

# Sex Difference in Oxidative Stress Parameters in Spinal Cord of Rats with Experimental Autoimmune Encephalomyelitis: Relation to Neurological Deficit

Mirjana Dimitrijević<sup>1</sup> · Jelena Kotur-Stevuljević<sup>2</sup> · Zorica Stojić-Vukanić<sup>3</sup> · Ivana Vujnović<sup>4</sup> · Ivan Pilipović<sup>4</sup> · Mirjana Nacka-Aleksić<sup>5</sup> · Gordana Leposavić<sup>5</sup>

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**Abstract** The study examined (a) whether there is sex difference in spinal cord and plasma oxidative stress profiles in Dark Agouti rats immunised for experimental autoimmune encephalomyelitis (EAE), the principal experimental model of multiple sclerosis, and (b) whether there is correlation between the oxidative stress in spinal cord and neurological deficit. Regardless of rat sex, with the disease development xanthine oxidase (XO) activity and inducible nitric oxide synthase (iNOS) mRNA expression increased in spinal cord, whereas glutathione levels decreased. This was accompanied by the rise in spinal cord malondialdehyde level. On the other hand, with EAE development superoxide dismutase (SOD) activity decreased, while  $O_2^-$  concentration increased only in spinal cord of male rats. Consequently, SOD activity was lower, whereas  $O_2^-$  concentration was higher in spinal cord of male rats with clinically manifested EAE. XO activity and iNOS mRNA expression were also elevated in their spinal cord.

Consistently, in the effector phase of EAE the concentration of advanced oxidation protein product (AOPP) was higher in spinal cord of male rats, which exhibit more severe neurological deficit than their female counterparts. In as much as data obtained in the experimental models could be translated to humans, the findings may be relevant for designing sex-specific antioxidant therapeutic strategies. Furthermore, the study indicated that the increased pro-oxidant–antioxidant balance in plasma may be an early indicator of EAE development. Moreover, it showed that plasma AOPP level may indicate not only actual activity of the disease, but also serve to predict severity of its course.

**Keywords** Experimental autoimmune encephalomyelitis · Sex differences · Multiple sclerosis · Oxidative stress · Spinal cord · Rat

## Introduction

Multiple sclerosis (MS) is the most common neuroinflammatory disease of the young adults. The disease is manifested as neurological deficit ranging from altered vision to serious physical disability and disturbances of urinary and bowel function. Depending on the clinical course, MS is classified as clinically isolated syndrome, relapsing–remitting disease and progressive disease [1]. Women show greater incidence of MS than men [2, 3]. However, a shorter time to progression in men than women [4] indicates that more severe forms of MS are attributable to man. We have recently shown a sexual dimorphism in the severity of EAE [5], the principal experimental model of MS [6]. Specifically, in male young adult DA rats clinical signs of EAE appeared later, but they developed more severe disease than females of the same strain and age [5].

✉ Mirjana Dimitrijević  
mirjana.dimitrijevic@ibiss.bg.ac.rs

<sup>1</sup> Department of Immunology, Institute for Biological Research “Siniša Stanković”, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia

<sup>2</sup> Department for Medical Biochemistry, Faculty of Pharmacy, University of Belgrade, 450 Vojvode Stepe, 11221 Belgrade, Serbia

<sup>3</sup> Department of Microbiology and Immunology, Faculty of Pharmacy, University of Belgrade, 450 Vojvode Stepe, 11221 Belgrade, Serbia

<sup>4</sup> Immunology Research Centre “Branislav Janković”, Institute of Virology, Vaccines and Sera “Torlak”, 458 Vojvode Stepe, 11221 Belgrade, Serbia

<sup>5</sup> Department of Physiology, Faculty of Pharmacy, University of Belgrade, 450 Vojvode Stepe, 11221 Belgrade, Serbia

Both MS and EAE are characterised by the central nervous system (CNS) lesions caused by blood-borne immune cells, mainly autoreactive T lymphocytes and inflammatory monocyte progeny, i.e. inflammatory macrophages and dendritic cells [6, 7]. They secrete cytokines, chemokines and reactive oxygen (ROS) and nitrogen species (RNS) and, consequently, promote tissue damage and inflammation [7]. In an inflammatory setting the CNS-residing cells (microglia and astrocytes) produce damaging mediators [7]. We found sex bias in the frequency of activated microglial/inflammatory monocyte-derived cells in spinal cords of DA rats immunised for EAE [5]. Additionally, we showed that their capacity to produce proinflammatory mediators correlate with the severity of clinical and histopathological picture of EAE [5].

A number of studies suggest that generation of RNS and ROS (mainly by inflammatory monocyte-derived cells) is the principal mechanism leading to the CNS tissue damage in MS and EAE [7–10]. The generation of these pro-oxidant mediators is followed by antioxidant defence system activation. Endogenous antioxidant defence system in the CNS comprises non-enzymatic (e.g., glutathione and uric acid) and enzymatic anti-oxidants, such as superoxide dismutases (SODs), glutathione peroxidase, catalase, heme oxygenases, quinone oxidoreductases and peroxiredoxins [11].

There are data indicating sex differences in the activity of pro-oxidant enzymes [12, 13]. Specifically, due to NADP(H)-oxidase up-regulation, superoxide anion ( $O_2^-$ ) generation is greater in microvessels of hypertensive male than female rats [13]. Antioxidant enzymes also exhibit sex-specific differences in activity [12, 14–16]. The activities of SOD (the enzymes catalyzing the dismutation of  $O_2^-$  into  $O_2$  and less damaging  $H_2O_2$ ) and catalase (the enzyme involved in  $H_2O_2$  degradation) in peritoneal macrophages are markedly lower in male than in female rats [12]. The sex bias in the magnitude of anti-oxidant enzyme activities has been related to the differences in circulating sex steroid hormone profile [14–16].

The study was designed to: (a) investigate whether there is sex difference in oxidative stress profile in spinal cord of DA rats immunised with homogeneous spinal cord homogenate in complete Freund's adjuvant to induce EAE and (b) ascertain whether there is correlation between oxidative stress in spinal cord and the neurological deficit in EAE. Considering that neurological deficit in this EAE model is mainly related to the inflammatory lesions in spinal cord [5], we examined spinal cord for xanthine oxidase (XO), the main enzyme involved in  $O_2^-$  generation in inflammatory settings [17], SOD activities, and  $O_2^-$  level. Additionally, spinal cord levels of glutathione (GSH), which reduces disulphide bonds formed within cytoplasmic proteins to cysteine by serving as an electron donor (thereby

preventing oxidative damage to important cellular components), were measured. Moreover, the total spinal cord pro-oxidant status (TOS) and total antioxidant status (TAS) in EAE were monitored. The severity of oxidative stress-induced spinal cord injury was estimated by examining generation of advanced oxidation protein products (AOPP) and the final products of polyunsaturated fatty acids peroxidation in the cells, as is malondialdehyde (MDA). In the same animals, various pro-oxidant and anti-oxidant parameters and their balance (PAB) were examined in plasma to determine whether there is correlation between spinal cord and plasma oxidative stress profile in EAE rats.

## Materials and Methods

### Animals

Three-month-old DA rats of both sexes from a breeding colony in the animal facility of the Immunology Research Centre “Branislav Janković” (Belgrade, Serbia) were used in the study. The animals were maintained under a 12-h light/dark cycle in a temperature-controlled environment, and were provided with standard laboratory food and tap water ad libitum. All experimental procedures and animal care were performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (revising Directive 86/609/EEC) and approved by the Institutional Animal Care and Use Committee.

### Induction and Clinical Assessment of EAE

EAE was induced as previously described [5]. Briefly, rats of both sexes received a single intradermal injection of 100  $\mu$ l rat spinal cord homogenate in complete Freund's adjuvant (containing 1 mg/ml heat-inactivated H37Ra strain *Mycobacterium tuberculosis*; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in the left hind paw, followed by a subcutaneous injection of 250  $\mu$ l *Bordetella pertussis* suspension ( $1 \times 10^9$  bacteria/ml; Institute “Torlak”, Belgrade, Serbia) on the dorsum of the same paw. Rats were daily examined for neurological signs of EAE. The signs were graded as: 0, no clinical signs; 0.5, distal tail atony; 1, complete tail atony; 2, paraparesis; 3, paraplegia; 4, tetraplegia, moribund state or death.

### Histopathological Analysis

For histopathological analysis, 13 days post-immunisation, the peak of EAE in DA rats [5], rats (3 per sex) were anaesthetized by an i.p. injection of 80:8 mg/kg of body weight of ketamine:xylozine anaesthetic solution (ketamine,

100 mg/ml Ketamidol, Richter Pharma AG, Wels, Austria; xylazine, 20 mg/ml Xylased, Bioveta, Ivanovice na Hané, Czech Republic and saline, mixed in a 1:0.5:8.5 ratio) and perfused with PBS followed by 4% buffered paraformaldehyde (PFA) (Sigma-Aldrich Chemie GmbH). Extracted spinal cords were fixed in 4% PFA overnight and embedded in paraffin. Five- $\mu\text{m}$  thick paraffin spinal cord sections stained with haematoxylin and eosin were used for the analysis of inflammation, graded as follows: 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, organisation of inflammatory infiltrates around blood vessels; 3, extensive perivascular cuffing with extension into adjacent subarachnoid space and spinal cord parenchyma [18].

### Tissue Sampling

For analyses of oxidative stress parameters spinal cords and blood were collected 7 days post-immunisation (inductive phase) and 13 days post-immunisation (effector phase) for EAE, as well as from naïve rats of both sexes. All rats were anaesthetised as for the histopathological analysis. Blood samples were collected directly in test tubes containing heparin and centrifuged at 2000g for 15 min at 4°C to separate the plasma. Plasma samples were stored at –80°C until they were analysed. Following removal, spinal cord samples were immediately frozen in liquid nitrogen and stored at –80°C until processed. Frozen tissues were homogenized in nine volumes of 0.1 M phosphate buffer (pH 7.4), containing 1.15% KCl, using Thomas tissue homogenizer (Thomas Scientific, Swedesboro, NJ, USA), fitted with a Teflon plunger. All the steps were carried out at 0–4°C. Homogenates were centrifuged at 12,000g for 40 min, and the supernatant was removed and used for biochemical analyses.

### Biochemical Assays

Chemicals were purchased from Sigma-Aldrich Chemie (Munich, Germany). Activity of XO was assayed spectrophotometrically by using the rate of urate formation from hypoxanthine [19]. One unit of XO activity is defined as forming of one  $\mu\text{mol}$  of urate per minute. Cu/Zn SOD activity was estimated according to the method based on the ability of the enzyme to inhibit autooxidation of epinephrine in alkaline medium [20]. One unit of SOD activity is defined as the activity that inhibits the auto-oxidation of epinephrine by 50%. Modified spectrophotometric methods using o-dianisidine [21], ABTS [22] and 3,3',5,5'-tetramethylbenzidine [23] as a chromogen were applied for evaluation of TOS, TAS and PAB, respectively [24]. PAB is expressed in arbitrary HK units, which correspond to the percentage of hydrogen peroxide in the standard solution. Levels of  $\text{O}_2^-$  were measured as a rate of reduction

of nitrobluetetrazolium according to the method by Auclair and Voisin [25]. The assay for assessment of GSH content was based on reaction with 5,5'-dithiobis-2-nitrobenzoic acid [26]. Concentration of MDA was determined using thiobarbituric acid reactive substances as previously described [27]. AOPP levels were estimated using a reaction with glacial acetic acid and potassium iodide as previously described [28].

Protein concentration in nervous tissue was quantified by Bradford method [29] and used to normalize biochemical parameters in the sample.

Spectrophotometric assays were done with a continuous spectrophotometer (Pharmacia LKB, Cambridge, UK) with the exception of assays for determination of TAS and TOS which were implemented on ILAB 300 Plus analyzer (Instrumentation Laboratory, Milan, Italy).

### RT-qPCR Quantification of iNOS mRNA

Spinal cord tissue samples were harvested using Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA, USA) and immediately stored at –70°C until RNA purification. Total RNA from spinal cord tissue homogenates was extracted using ABI Prism 6100 Nucleic Acid PrepStation system (Applied Biosystems) and Total RNA Chemistry Starter Kit (Applied Biosystems). The procedure included DNase treatment to ensure that no genomic DNA contamination was present.

Isolated RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), in 20- $\mu\text{l}$  reactions ran under optimal conditions (10 min, 25°C; 120 min, 37°C; 5 s, 85°C).

For the analysis of iNOS expression, triplicate 25- $\mu\text{l}$  RT-qPCR reactions, containing 1 $\times$  TaqMan Gene Expression Master Mix (Applied Biosystems), 1 $\times$  mix of pre-made primer and hydrolysis probe sets (TaqMan Gene Expression Assays, Applied Biosystems) and 5  $\mu\text{l}$  of cDNA template, were ran under the default Applied Biosystems 7500 Real-Time PCR System conditions (2 min at 50°C, 10 min at 95°C, followed by 40 cycles consisted of 15 s at 95°C and 1 min at 60°C incubations each). Relative quantification of iNOS level was done using SDS v1.4.0. software (Applied Biosystems) and  $2^{-\Delta\Delta\text{Ct}}$  method with  $\beta$ -actin as a normalizer.

### Statistical Analysis

Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used for statistical analysis of the parameters of oxidative stress. Data were presented as mean  $\pm$  SEM. Parametric correlations were assessed using Pearson correlation coefficients. All statistical analyses were performed using GraphPad Prism 5 software

(GraphPad Software, Inc., La Jolla, CA, USA). Values of  $\leq 0.05$  were considered significant.

## Results

### Male Rats Developed More Severe EAE than Female Rats

As previously described [5], DA rats developed monophasic EAE characterised by an inductive phase without apparent clinical signs of the disease followed by an effector phase of EAE with manifested neurological deficit of different intensities, and a spontaneous recovery phase (Fig. 1a). Rats of both sexes demonstrated high susceptibility to EAE, with the incidence of 100% ( $n=32$ ) and 85.6% ( $n=35$ ) in females and males, respectively (Fig. 1a). The first clinical signs of EAE appeared 1 day later ( $11.3 \pm 0.2$  in males vs.  $9.8 \pm 0.2$  in females;  $p < 0.001$ ) in males, but in rats of both sexes the disease reached the peak on the day 13th post-immunisation. Male rats developed more severe neurological deficit than female ones, as indicated by the greater overall maximal clinical score ( $2.9 \pm 0.2$  in males vs.  $2.1 \pm 0.1$  in females;  $p < 0.01$ ). However, the disease was of shorter ( $p < 0.001$ ) duration in male ( $4.2 \pm 0.5$  days) than in female rats ( $6.7 \pm 0.5$ ). The clinical signs of EAE in rats examined for oxidative stress parameters are shown in Fig. 1b. Histological analysis of the spinal cord

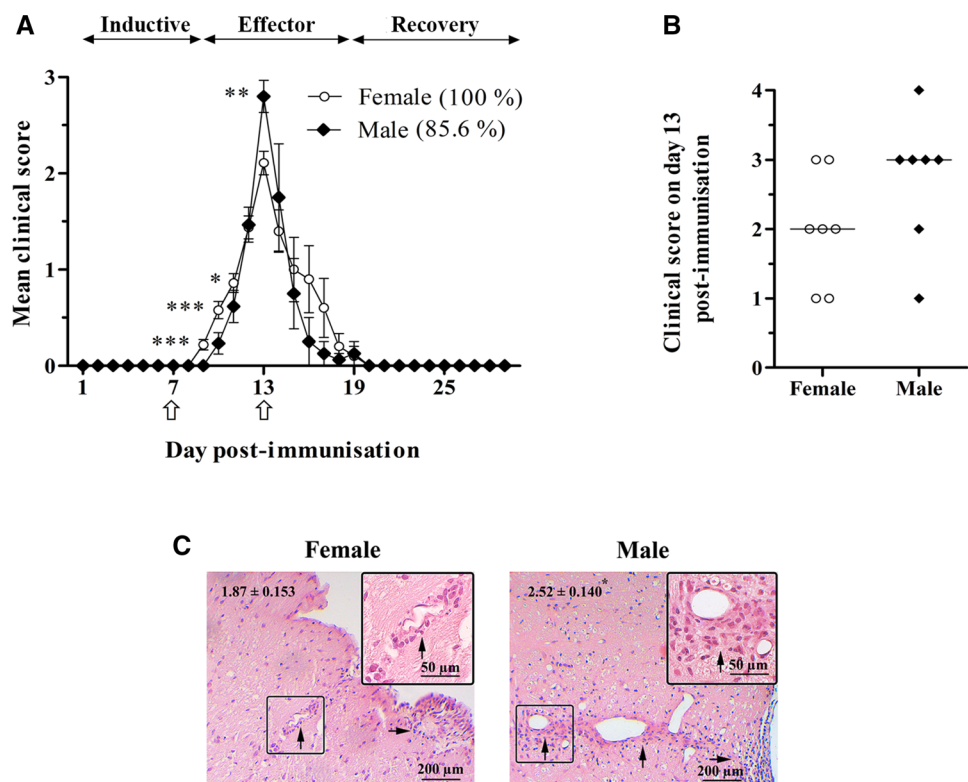
for the inflammation score showed more severe ( $p < 0.05$ ) inflammation in male than in female rats on the 13th day post-immunisation (Fig. 1c). Specifically, the perivascular cuffs and parenchymal infiltration of mononuclear cells were more pronounced in male rat spinal cord. Irrespective of sex, as in other models of acute rat EAE [30], there were no clear signs of demyelination in the spinal cord.

### Redox Status in Spinal Cord Tissue Upon EAE Induction

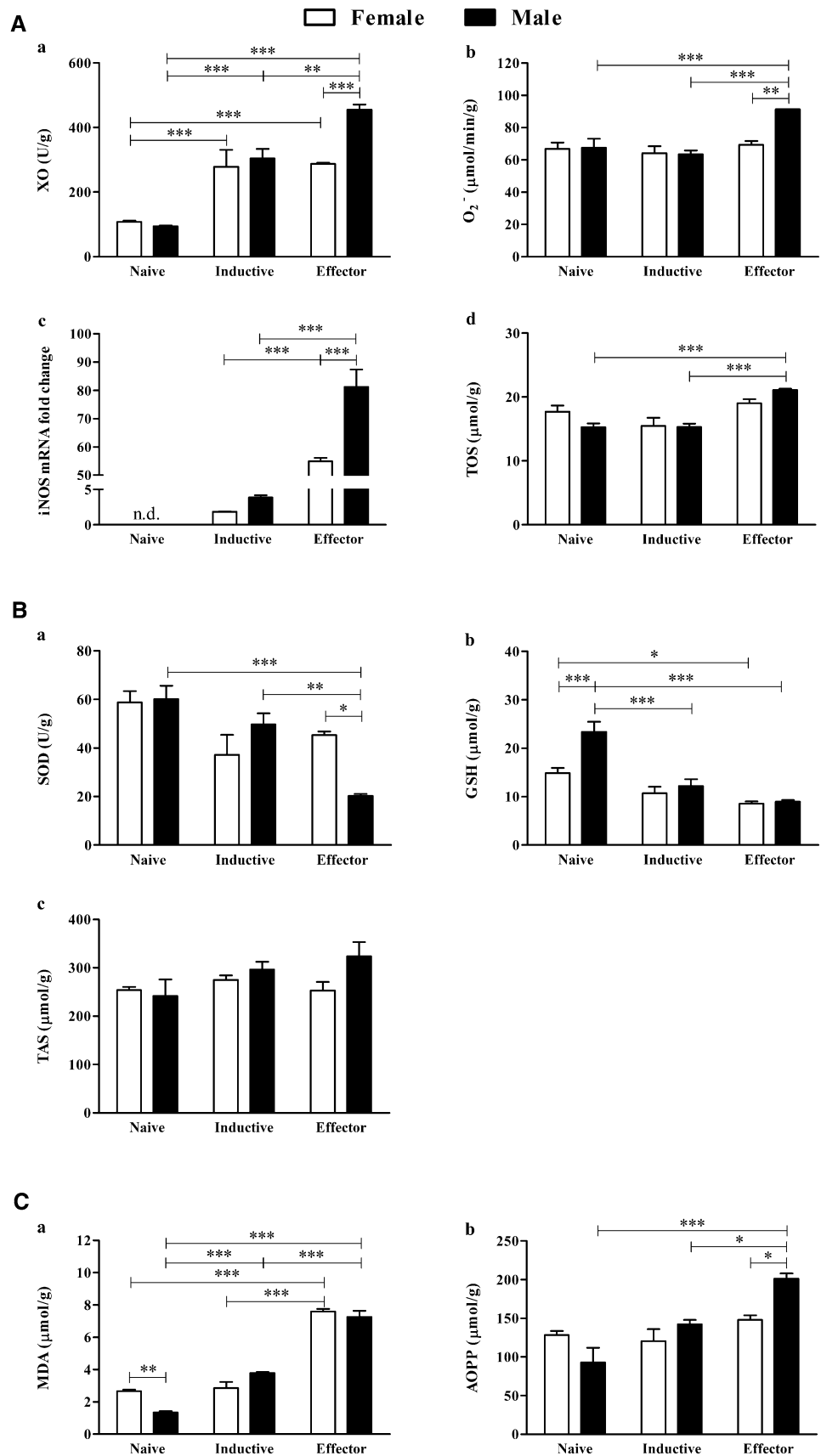
Next, we examined activity of XO, the main enzyme involved in  $O_2^-$  generation in inflammatory setting [17]. In rats of both sexes, in the inductive phase of EAE, XO activity was elevated ( $p < 0.001$ ) in spinal cord when compared with sex-matched naïve rats (Fig. 2Aa). With further development of the disease XO activity increased ( $p < 0.01$ ) in males, but not in females. Consequently, in the effector phase of EAE its activity was greater ( $p < 0.001$ ) in male than female rat spinal cord (Fig. 2Aa).

However, the level of  $O_2^-$ , a central pro-oxidant generated in several enzymatic and non-enzymatic reactions, changed only in male rat spinal cord. In the effector phase of EAE, its level was greater ( $p < 0.001$ ) than in the inductive phase of EAE and in naïve rats (Fig. 2Ab). Consequently, spinal cord level of  $O_2^-$  was greater ( $p < 0.01$ ) in male than in female rats with clinically manifested EAE (Fig. 2Ab).

**Fig. 1** Clinical and histopathological characteristics of EAE in female and male rats. **A** Clinical course and the incidence (in brackets) of EAE. **B** EAE clinical scores on day 13 post-immunisation of rats used for analyses of oxidative stress parameters. Horizontal bars indicate median value for clinical scores in each sex group. **C** Photomicrographs of mononuclear cell infiltrations in spinal cord sections from female (left) and male (right) rats with clinically representative EAE on day 13 post-immunisation ( $n=3$  rats/sex). Arrows designate perivascular and perimeningeal cell infiltrates. Data are presented as means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  (Mann–Whitney *U* test)



**Fig. 2** Parameters of oxidative stress in the spinal cord of female and male rats. **A** Pro-oxidative parameters: xanthine oxidase (XO) activity (a),  $O_2^-$  level (b), expression of mRNA for iNOS (c) and total prooxidant status (TOS) (d); **B** anti-oxidative parameters: superoxide dismutase (SOD) activity (a), glutathione (GSH) level (b) and total antioxidant status (TAS) (c); and **C** parameters of tissue damage: malondialdehyde (MDA) level (a) and advanced oxidation protein products (AOPP) level (b), in the spinal cord of non-immunised (naïve) and EAE-immunised rats on day 7 (inductive) and on day 13 (effector) post-immunisation. Data are presented as means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  (ANOVA, Bonferroni post-hoc test)



Irrespective of sex, the expression of mRNA for iNOS was below the level of detectability in the spinal cord of naïve rats (Fig. 2Ac). Immunisation for EAE up-regulated the expression of mRNA for this enzyme in the spinal cord of rats of both sexes, but this increase reached statistical significance only in the effector phase of the disease. Additionally, in this phase the expression of mRNA for iNOS was greater ( $p < 0.001$ ) in male than in female rat spinal cord.

In accordance with the previous findings, TOS, reflecting the overall pro-oxidant tissue potential, and consequently assumed to be a comprehensive marker of the tissue oxidative stress burden, increased ( $p < 0.001$ ) with the development of clinically manifested disease only in male rats (Fig. 2Ad).

Next, we examined the anti-oxidative status of spinal cord upon EAE induction. The results are presented at the Fig. 2B.

Given that  $O_2^-$  is a substrate for enzymatic SOD activity, concomitantly with the rise in  $O_2^-$  level in male rats, the fall in this protective anti-oxidant activity was registered. Consequently, SOD activity was lower in the effector phase of EAE than in the inductive phase ( $p < 0.01$ ) of the disease and in naïve rats ( $p < 0.001$ ) (Fig. 2Ba). However, in female rat spinal cord SOD activity did not change following immunisation for EAE (Fig. 2Ba).

The analysis of GSH, another important molecule of cellular anti-oxidant defence system, showed greater ( $p < 0.001$ ) level in male than female naïve rats (Fig. 2Bb). However, with EAE induction the level of GSH diminished ( $p < 0.001$ ) in male rat spinal cord, but it did not statistically significantly change in spinal cord of their female counterparts (Fig. 2Bb). At the peak of EAE, spinal cord GSH level decreased in male ( $p < 0.001$ ) and female ( $p < 0.05$ ) rats when compared with sex-matched naïve animals.

Next, spinal cord was examined for TAS as a comprehensive measure of the total anti-oxidant potential. In rats of both sexes, irrespective of EAE phase, TAS concentration was similar in naïve and immunised rats (Fig. 2Bc).

Finally, we investigated spinal cord for the concentrations of MDA, a marker of lipid oxidative degradation, and AOPP, a marker of oxidative protein damage. In male rat spinal cord the concentration of MDA gradually increased with the disease progression; being approximately three-fold (in the inductive phase)—five-fold (in the effector phase) greater ( $p < 0.001$ ) when compared with naïve rats (Fig. 2Ca). However, its generation in female rats was slower, so it was approximately three-fold greater ( $p < 0.001$ ) in the effector phase than in sex-matched naïve rats (Fig. 2Ca).

As a final point, with EAE development, spinal cord AOPP level increased in male, but remained unaffected in female rats. In males, in the effector phase of EAE,

AOPP level was greater than in the inductive phase of the disease ( $p < 0.05$ ) and in naïve animals ( $p < 0.001$ ) (Fig. 2Cb). Consequently, in this phase of EAE AOPP level was greater ( $p < 0.05$ ) in male than in female rat spinal cord.

### Redox Status in Rat Circulation Upon EAE Induction

Additionally, the redox status was assessed in circulation of rats immunised for EAE.

In rats of both sexes, with the development of clinical EAE, the plasma level of  $O_2^-$  was markedly increased ( $p < 0.001$ ) compared with those in the inductive phase and in naïve rats (Fig. 3Aa). Thus, it seems that the development of the central pro-oxidant changes was rather slow and/or that the protective mechanisms were sufficient to neutralise the initial changes. Despite these findings, TOS, as a comprehensive measure of pro-oxidant potential in blood, did not significantly change in either male or female rats developing EAE (Fig. 3Ab).

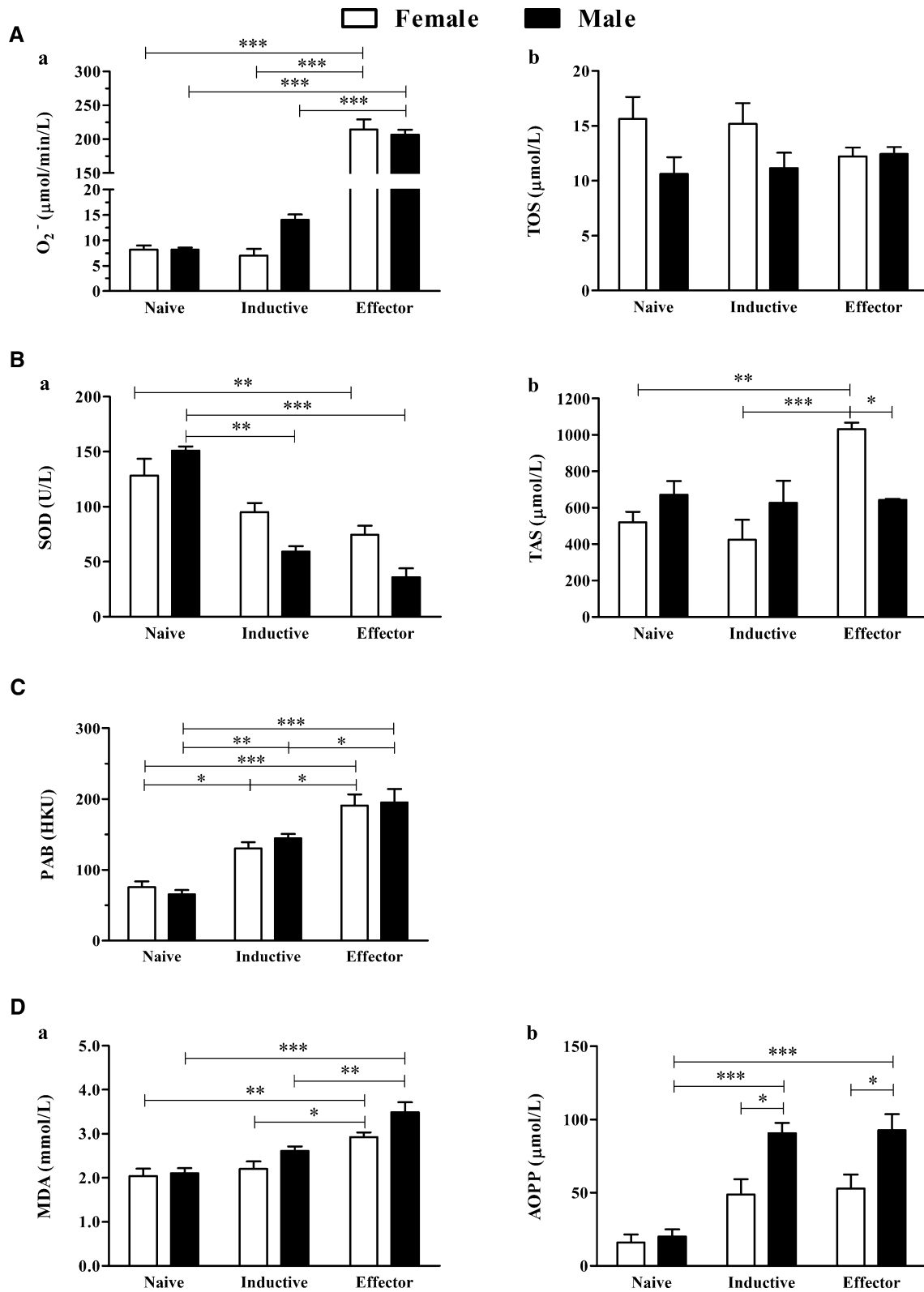
In male and female rats, the enzymatic part of anti-oxidative protection in circulation substantiated in SOD activity was compromised in both the phases of EAE. Following EAE induction the fall in SOD protective activity occurred earlier and was sharper ( $p < 0.001$ ) in male rat circulation (Fig. 3Ba).

However, we failed to register any changes following the immunisation in the concentration of TAS in plasma of male rats, while it increased in plasma of female rats in the effector phase of EAE ( $p < 0.001$  and  $p < 0.01$  compared with the inductive phase and naïve rats, respectively) (Fig. 3Bb). Consequently, in effector phase of EAE the concentration of this marker was lower ( $p < 0.05$ ) in male than in female rat plasma (Fig. 3Bb).

The analysis of PAB showed its progressive increase with EAE development in rats of both sexes. Irrespective of phase of the disease, PAB did not differ between female and male rats (Fig. 3C).

Finally, differently from the inductive phase, in the effector phase of EAE the concentration of MDA increased in circulation of both male ( $p < 0.001$ ) and female ( $p < 0.01$ ) rats (Fig. 3Da).

On the other hand, in male rats, differently from their female counterparts, the plasma concentration of AOPP, markedly increased in the inductive phase of EAE ( $p < 0.001$ ), and this increase persisted in the effector phase (Fig. 3Db). However, the mild increase in AOPP level in plasma of female rats during the effector phase did not reach statistical significance. Consequently, plasma AOPP level in both the phases of EAE was greater ( $p < 0.05$ ) in male than in female rats (Fig. 3Db).



**Fig. 3** Parameters of oxidative stress in the plasma of female and male rats. **A** Pro-oxidative parameters:  $O_2^-$  level (a) and total prooxidant status (TOS) (b); **B** anti-oxidative parameters: superoxide dismutase (SOD) activity (a) and total antioxidant status (TAS) (b); **C** prooxidative-antioxidative balance (PAB) and **D** parameters of tissue damage: malondialdehyd (MDA) level (a) and advanced oxidation

protein products (AOPP) level (b), in the plasma of non-immunised (naïve) and EAE-immunised rats on day 7 (inductive) and on day 13 (effector) post-immunisation. Data are presented as means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  (ANOVA, Bonferroni post-hoc test)

## Correlation Between Redox Status in Spinal Cord and Circulation in Rats with EAE

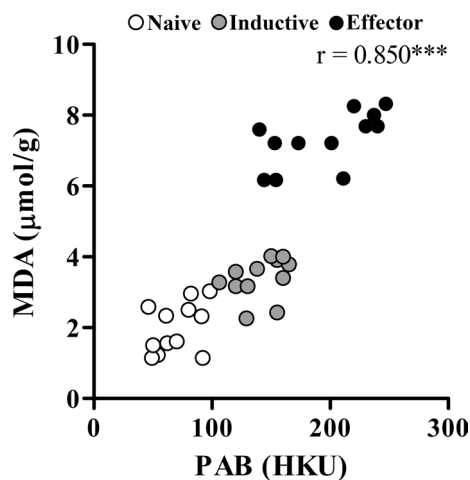
There were multiple correlations between parameters of oxidative stress in plasma and spinal cord (data not shown). However, PAB, a measure of capability of different mechanisms to maintain redox balance in circulation, was identified as the only parameter which value increases already in the inductive phase of EAE, and augments with the disease development in both sexes (Fig. 3C). Additionally, a strong positive correlation (Pearson's  $r=0.850$ ,  $p<0.001$ ) between PAB value in plasma and MDA level in the spinal cord was found (Fig. 4), suggesting the connection between redox status in the target tissue and systemic circulation.

## Discussion

The present study extended our previous finding indicating that young adult male DA rats inoculated with spinal cord emulsion exhibit the more severe neurological deficit than their female counterparts [5] by showing sex bias in the spinal cord oxidative damage. In favour of the imbalance between anti-oxidant and pro-oxidant-generating systems resulting in the greater oxidative stress in male than female rat spinal cord in the effector phase of EAE spoke the greater level of  $O_2^-$  and enhanced expression of iNOS (suggesting greater NO generation) in male rat spinal cord. This was in agreement with data indicating (a) pro-inflammatory cytokine-induced up-regulation of iNOS in the CNS lesions in both EAE and MS [31] and (b) the greater

expression of pro-inflammatory cytokines in male than female rat spinal cord mononuclear cells [5]. Given that there is a clear association between NO production in Th lymphocytes infiltrating spinal cord and EAE severity [32], the greater iNOS expression in male EAE rat spinal cord is also consistent with greater number of infiltrating Th lymphocytes in their spinal cord [5]. Furthermore, it should be emphasized that NO can react with  $O_2^-$  to form peroxynitrite ( $ONOO^-$ ), which exhibits stronger pro-oxidant potential than  $O_2^-$  [33, 34]. The sex bias in iNOS expression seems to be particularly important in light of data indicating that in the EAE models characterised by the absence of demyelination, iNOS expression in perivascular macrophages, at the peak of the disease, is predominantly associated with transient functional disturbances of axons [35]. In this context, it should be pointed out that the increased iNOS expression was followed by diminished arginase mRNA expression at the peak of EAE ( $1 \pm 0.19$  in female vs.  $0.02 \pm 0.023$  in male;  $p<0.01$ ), as it is shown that with the substrate limitation, iNOS may become uncoupled and produce ROS [36]. To suggest pathogenic significance of the above described sex differences is the greater spinal cord level of AOPP in male than female rat spinal cord in the effector phase of EAE.

Multiple pathways that take part in the generation of  $O_2^-$  comprise several oxidase enzymes (NADPH oxidase and cytochrome P-450, XO), but XO is a major enzyme generating ROS in the context of inflammation [17]. The expression of XO, and consequently its  $O_2^-$  generation capacity are shown to be substantially augmented in the presence of proinflammatory cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) [37, 38]. Consistently, XO activity increases in mouse brain at the peak of EAE, and this increase is implicated in the pathogenesis of the disease [17, 39]. Thus, the greater XO activity in spinal cord from male than female rats with EAE could be related to the greater expression of pro-inflammatory cytokines in male rat spinal cord mononuclear cells [5]. Inflammatory monocyte-derived cells (macrophages and dendritic cells) and activated microglia are thought to be the major source of XO in the CNS in EAE models and MS [17, 40]. Thus, the sex bias in spinal cord XO activity is consistent with the greater frequency of CD45<sup>hi</sup>CD11b<sup>+</sup> cells, presumably highly activated microglial/inflammatory monocyte-derived cells [41], in spinal cord of male rats immunised for EAE compared with their female counterparts [5]. The greater frequency of CD45<sup>hi</sup>CD11b<sup>+</sup> cells in male than female rat spinal cord could be related to the greater frequency of Th17 cells infiltrating male rat spinal cord. Although exact mechanism is not clear, it has been suggested that IL-17-stimulated production of ROS in spinal cord endothelial cells increases secretion of inflammatory monocyte chemoattractant protein-1 and facilitates inflammatory monocyte transmigration across blood–brain



**Fig. 4** Correlation between prooxidative-antioxidative balance (PAB) in the plasma and concentration of malondialdehyde (MDA) in the spinal cord. Plasma and spinal cords were obtained from non-immunised (naïve) and EAE-immunised rats of both sexes on day 7 (inductive) and on day 13 (effector) post-immunisation. Pearson's  $r$  value is shown in the graph \*\*\* $p<0.001$



barrier and their accumulation in spinal cord [42]. Furthermore, given that recently it has been shown that NADPH oxidases activity up-regulates in the peak of EAE [43], the contribution of sex differences in their expression to the greater spinal cord  $O_2^-$  level could also not be excluded. To support this option was greater expression of this enzyme in (a) male compared with female porcine vascular tissue [44] and (b) in male than female human cerebral blood vessels [45].

Given that differences in the efficiency of anti-oxidant mechanisms could contribute to greater spinal cord  $O_2^-$  level, the activity of SOD, the enzyme representing the first and most important line of defence against  $O_2^-$  burden, was examined. Although the activity of this enzyme did not differ between sexes in spinal cord of naïve rats, with the disease development its activity diminished only in male rats. Consequently, in the effector phase of EAE SOD activity was lower in spinal cord of male than female rats. To the best of our knowledge there are no data on sex difference in SOD activity in spinal cord of rats with EAE. However, it has been shown that SOD activity decreases in the brain homogenate from female Sprague–Dawley rats following immunisation with myelin basic protein [46]. This suggest strain and/or brain region differences in SOD activity in rats with EAE. However, there is evidence that in mice the impaired Cu/Zn-SOD activity contributes to more severe oxidative brain damage [47]. Additionally, SOD1-deficient mice develop more severe EAE than wild type animals [48]. Thus, lower SOD activity in male than female rat spinal cord could also be associated with more severe disease in male rats [5].

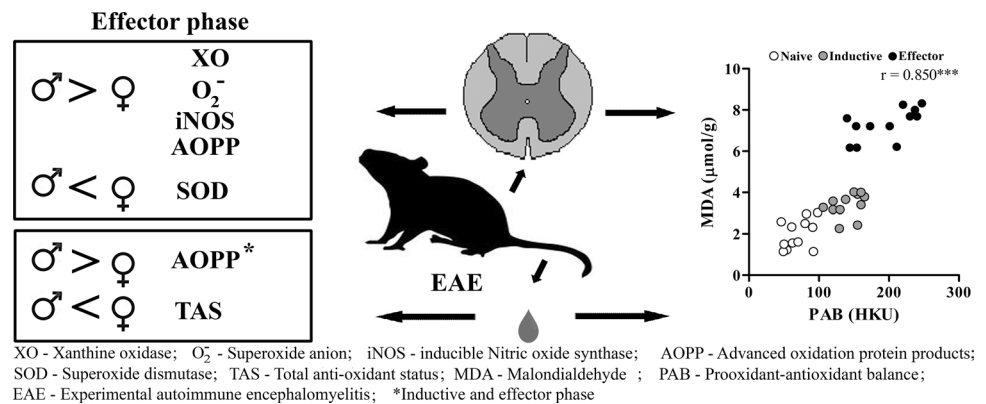
The present also study demonstrated sex differences in GSH level in the spinal cord of naïve rats, i.e. male rats exhibited higher level of GSH than female rats. However, in both sexes the immunisation for EAE lowered GSH spinal cord level, but to a greater extent in male than in female rats, so that its level was comparable in male and female immunised rats. The decrease in level of GSH, the major brain anti-oxidant [49], in spinal cord of rats with EAE is consistent with findings indicating: (a) diminished GSH levels in the brain and spinal cord of mice developing EAE [50, 51], (b) lessened cerebrospinal fluid GSH level and impaired balance of glutathione-related enzyme activities in MS patients [52], and (c) depressed GSH concentrations in the frontoparietal brain region of MS patients regardless of sex [53].

In the spinal cord of naïve rats, sex difference in the level of MDA was consistent with sex bias in the level of GSH. Specifically, in spinal cord of male rats which exhibited higher GSH level than their female counterparts, lower MDA level was observed. Inverse relationship between concentrations of MDA and GSH has been acknowledged [51, 54]. Following the immunisation, contrary to GSH

level, MDA level increased in spinal cord of rats of both sexes. The increase in MDA level has already been shown in spinal cord of EAE rats [39] and cerebrospinal fluid of MS patients [55]. However, this EAE-related increase was markedly more pronounced in male than in female rats, so MDA level was comparable in male and female rats at the peak of disease. On the other hand, with EAE development AOPP level increased only in male rats. This finding in conjunction with data indicating that: (a) auto-reactive Th cells instigate the CNS inflammation and consequently the tissue damage by acting on myeloid cells via the production of granulocyte–macrophage colony-stimulating factor (reviewed in [56]); (b) myeloid cells are implicated in both the inflammatory process in EAE/MS and as executors of tissue damage in the CNS in these pathologies (reviewed in [56]); (c) activated myeloid cell (mainly inflammatory monocyte progeny)-derived ROS and RNS represent the major CNS tissue damaging mediators in EAE and MS (reviewed in [56]), it may be assumed that that the greater oxidative stress-induced spinal cord protein damage in male rats contributed to the progression of EAE, and the greater neurological deficit in this sex at the peak of the disease. On the other hand, considering oxidative stress profile in naïve rats and in the inductive phase of EAE, it does not seem likely that distinct degree of oxidative stress contributed to sex difference in the incidence of EAE. This is consistent with data indicating a strong correlation between infiltration of inflammatory monocytes (as major source of ROS and RNS in spinal cord tissue) and progression to the paralytic stage of EAE [57]. It should be pointed out that: (a) multiple sclerosis also exhibits lower incidence, but a more severe clinical course in males than females [58] and (b) this phenomenon is assumed to arise as a result of intricate interactions between the immune mechanisms and a significant number of nervous tissue protective (including blood flow, apoptosis inhibition)/repair mechanisms [58–62] and (c) sex-related factors in MS, and possibly EAE play different, and opposing, roles in the immune system vs. the CNS [58].

To the best of our knowledge the parameters of oxidative stress in blood in rodent models of monophasic EAE have not been systematically investigated. Our study indicated that, irrespective of sex, in the effector phase of EAE plasma levels of  $O_2^-$ , PAB and MDA were increased compared with sex-matched naïve animals. Correspondingly, the elevated levels of MDA have been found in sera from MS patients [55, 63, 64]. Furthermore, analyses of correlation between spinal cord and plasma oxidative stress parameters revealed that the levels of  $O_2^-$ , PAB, MDA and AOPP in plasma mirrored the magnitude of oxidative stress in the spinal cord (data not shown). Among these parameters, plasma PAB appeared to be the best predictor for the oxidative damage in the

**Fig. 5** Graphical summary of the changes in spinal cord and plasma of male and female rats immunised for EAE



spinal cord of EAE rats due to substantial correlation with the levels of MDA in the nervous tissue. On the other hand, the greater level of AOPP in the plasma of male than female rats in the effector phase of the disease correlated with its greater level (suggesting more severe protein damage) in male than female spinal cord. In male rat exhibiting more severe neurological deficit this rise also coincided with lower plasma TAS. The sex bias in plasma TAS level has been registered in MS, but its value was lower in females than in males [65]. However, as the authors did not provide data on sex differences in the clinical picture of the disease [65], it is not possible to correlate the previous findings with the clinical severity of the disease. In the inductive phase of EAE, AOPP level was also greater in plasma of male than female rats. Thus, it seems that plasma AOPP level not only correlate with the disease severity in the effector phase, but also that its greater value in the inductive phase predicts clinically more severe EAE.

In conclusion, our study suggests that sex bias in the oxidative stress contribute to more severe neuroinflammation, and consequently more pronounced neurological deficit in male than in female rats immunised for EAE. Specifically, it revealed more pronounced generation of both ROS and RNS in spinal cord of male rats (Fig. 5). This may also suggest distinct, sex-based therapeutic effectiveness of antioxidant agents in EAE, and possibly MS. The analysis of oxidative stress parameters in plasma of rats immunised for EAE (Fig. 5) indicated that PAB may be considered as a predictor of the disease development, and suggested that plasma AOPP level, as in ulcerative colitis [66], may be used as a simple plasma marker to assess disease activity, predict the severity of disease course, and perhaps response to therapy.

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### Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical Approval** All applicable international, national and institutional guidelines for the care and use of animals were followed.

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