochemistry, Niš, Serbia**

Abstract

Background/Aim. Experimental autoimmune encephalomyelitis (EAE) is a model of multiple sclerosis (MS), in which we investigated the neuroprotective effect of agmatine (AGM), known as a primary amine produced via the decarboxylation of L-arginine. **Methods.** Dark Agouti rats were divided into groups: control (C), Complete Freund`s Adjuvant (CFA), EAE rats decapitated on the 13th day *post* immunization (dpi) (EAE13) and on the 20th dpi (EAE20), EAE animals given three (EAE+AGM13) and 10 (EAE+AGM20) doses of AGM, and healthy animals administered three/10 doses of AGM (AGM). Thiobarbituric acid-reacting substances (TBARS), SH groups (SH), total glutathione (GSH), glutathione peroxidase activity (GPx), superoxide dismutase activities (tSOD, MnSOD, CuZnSOD) and nitrite/nitrate concentration (NO_2+NO_3) were assessed in plasma and brain structures [whole encephalitic mass (WEM) and brainstem (BS)]. **Results.** The obtained results showed that AGM treatment successfully attenuated severe clinical deficits in EAE. Applications of AGM in EAE rats induced normalized TBARS, SH, GSH, GPx and NO in WEM. In BS, AGM expressed less prominent effects, inducing normalized TBARS, GPx and NO, but no effect on SH and GSH. In both brain structures, tSOD activity lowered and normalized at the peak and in the remission phase of the disease, post-AGM treatment. The effect of AGM on the MnSOD in EAE was expressed in WEM/BS only in the remission phase as a reduced activity. **Conclusion.** Milder clinical form of developed EAE in rats indicates promising therapeutic effect of AGM in MS. The activated antioxidant system and suppressed oxidative/nitrosative stress development may denote a successful blockade of neuroinflammation initiated by EAE immunization. The study implies the capability of AGM to attenuate oxidative/nitrosative damage at the peak of EAE by modulating antioxidative defense capacity during the time-course of the disease. Thus, AGM may be considered as an agent with a beneficial effect on neuroinflammation in EAE.

Key words:
agmatine; antioxidants; encephalomyelitis; multiple sclerosis; neuroprotective agents; oxidative stress; rats; treatment.

Apstrakt

Uvod/Cilj. Eksperimentalni autoimunski encefalomijelitis (EAE) je model multiple skleroze (MS) u kome su ispitivani neuroprotektivni efekti agmatina (AGM), poznatog primarnog amina koji se dobija dekarboksilacijom L-arginina. **Metode.** Dark Aguti pacovi su podeljeni u grupe: kontrola (C), kompletni Frojdov adjuvans (CFA), EAE pacovi dekapitovani 13 dana (EAE13) i 20 dana (EAE20) nakon imunizacije, EAE životinje sa tri (EAE+AGM13) i 10 (EAE+AGM20) doza AGM i zdrave životinje sa tri/10 doza AGM (AGM). Reaktivne supstance koje reaguju sa tiobarbiturnom kiselinom (TBARS), SH grupe (SH), koncentracija ukupnog glutationa (GSH), aktivnost glutation peroksidaze (GPx), aktivnost superoksid dizmutaza (tSOD, MnSOD, CuZnSOD) i koncentracija nitrita/nitrata (NO_2+NO_3) su određivani u plazmi i moždanim strukturama [kompletna encefalitična masa (WEM) i produžena moždina (BS)]. **Rezultati.** Dobijeni rezultati su pokazali da tretman sa AGM uspešno smanjuje teški klinički deficit u EAE. Aplikacija AGM kod EAE pacova je normalizovala TBARS, SH, GSH, GPx i NO u WEM. U BS, AGM je doveo do manje izraženih efekata normalizacijom TBARS, GPx i NO, ali je bio bez efekata na SH grupe i GSH. U obe moždane strukture, tSOD je bila smanjena i normalizovana u piku i remisiji bolesti nakon tretmana sa AGM. Efekat AGM na MnSOD u EAE je bio izražen u WEM/BS samo u toku remisije bolesti i manifestovao se kao redukcija

Correspondence to: Ivana Stevanović, University of Defence, Faculty of Medicine of the Military Medical Academy, Crnotravska 17, 11 000 Belgrade, Serbia. E-mail: ivanav13@yahoo.ca

aktivnosti enzima. **Zaključak.** Blaža forma razvijenog EAE pokazuje izraženi terapijski efekat AGM kod MS. Aktivirani antioksidativni sistem i supresija razvoja oksidativnog/ nitrozativnog stresa mogu predstavljati uspešnu blokadu neuroinflamacije indukovanu EAE imunizacijom. Studija pokazuje sposobnost AGM da ublaži oksidativno/ nitrozativno oštećenje u piku EAE modulacijom kapaciteta antioksidativne odbrane u toku trajanja bolesti. U tom smislu, AGM se može smatrati agensom za antioksidativno lečenje i prevenciju neuroinflamacije u EAE.

Ključne reči:
agmatin; antioksidansi; encefalomijelitis; multipla skleroza; neuroprotekstivi; stres, oksidativni; pacovi; lečenje.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune neuroinflammatory disease, concerning the animal model used to study human multiple sclerosis (MS). The autoimmune molecular target(s), which have been identified and used in biological models, seem to be proteins expressed by myelin-producing oligodendrocytes [1].

Neuroinflammation in EAE targets the spinal cord/ cerebellum, triggering so-called flaccid paralysis characterized by reduced muscle tone that progress from the tail upward along the body [2]. Dark Agouti (DA) rats are genetically susceptible to EAE [3]. The disease follows a predictable clinical course, characterized by a prodromal period of 10–15 days followed by ascending paralysis beginning in the tail and hind limbs and progressing to the fore-limbs concurrent with weight loss. Progressive hind-limb paralysis during EAE is the consequence of demyelination and damaged axonal conduction followed by inflammation [4].

The control of reactive oxygen and nitrogen species (ROS, RNS) and their cytotoxic bioproducts in affected cells are performed through scavenging enzymes or thiols, which participate in tissue repair and survival during EAE. Oxidative and nitrosative stress play an important role in myelin loss and degeneration of nerve tissue in MS [5]. It is one of the critical steps in the progression of neurodegenerative diseases and conditions where oxidative stress causes damage to mitochondria, with consequent energy failure, through the production of ROS/RNS [6, 7].

Nitric oxide (NO•) seems to be a target for new therapies in human demyelinating disease [8]. In physiological conditions, NO• was created from the oxidation of the terminal guanidine nitrogen of arginine by the enzyme NO• synthase (NOS) presented in three isoforms (neuronal-nNOS, endothelial-eNOS, and inducible-iNOS) [9]. Besides its role as a transsynaptic retrograde messenger in the brain, in EAE and MS, NO• promotes neuronal injury, inducing mitochondrial dysfunction, lipid peroxidation (LPO), as well as nitration of key protein and ion channel disability [10]. Suppressed NO• metabolism seems to express beneficial effects during the course of experimental MS [11].

Malondialdehyde (MDA) is an end-product of polyunsaturated fatty acids peroxidation and represents the severity of oxidative stress-induced brain injury [12]. An indicator of LPO was determined by measurable parameter thiobarbituric acid reactive substances (TBARS) [13]. Antioxidant defense against free radical-induced toxicity include enzymes that scavenge ROS/RNS, such as catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD), but also non-enzymatic biomolecules: glutathione (GSH), vitamins C and E, uric acid [14]. Their synergistic action regulates oxido-reductive balance and thus suppresses oxidative injury [15].

The present study examined potentially beneficial effect of agmatine – AGM (4-aminobutyl) guanidine, on oxidative and/or nitrosative stress development in the EAE – the model of MS. AGM is an amine that is formed by decarboxylation of L-arginine with the enzyme arginine decarboxylase and hydrolyzed through the enzyme agmatinase to putrescine [16]. It exerts neuromodulation properties, particularly assuring the control and modulation of NO pathway, influencing glutamate N-methyl-D-aspartate (NMDA) receptors and limiting oxidative/ nitrosative stress development. By its antioxidant activities, AGM reveals neuroprotective outcomes [17]. We proposed that treatment with AGM administered during the acute phase of EAE would attenuate disease severity, both clinically and biochemically. Based on the presented data, the aim of this study was to examine the role of ROS/RNS and the effectiveness of AGM treatment on rats' whole encephalitic mass (WEM) as well as on brainstem (BS) in EAE.

Methods

Animals

A permission of the Ethics Committee for the welfare of experimental animal's No. 119-01-5/14/2017-09 was obtained from the Ministry of Agriculture and Environmental Protection – the Veterinary Directorate of the Republic of Serbia. Inbred two-month-old female DA rats were kept in cages under standardized housing conditions (ambient temperature: 23 ± 2 °C, relative humidity: 55% ± 3% and a light/dark cycle: 13/11 h) and had free access to standard laboratory pellet food and tap water. All the experiments were performed after 7 days of adaptation to laboratory conditions and were carried out between 9 a.m. and 1 p.m. For the principle of welfare, during the period of paralysis, water and food were given manually.

Experimental procedure

Experimental autoimmune encephalomyelitis was induced by the subcutaneous (sc.) injection of 0.1 mL solution of rat spinal cord tissue homogenate (50% w/v in saline) dissolved in Complete Freund`s Adjuvant (CFA; Sigma, St. Louis, MO, USA) in the right hind footpads. The dose of

AGM (75 mg/kg bw.; dissolved in water) was selected on the data based on our previous studies with other models, which showed that a dose of 75 mg/kg bw. was not toxic (no change in body weight or food intake in rats, and no visible morphological changes) [18]. EAE-untreated rats received an equal volume of saline or CFA. From the day *post* immunization (dpi), the rats were daily monitored for the clinical score. Applied protocols with AGM in all animals were performed starting from the 10th day; by default, every day intraperitoneal application. The time point decapitation was performed on the 13th dpi (EAE13; the peak of the disease; animals received three doses of AGM) and on the 20th dpi (remission of the disease; animals received 10 doses of AGM). Before the immunization, as well as before the decapitation, all animals were intraperitoneally anesthetized (sodium-pentobarbital 45 mg/kg bw.). Clinical evaluation of EAE was undertaken daily in a double-blind manner 20 dpi [19].

The animals were randomly divided into experimental groups: control group (C, n = 8); group treated with Complete Freund`s Adjuvant (CFA, n = 8); EAE group that was decapitated on the 13th dpi (EAE13, n = 8); EAE group that was decapitated on the 20th dpi (EAE20, n = 8); EAE group treated with three doses of AGM (EAE+AGM13, n = 8); EAE group treated with 10 doses of AGM (EAE+AGM20, n = 8), as well as healthy animals treated with three (n = 8) and 10 (n = 8) doses of AGM integrated into a common group (AGM, n = 16).

The decapitation was performed 24 hours after the last AGM application or at the appropriate time point. The animals' brains were immediately put on liquid nitrogen and stored at -20 °C until analysis.

Clinical evaluation

All animals were scored daily according to the clinical signs on a scale of 0–5. EAE clinical expression was considered as 0 = no abnormalities; 0.5 = partial loss/ reduced tail tone and inability to rotate the back end of the tail; 1 = tail atony; 1.5 = slightly/ moderately unsteady gait and reduced straightening up ability or combination; 2 = hind limb weakness; 2.5 = partial hind limb paralysis; 3 = complete hind limb paralysis; 3.5 = complete paralysis of hind limbs and forelimb weakness; 4 = quadriplegic with breathing difficulties; 5 = moribund state or death [19]. Numerous parameters of the disease were observed to estimate the severity of EAE: mean clinical score (average clinical scores for all rats within a group on a specified day); mean maximal severity score (the mean of the maximal clinical score that each animal in a group extended over the course of the experiment); duration of paralysis (the mean number of days for which the rats had a score of 2 or more).

Measurement of oxidative/nitrosative status indicators in plasma and brain homogenates

Blood samples for determining oxidative/nitrosative status parameters were collected from the external iliac vein into vials containing heparin and EDTA.

The brain structures (WEM and BS) were dissected on ice, and 0.1 g of each tissue slice was transferred into 0.9% sodium chloride (normal saline). Homogenization was performed on ice by a homogenizer (Tehtnica, Zelezniki, Slovenia) at 800 rotations/ min. The homogenates were centrifuged (1,000 × g, 15 min, 4 °C), the precipitates were redispersed in sodium chloride, centrifuged (2,500 × g, 30 min, 4 °C) and the obtained precipitates were dissolved in 1.5 mL of deionized water. The samples were centrifuged again (2,000 × g, 15 min, 4 °C) and the supernatants (crude mitochondrial fractions) were stored at -70 °C [20]. The total protein concentration was determinate by Lowry et al. [21] in WEM and BS.

Lipid peroxidation analysis in the plasma and WEM/BS was measured as thiobarbituric acid reactive substances (TBARS) production using the method described by Girotti et al. [22]. The results are expressed as μM/L in plasma and μM/mg proteins in brain homogenates.

The determination of total SH groups in plasma and WEM/BS was carried out according to the method of Elman [23]. The results are expressed as mM/L in plasma and nM/mg proteins in brain homogenates.

The total glutathione (GSH+1/2GSSG, in GSH equivalents) content was established by the DTNB-GSSG reductase recycling assay, spectrophotometrically at 412 nm [24]. The results were expressed as nM/mg proteins.

Glutathione peroxidase analysis (Randox Laboratories, USA) was performed spectrophotometrically at 340 nm [25]. The unit of enzyme activity of GPx is defined as the number of micromoles of NADPH oxidized per min (μM NADPH/min). The results were expressed as U/mg proteins.

Superoxide dismutase (EC 1.15.1.1.; SOD) activity was measured spectrophotometrically, as inhibition of epinephrine spontaneous auto-oxidation for 10 min at 480 nm [26]. Cytosolic SOD (CuZnSOD) was calculated as the difference of total (tSOD) and mitochondrial (MnSOD) enzyme activity. All three isoforms of SOD were expressed as U/mg proteins.

Nitrite and nitrate (NO_2 + NO_3) accumulation, as an indirect measure of NO release, was determined in WEM/BS, using the colorimetric method of Griess at 492 nm [27]. The results were expressed as nM/mg proteins.

Statistical analysis

One-way ANOVA and Tukey`s *post hoc* tests were used (software GraphPad Prism, version 5.03) for statistical data analysis. Values are presented as means ± standard deviation (SD). The linear regression analysis was performed to determine the relation between the obtained values of parameters, using the statistical program GraphPad Prism. Differences were considered statistically significant for $p < 0.05$.

Results

All immunized animals developed clinical signs of the disease (100% incidence) (Table 1).

Most AGM treated animals developed a milder form of EAE in comparison to EAE rats and completely recovered at the end of the observing interval (Figure 1). The mean

Table 1

The influence of agmatine (AGM) on the extent of induced experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13) and in the remission of EAE (EAE20), as well as after three (EAE+AGM13) and 10 (EAE+AGM20) doses of AGM in EAE animals

Parameter	EAE13	EAE+AGM13	EAE20	EAE+AGM20
Animals, n	8	8	8	8
Incidence, n	8/8	8/8	8/8	8/8
Mean maximum severity score	2.79 ± 0.57	1.50 ± 0.71†	2.50 ± 1.12	1.00 ± 0.50‡
Duration of paralysis in days	2.13 ± 0.35	1.38 ± 0.52†	4.00 ± 0.45	1.43 ± 0.79‡
Mortality rate	1/8	0/8	3/8	1/8

Data are presented as mean ± standard deviation (SD).
†Indicates a statistically significant difference from the EAE group at the peak of the disease (EAE13); ‡Indicates a statistically significant difference from the EAE group in the remission of the disease (EAE20).
***p* – values were obtained by one-way ANOVA followed by Tukey`s test ($p < 0.05$).**

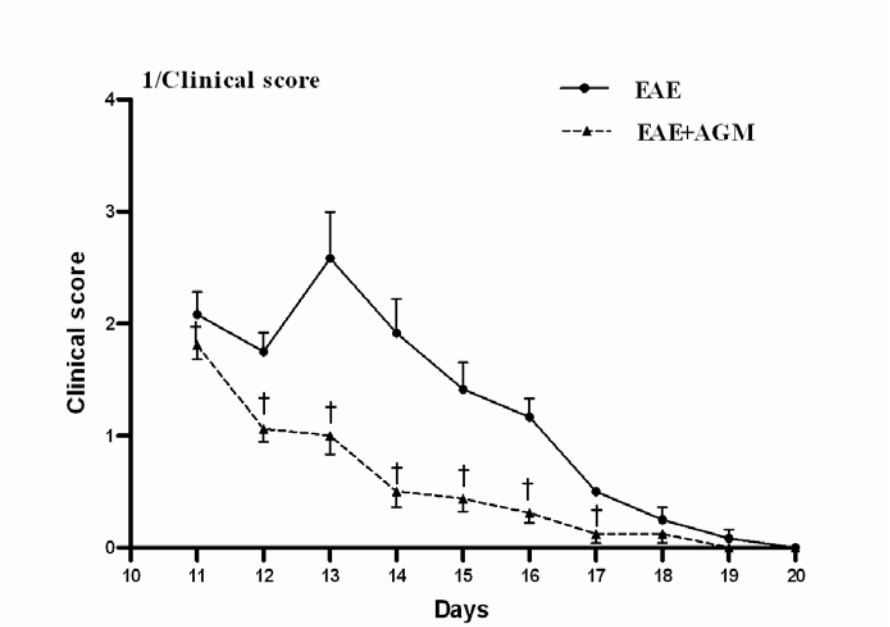


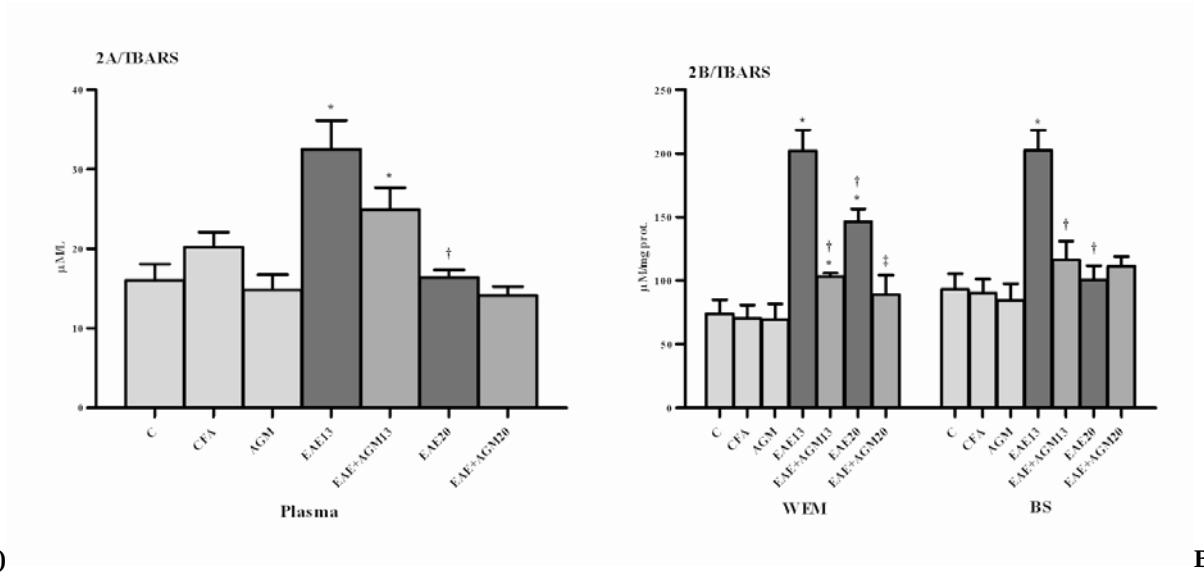
Fig. 1 – The effect of agmatine (AGM) treatment on the experimental autoimmune encephalomyelitis (EAE) clinical signs from 11th – 20th day post immunization (dpi). Results are given as mean ± standard deviation (SD).
†$p < 0.05$ statistically significant difference compared to the EAE group.

maximal severity score was significantly lower in the AGM treated EAE group at the peak of the disease (1.50 ± 0.71 vs. 2.79 ± 0.57, $p < 0.05$) and in the remission of EAE (1.00 ± 0.50 vs. 2.50 ± 1.12, $p < 0.05$) compared to the appropriate EAE group. In addition, the duration of paralysis was significantly shorter in the EAE+AGM13 (1.38 ± 0.52 vs. 2.13 ± 0.35, $p < 0.05$) and EAE+AGM20 (1.43 ± 0.79 vs. 4.00 ± 0.45, $p < 0.05$) groups in comparison to the EAE rats. Additionally, mortality rate was lower in the EAE+AGM13 group compared to the EAE13 group (12.5%) and in the EAE+AGM20 group (12.5%) compared to the EAE20 group (37.5%).

Concentrations of TBARS in plasma increased in the the EAE13 and EAE+AGM13 groups compared to the control group. In the remission of the disease, TBARS concentration lowered compared to the EAE13 (Figure 2A). In tissue, compared to controls, TBARS increased in WEM of the EAE13 and EAE20 groups, while in BS, TBARS increased at the peak of the disease (EAE13) (Figure 2B). Compared to the appropriate EAE group (EAE13/EAE20), the administration of AGM reduced TBARS in WEM (EAE+AGM13/20), while in BS, AGM lowered TBARS at the peak of the disease (EAE+AGM13).

Among all groups, total SH groups content in plasma was decreased only at the peak of EAE (EAE13) compared to the control (Figure 3A). A similar trend of results was registered in tissue: decreased SH in WEM and BS at the peak of EAE (EAE13) and normalized SH in WEM in the remission of EAE, as well as post AGM application. Assuming higher control values in BS, SH depleted in both investigated time points of the disease (EAE13 an EAE20) and after AGM administration at the peak of the disease (EAE+AGM13) (Figure 3B).

The results showed a decreased GSH content in WEM of the EAE13 group, while in the remission of the disease, GSH values normalized to controls in the EAE20 group and



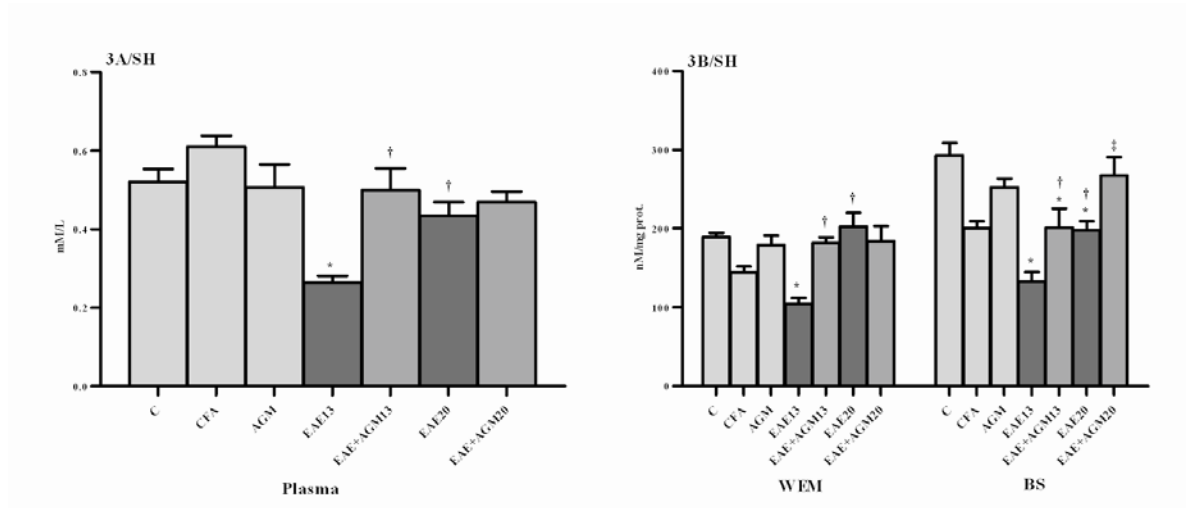
A) **B)**

Fig. 2 –Thiobarbituric acid reactive substances (TBARS) in (A) plasma (μM/L), and (B) brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; μM/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).

Values are expressed as mean ± standard deviation (SD).

The labels of significance: compared to *the control group (C), compared to †the EAE group at the peak of the disease (EAE13) and compared to ‡the EAE group in the remission of the disease (EAE20).

Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).



A) **B)**

Fig. 3 – Total SH groups (SH) in (A) plasma (mM/L), and (B) brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; nM/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), EAE at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).

Values are given as mean ± standard deviation (SD).

The labels of significance: compared to *the control group (C), compared to †the EAE group at the peak of the disease (EAE13) and compared to ‡the EAE group in the remission of the disease (EAE20).

Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).

both the EAE+AGM13 and EAE+AGM20 groups (Figure 4). In BS, similar to SH groups, GSH content was higher in the control groups (C, CFA, AGM) and markedly reduced at the peak and the remission of EAE (EAE13 and EAE20), but also after 10 administered doses of AGM in EAE (EAE+AGM20). Three doses of AGM at the peak of EAE induced GSH repair in BS.

The activity of GPx in both WEM and BS decreased at the peak of the disease (EAE13), while in all other investigated groups, it restored to controls (Figure 5).

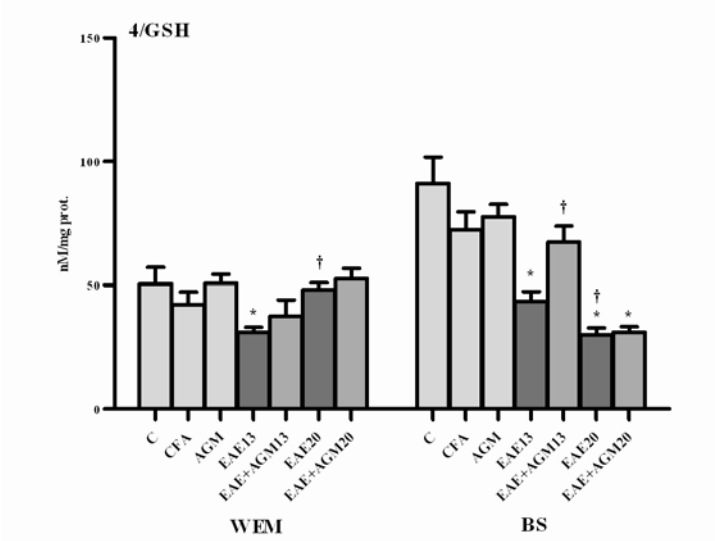


Fig. 4 – The total glutathione content (GSH) in brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; nM/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).
Values are given as mean ± standard deviation (SD).
The labels of significance: compared to *the control group (C), compared to †the EAE group at the peak of the disease (EAE13) and compared to ‡the EAE group in the remission of the disease (EAE20).
Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).

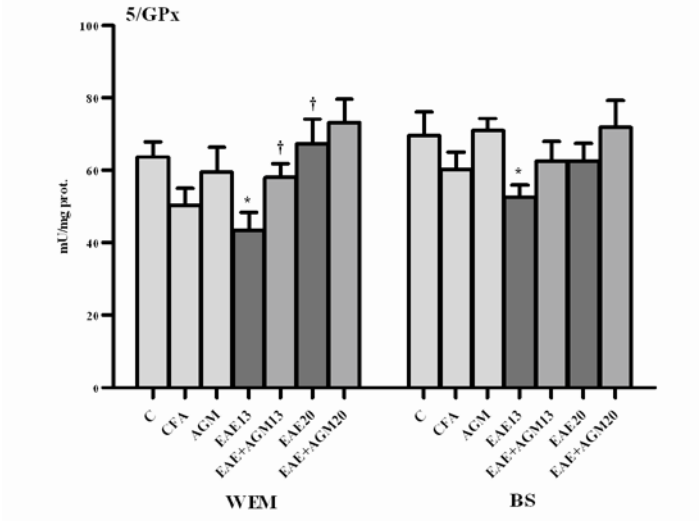


Fig. 5 – The glutathione peroxidase activity (GPx) in brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; mU/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).
Values are given as mean ± standard deviation (SD).
The labels of significance: compared to *the control group (C), compared to †the EAE group at the peak of the disease (EAE13) and compared to ‡the EAE group in the remission of the disease (EAE20).
Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).

The activity of tSOD in WEM increased in the EAE13 and EAE20 groups compared to the control, while a reduced tSOD was registered in the EAE+AGM13 and AGM groups (Table 2). In BS, we noted the increased tSOD activity at the peak (EAE13) and at the remission (EAE20) of the disease compared to the control, and a significantly decreased enzyme activity after AGM treatment at both intervals of the disease (EAE+AGM13, EAE+AGM20) compared to the appropriate EAE group.

Mitochondrial SOD activity increased in both WEM and BS at the peak of EAE (EAE13 and EAE+AGM13) as well as in the remission of EAE (EAE20 and EAE+AGM20) compared to the control (Table 2).

Cytosolic SOD activity in WEM increased at the peak of EAE (EAE13) and significantly decreased after AGM treatment (EAE+AGM13) compared to the control. In BS, we noted the elevated CuZnSOD activity at the peak (EAE13) and at the remission (EAE20) of the disease compared to control values, however, AGM treatment significantly decreased enzyme activity at the peak of EAE (EAE+AGM13) compared to the EAE13 group of animals (Table 2).

The results showed the increased NO_2+NO_3 concentrations in both WEM and BS of the EAE13 group and normalized $NO^{\bullet}$ level after AGM treatment at the peak of EAE (EAE+AGM13). In the remission phase of the disease (EAE20), NO_2+NO_3 lowered in both WEM and BS compared to the peak of the disease (EAE13). Ten-day application of AGM induced additional reduction of NO_2+NO_3 in WEM (EAE+AGM20) compared to EAE20 and a significant elevation of NO_2+NO_3 in BS compared to EAE20 (Figure 6).

Table 2

Activities of superoxide dismutases [total superoxide dismutase (tSOD), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD)] in whole encephalitic brain (WEM) and brainstem (BS) at the peak of the disease (experimental autoimmune encephalomyelitis – EAE13) and in the remission of EAE (EAE20), as well as after three (EAE+AGM13) and 10 (EAE+AGM20) doses of agmatine (AGM) in EAE animals

Superoxide dismutases	Brain structure	Groups						
		C	CFA	AGM	EAE13	EAE+AGM13	EAE20	EAE+AGM20
tSOD	WEM	727 ± 121	850 ± 112	514 ± 115*	1,743 ± 343*	574 ± 101*,†	909 ± 105*,†	756 ± 178
	BS	1052 ± 85	1152 ± 114	1,038 ± 217	2,189 ± 402*	1,190 ± 349†	1,716 ± 464*	1,193 ± 287‡
MnSOD	WEM	71 ± 12	102 ± 23	73 ± 9	210 ± 42*	169 ± 37*,†	145 ± 28*,†	99 ± 4*,‡
	BS	102 ± 23	115 ± 25	125 ± 16*	324 ± 10*	228 ± 37*,†	232 ± 72*,†	153 ± 41*
CuZnSOD	WEM	656 ± 122	812 ± 122	438 ± 130*	1,533 ± 348*	406 ± 106*,†	764 ± 109†	628 ± 108
	BS	950 ± 91	1115 ± 115	913 ± 212	1,864 ± 404*	879 ± 212†	1,484 ± 404	1,039 ± 287

Data are presented as mean ± standard deviation (SD).
C – control (saline); CFA – Complete Freund`s Adjuvant.
*Indicates a statistically significant difference from the control group of animals; †Indicates a statistically significant difference from the EAE group at the peak of the disease (EAE13); ‡Indicates a statistically significant difference from the EAE group in the remission of the disease (EAE20).
***p* – values were obtained by one-way ANOVA followed by Tukey`s test ($p < 0.05$).**

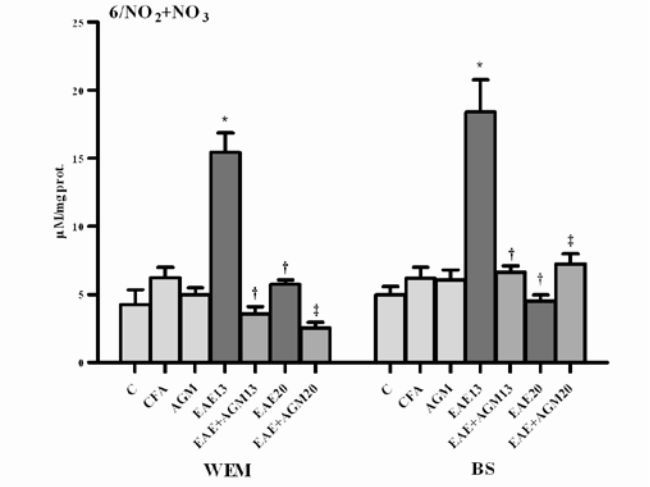


Fig. 6 – The nitrite and nitrate concentration (NO_2+NO_3) in brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; μM/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).
Values are given as mean ± standard deviation (SD).
The labels of significance: compared to *the control group (C), compared to †the EAE group at the peak of the disease (EAE13) and compared to ‡the EAE group in the remission of the disease (EAE20).
Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).

Discussion

The obtained results showed that AGM treatment successfully attenuated severe clinical deficit and suppressed oxidative/ nitrosative stress in EAE. The most severe clinical score was revealed around 13 dpi, followed by the signs of a recovery (Figure 1). This biological model of EAE is complementary with the previously published results in mice EAE, where maximum clinical signs appeared around 20 dpi [18]. During the disease expansion, AGM expressed a strong protective effect, reducing the clinical score in rats with EAE (EAE+AGM) at the peak of the disease compared to the EAE group (Table 1). Also, in the remission of the disease, EAE neurological signs in EAE animals were significantly more severe when compared to the EAE+AGM rats, suggesting the importance of NO metabolism in EAE pathogenesis. The better clinical score in the EAE+AGM group compared to the EAE animals suggests that AGM suppressed inflammation in EAE, which is in accordance with the previously published results [28, 29].

Oxidative stress in MS is a toxic condition in which the excessive production of ROS overcomes the intrinsic antioxidant capacities [30]. Many studies revealed the existence of oxidized phospholipids and MDA in the myelin membranes of apoptotic oligodendrocytes, together with oxidized DNA in oligodendrocyte nuclei [31]. An increase of TBARS in the plasma of EAE rats indicates the increased oxidative damage of lipids with systemic generalized expression (Figure 2A). Following the immunization, we showed the increase in TBARS in brain homogenates (WEM, BS) of EAE rats at the peak of the disease (Figure 2B) which is in accordance with the previous studies [32]. Agmatine treatment, which is known for its immunomodulatory and antioxidative effects, is followed by a significant decrease in TBARS (EAE+AGM20) compared to EAE13 in plasma and in WEM/BS (EAE+AGM13/20) (Figure 2) [33].

Although protein SH groups is determined by the structure and function of proteins, non-protein thiols are predominant in the cellular defense against oxidative stress. The key antioxidant reserve corresponds to several thiol groups, but additionally they can be the major targets for ROS and RNS. Membrane SH groups increase membrane permeability to calcium ions (Ca^{2+}) following excitotoxicity, as well as the production of ROS/RNS that leads to the increase in lipid peroxidation and the decrease in SH groups in plasma of EAE animals at the peak of the disease (Figures 2A, 3A). Plasma SH is modified in MS patients, indicating that SH content is a useful biochemical marker of *in vivo* redox reactions [34]. The results of decreased total SH groups in plasma and WEM/BS homogenates in the EAE13 group (Figures 3A, B) denote the excessive redox-dependent changes in EAE, which, additionally, affect the activity of the mitochondrial respiratory chain complex [35]. The demyelinating condition is associated with the increased protein SH groups nitrosylation, leading to the total SH content depletion [36]. In contrast, during AGM therapy, which led to repairing the resulting oxidative damage, total SH in plasma and tissue homogenates (WEM, BS) were restored to control values (Figures 3A, B).

Depleted GSH in WEM/BS is in accordance with the decreased SH groups at the peak of the disease (EAE13), while in the remission phase of EAE (EAE20), SH groups and GSH restore in WEM (Figure 4). Assuming that GSH is the major thiol present in brain tissue as redox buffer and the fact that MDA is considered as a good marker of LPO, inverse relationship between GSH level and MDA concentration in the EAE13 group indicate the current state of oxidative stress [37]. In EAE20, together with normalized TBARS in BS, SH and GSH were not restored, meaning the prolonged pro-oxidative loads. The treatment by AGM induced the repair of GSH in BS at the peak of the disease, sustaining its antioxidant profile [18]. Normalized TBARS at the peak of EAE (EAE13) and on the 20th dpi indicate the better antioxidant outcome of AGM on lipid components than on proteins.

The result of toxic oxygen metabolites in the brain, generated by neurons and glial cells is the production of superoxide anion ($O_2^{\bullet-}$), which is dismutated to hydrogen peroxide (H_2O_2) as a precursor of high destructive hydroxyl radical ($^{\bullet}OH$). They are all able to react with membrane lipids and cause LPO [38]. The destructive effects of these ROS are interrupted with GPx/GSH enzyme system, which is one of the crucial cell redox pathways [39]. Lower GPx activity in WEM of EAE13 rats revealed reduced antioxidative capacity against oxidative stress (Figure 5), which may result from its inhibition and inactivation by ROS in the presence of $O_2^{\bullet-}$ [40]. As a result of the increased oxidative stress in the EAE13 group, we registered a significantly reduced GPx, followed with reduced GSH in WEM and BS (Figures 4, 5). It could mean that tissue oxidative stress during EAE is predominantly induced by the suppressed non-enzymatic role of GSH rather than by its cofactor function in GPx. The treatment with AGM after three doses in EAE animals (EAE+AGM13) led to restored GPx in WEM (Figure 5). The AGM pathway not only seems to promote neuronal health through its transformation to polyamines, but additionally suppresses inflammation and excitotoxicity, affecting the glutamatergic transmission, reducing glutamate release and inhibiting NOS activity [33]. These multifaceted aspects of the AGM pathway can clarify the restored GPx activity in EAE groups with AGM, as well as their possible therapeutic potential and drug discovery.

The elevated MnSOD and CuZnSOD, as well as total SOD activity in WEM and BS of EAE13 and EAE20, denoted the compromised oxidative balance (Table 2). The application of AGM in EAE13 induced a decreased total SOD and cytosolic SOD in WEM, contrary to significantly increased MnSOD compared to controls, meaning that AGM in WEM influenced predominantly cytosolic and extracellular SOD. At the peak of EAE, the AGM application induced normalized total SOD and CuZnSOD in BS, while MnSOD stayed elevated (same as in EAE). The remission of EAE in both structures (WEM, BS) is characterized by depressed SOD isoforms (tSOD, MnSOD, CuZnSOD) compared to the EAE13 group. AGM induced normalized activity of tSOD and CuZnSOD in the remission of the disease in WEM and BS, with a mild elevation of MnSOD compared to controls.

Such results indicate AGM-induced predominant influence on mitochondrial SOD in BS, throughout EAE, which could be promising, assuming that the mitochondria respiratory chain is an important source of ROS [41].

Besides its beneficial neuro/immunomodulatory effects, $NO^{\bullet}$ participates in the disruption of the blood-brain barrier (BBB), promoting inflammatory, and cytotoxic effects, and inducing oligodendrocyte injury and demyelination, axonal degeneration, and impairment of axonal conduction [42, 43]. Elevated NO_2+NO_3 in EAE13 are followed by AGM-induced normalized NO in both WEM and BS (Figure 6). Astrocytes in MS plaques express high levels of constitutive NOS, producing NO, which interact with $O_2^{\bullet-}$ and produce highly reactive peroxynitrite ($ONOO^-$). The reaction rate between NO and $O_2^{\bullet-}$ is three times higher than superoxide dismutation by SOD, which may harm oligodendrocytes and axons [44]. The mechanisms underlying AGM inhibitory action on astrocytes might, therefore, include the inhibition of $NO^{\bullet}$ production at the peak of the disease and subsequent pathological effects of $NO^{\bullet}$ hyperproduction [45]. The significant positive correlation between MnSOD activity and NO_2+NO_3 ($r = 0.8095$, $p < 0.05$) in WEM of the EAE+AGM13 rats at the peak of the disease may suggest mitochondria as a crucial generator of ROS/RNS in EAE and as a place of the beneficial effects of AGM.

Depleted NO in both WEM and BS after the AGM treatment may suggest that NOS interconnects its effect with AGM, accomplishing physiological effects in the brain [46]. Being structurally similar to L-arginine, AGM is a competitive NOS inhibitor [47]. It also protects neurons against glutamate toxicity and this effect was mediated by NMDA receptor blockade, with AGM interacting at a site located within the NMDA channel pore [48]. The increased NO_2+NO_3 level in the inverse proportion to the GSH content in EAE rats can be explained by the oxidation of GSH within free radical neutralization, as well as its lower synthesis, resulting from a decreased production of cysteine as the limiting GSH precursor. The beneficial effect of AGM could be related to its ability to inhibit iNOS or to block NMDA receptors and/or voltage-dependent Ca^{2+} channels [49]. A possible explanation for the decrease NO and TBARS concentrations might be that NO acts as ROS scavenger, protects cells from LPO and, consequently, prevents the progressive increase of TBARS level.

Conclusion

These results indicate that LPO might have a more important role in protection after AGM administration in EAE animals. Additionally, the AGM treatment in EAE rats which caused a significant decrease in TBARS concentration, suggested the activation of the antioxidant system, resulting in an aggressive oxidative mechanism blockade initiated by EAE immunization. The neuroprotective roles of AGM were acknowledged through the oxidative stress development indicators, such as decreased TBARS and increased GSH concentration.

The study denotes that AGM can attenuate oxidative/nitrosative stress at the peak of EAE by modulating antioxidative defense capacity during the time-course of the disease. Several studies that have investigated the neuroprotective effects of AGM, through its ability to reduce oxidative stress, suggested the beneficial effect of AGM in the treatment of neuroinflammation in MS experimental models. The precise mechanisms of AGM action remain to be elucidated.

Acknowledgement

This work was supported by the Faculty of Medicine of the Military Medical Academy, University of Defence (grant number MFVMA/1/18-20) and the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant number 41018). No conflict of interest exists for any of the authors of this article.

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Received on July 7, 2019
Revised on December 6, 2019
Accepted on December 19, 2019
Online First December, 2019