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Redox imbalance in peripheral blood of type 1 myotonic dystrophy patients

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Objectives: The aim of our study was to determine if redox imbalance caused by the activities of antioxidant enzymes existed in erythrocytes of type 1 myotonic dystrophy (DM1) patients.
Methods: The activities of erythrocyte superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase were measured in 30 DM1 patients and 15 healthy controls (HCs). The obtained values were correlated with the Muscular Impairment Rating Scale (MIRS) score and creatine kinase (CK).
Results: Superoxide dismutase and catalase activities were lower in DM1 patients compared to HCs. A positive correlation was found between disease duration and MIRS score as well as with glutathione reductase activity. In DM1 patients, there were positive correlations between catalase, glutathione peroxidase, and glutathione reductase activities. After sub-dividing DM1 patients according to CK levels, superoxide dismutase activity was still statistically different from HCs. However, catalase activity was significantly lower only in DM1 patients with increased CK.
Discussion: Undesirable alterations in antioxidant enzyme activities during DM1 disease progression may result in conditions favoring oxidative stress and changes in metabolism which together could contribute to muscle wasting.

Keywords: Catalase, Erythrocytes, Glutathione reductase, Glutatione peroxidase, Type 1 myotonic dystrophy, Superoxide dismutase

Introduction

Type 1 myotonic dystrophy (DM1) is the most common form of muscular dystrophy in adults with a worldwide prevalence of 1–35 patients per 100 000 inhabitants.[1] DM1 is an autosomal-dominant disease caused by the expansion of Cytosin, Thymine, Guanine (CTG) trinucleotide repeats within the non-coding 3′-untranslated region of the gene encoding dystrophia myotonica-protein kinase on chromosome locus 19q13.3.[2] This mutation has a trans-dominant effect through the regulation of alternative splicing of pre-messenger RNA for various proteins, mRNA translation and mRNA stability, which contribute to the multiple features of DM1.[3] However, the alternative splicing of many different transcripts fails to fully explain muscle wasting in DM1 patients. DM1 is a multi-systemic disease that affects many organs and tissues besides muscle.[4] DM1 has been described as a disease characterized by premature aging, which involves oxidative stress in its pathogenesis.[5] Reduced or increased cellular antioxidant activity and increased oxidative stress parameters have been reported in DM1, which may be associated with muscular and extra-muscular signs of the disease.[5–8] There is considerable evidence suggesting that reactive oxidative species (ROS) and reactive nitrogen species, such as superoxide ($O_2^{\cdot-}$) and nitric oxide ($NO^{\cdot}$), can play roles in age-related loss of muscle mass and function.[9] The inability of aged muscle to respond to stress is explained by deteriorating redox signaling processes and redox-mediated adaptation.[10] Current knowledge supports the likelihood that interactions between the primary genetic defect and disruptions in the normal production of free radicals contribute to muscle atrophy in muscular dystrophies.[10] The inability of muscle regeneration to keep up with apoptotic and necrotic events following oxidative stress during normal muscular exercise may underlie muscle atrophy in DM1.[11] In sporadic and familiar amyotrophic lateral sclerosis, also characterized by muscle wasting, decreased levels of both copper–zinc superoxide dismutase (SOD1) and catalase (CAT)

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activities in erythrocytes have been described.[12] Thus, circulating oxidative stress could be related to skeletal muscle wasting in DM1.

The aim of our study was to analyze the levels of antioxidant defense enzymes in erythrocytes of patients with DM1, to compare them with those in healthy controls (HCs) and to correlate individual antioxidant enzyme activity with DM1 clinical data, Muscular Impairment Rating Scale (MIRS) scores and creatine kinase (CK) levels. Erythrocytes are a good model for studying systemic oxidative stress and have been exploited in other studies.[12]

Methods

Patients selection

This cross-sectional study comprised 30 (14 males and 16 females) adult patients aged 41 ± 5 years primarily diagnosed with DM1, who were examined in the outpatient and inpatient units of the Neurology Clinic in Belgrade from 1 September until 31 December 2011 and 15 HCs matched for gender (seven males and eight females) age 36 ± 5 years. Patients with congenital and late adult form of DM1 were not included. In addition to standard clinical and electromyography data, genetic diagnosis of CTG repeat expansion was obtained from all the patients (mean was 691 ± 57). The study was approved by the Ethical Board of the Neurology Clinic, University of Medical Sciences, Belgrade, N° 29/X-5. All patients gave informed written consent to participate in the study.

The severity of DM1 was assessed by the MIRS (mean was 3.4 ± 0.2).[13] The MIRS is an ordinal five-point rating scale established in accordance with the clinically recognized distal to proximal progression of muscular involvement in DM1. The man level of serum CK in patients was 251 ± 50 IU/l.

Biochemical analysis

Each study participant donated a 5 ml blood sample obtained by venous puncture between 8 and 9 a.m. after an overnight fast and a period of tobacco abstinence (overnight 12 hours). Erythrocyte lysates were prepared as follows. After the heparin-treated blood samples were centrifuged at $2000 \times g$ for 15 minutes at 4°C, the plasma was discarded. The separated erythrocytes were washed three times with 0.9%, w/v NaCl. Washed cells (0.5 ml) were then lysed by adding 3 ml of ice-cold distilled water followed by thorough mixing.

Hemoglobin (Hb) concentration

The total Hb content of the hemolysates was measured as cyanmethemoglobin using the Drabkin method.[14]

Hemoglobin removement

To remove Hb 1.0 ml of an ethanol/chloroform (1:1, v/v) mixture was added to an aliquot (0.5 ml) of the hemolysate cooled on ice.[15] This mixture was stirred constantly for 15 minutes before being diluted with 0.5 ml of distilled water. After centrifugation for 10 minutes at $1600 \times g$, the pale-yellow supernatant was separated from the protein precipitate and was used to assay SOD enzyme activity spectrophotometrically and for native polyacrylamide gel electrophoresis (PAGE).

Spectrophotometric measurements

Antioxidant enzyme activities were measured as previously described.[12]

SOD1 activity was determined by the epinephrine method, which is based on the capacity of SOD1 to inhibit autoxidation of adrenaline to adrenochrome. Reaction mixtures consisted of 3×10^{-4} M adrenaline, 1×10^{-4} M EDTA, and 0.05 M Na_2CO_3, pH 10.2. One unit of SOD is defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline.

CAT activity is defined as the amount of enzyme, which decomposes 1 mmol hydrogen peroxide (H_2O_2) in 1 minute at pH 8.0 in 1 M Tris–5 mM EDTA buffer measured at 230 nm.

GSH-Px—glutathione peroxidase activity was measured according to NADPH consumption (NADPH oxidation by glutathione reductase) monitored at 340 nm. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM GSH, 1 mM sodium azide, 1 U/ml glutathione reductase, 0.2 mM NADPH, and 3 mM t-butyl hydroperoxide.

GR—glutathione reductase activity measurement is based on NADPH oxidation concomitant with GSH reduction. The reaction mixture consisted of 0.5 M sodium phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.1 mM NADPH, and 0.1 mM GSSG. Enzyme activity is expressed in U/g of soluble protein. One unit of enzyme activity is defined as the amount of enzyme required to transform 1 μmol of substrate per minute under the above described assay conditions.

Native PAGE

Was performed according to Laemmli, 1970 using 12% acrylamide under non-denaturing conditions.[16] Prior to PAGE, aliquots of SOD1 from erythrocytes were incubated for 2 hours at 37°C with gentle stirring in the absence (control) and presence of 5 mM H_2O_2. Incubated SOD samples were diluted to 2 U/ml using a solution containing 12% glycerol, 0.5 mM Tris–HCl (pH 6.8), and 0.2 M EDTA before loading 50 μl per well. SOD1 bands (achromatic zones on an otherwise uniformly violet-blue gel) were visualized using the activity staining procedure described by Beauchamp and Fridovich[17] requiring the reduction of nitroblue tetrazolium (NBT) with superoxide produced by

photochemical reduction of riboflavin with *N*,*N*,*N*′,*N*′,-tetramethylethylenediamine. To quantify SOD activity, the gels were scanned using a densitometer (GS-700, Bio-Rad) and the tiff image files were analyzed using Multi-Analyst 1.0.2 software (Bio-Rad).

Statistical analysis

Statistical analyses were performed according to the protocols originally described by Manley[18] and Blagojević *et al.*,[19] which have been subsequently applied to study other pathophysiological conditions.[12,20] Differences in antioxidant enzyme activity between DM1 patients and controls were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Student's *t*-test. Spearman's coefficient was used for correlation of two variables. Since correlation analysis calculates relationships between individual components, we performed canonical discriminant analysis that calculated differences between groups, taking into consideration the complete correlation matrix (i.e. composition of antioxidant defense) of separate patient groups. In all analyses, $P < 0.05$ was considered as significant.

Results

The activities of SOD1 and CAT were lower in DM1 patients compared to HCs (Fig. 1).

Other measured antioxidant enzymes (GSH-Px and GR) showed no differences. In-gel SOD activity showed the same result (lower activity of SOD1 in DM1 patients compared to HCs). Figure 2 illustrates a representative SOD1 activity profile in non-denaturing PAGE conditions. In addition, SOD1 in HCs was more active (45% of activity remaining; $P < 0.05$) after partial inhibition with equimolar concentration of H_2O_2, compared to that in DM1 patients (25% activity remaining; $P < 0.001$).

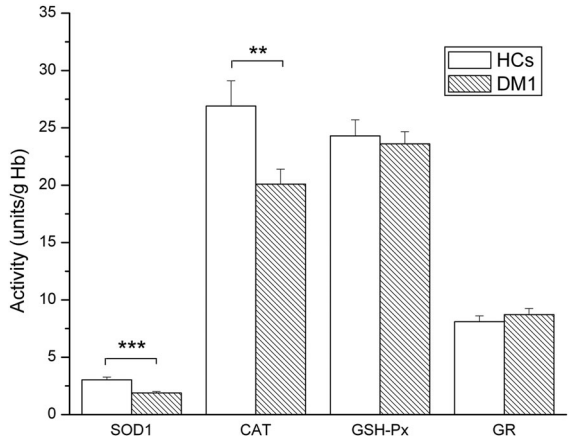

Figure 1. Antioxidant enzyme activities in erythrocytes from DM1 patients ($n = 30$) and HCs ($n = 15$). Results are expressed as mean ± SE and compared by *t*-test. ***$P < 0.001$, **$P < 0.01$.

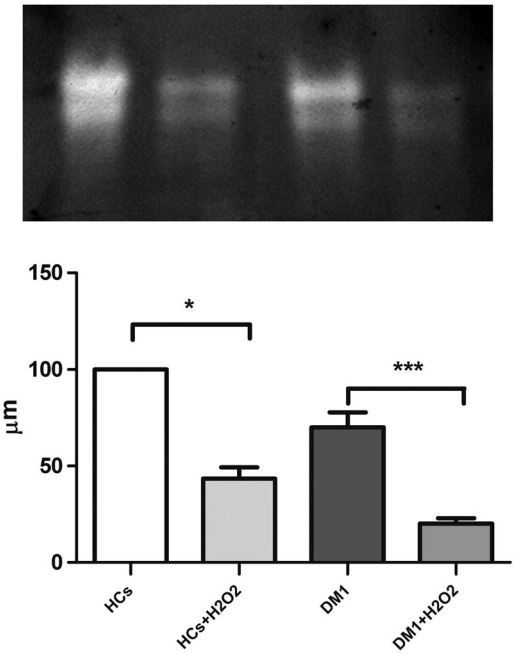

Figure 2. Native PAGE gel stained for SOD1 enzyme activity with NBT. Lanes, from left to right: HCs; HCs + H_2O_2; DM1 and DM1 + H_2O_2. Histogram presents quantitative analysis of SOD activity. Graphs represent % from SOD activity HCs taken as 100% (±SE). Statistical analysis showed a more pronounced reduction in activity after H_2O_2 treatment of DM1 SOD compared with to HC SOD (*$P < 0.001$; $n = 7$, vs. *$P < 0.05$; $n = 5$). Note the difference between samples with respect to relative band intensity (in-gel SOD1 activity). For details, see 'Methods' section.**

When DM1 patients were segregated according to MIRS grade score, SOD1 activity in erythrocytes of DM1 patients with MIRS grades III and IV was significantly lower than HCs (Table 1). Differences in CAT activity were just above the threshold for a statistically acceptable ANOVA *P* value (therefore the *post hoc* test showed no statistical significance). The activities of GSH-Px and GR were not different compared to those found in HCs.

Table 1 The activity of antioxidant enzymes in DM1 patients with different severity of muscle impairment

	SOD1	CAT	GSH-Px	GR
HCs ($n = 15$)	3.0 ± 0.2a	26.9 ± 2.2	24.3 ± 1.4	8.1 ± 0.5
DM1 MIRS II ($n = 4$)	2.5 ± 0.2ab	18.9 ± 3	22.3 ± 1.4	7.9 ± 0.7
DM1 MIRS III ($n = 11$)	1.6 ± 0.2b	20.8 ± 2.3	24.5 ± 2.1	8.8 ± 1.0
DM1 MIRS IV ($n = 11$)	1.9 ± 0.2b	20.7 ± 2.2	22.7 ± 1.7	9.0 ± 1.0
DM1 MIRS V ($n = 4$)	2.3 ± 0.3ab	15.7 ± 0.2	26.3 ± 1.2	8.6 ± 1.0
ANOVA	$P < 0.001$	N.S.	N.S.	N.S.

Results are expressed as mean ± SE and tested by one-way ANOVA followed by Tukey's HSD test (different letters (a) or (b) mean statistical significant difference between groups; $(_{ab})$ means no significant difference in comparison with the other groups). N.S. — not significant.

Table 2 Comparison of antioxidant defense in HCs and DM1 patients with normal and increased CK values

	SOD1	CAT	GSH-Px	GR
HCs ($n = 15$)	3.04 ± 0.24a	27 ± 2a	24.3 ± 1.4	8.1 ± 0.5
DM1 patients – CK normal	1.88 ± 0.21b	22.2 ± 2.2ab	22.7 ± 2	9.2 ± 1.2
DM1 patients – CK increased	1.91 ± 0.18b	19.8 ± 1.8b	25.6 ± 1.3	8.4 ± 0.5
ANOVA	$P < 0.001$	$P < 0.05$	N.S.	N.S.

Results are expressed as mean ± SE and tested by one-way ANOVA followed by Tukey's HSD test (different letters (a) or (b) mean statistical significant difference between groups; (ab) means no significant difference in comparison with the other groups). N.S. – not significant.

After segregating the patients according to their CK levels, normal (60–174 IU/l) or increased (>180 IU/l), SOD1 was still statistically lower in both groups of DM1 patients compared to HCs. However, CAT activity was significantly lower only in DM1 patients with increased CK (Table 2). GSH-Px and GR activities were not different compared to HCs.

Inter-correlations between the activities of different antioxidant enzymes and clinical parameters as well as MIRS score, CK levels, and CTG number in DM1 patients are shown in Table 3.

There were no significant correlations between the level of measured antioxidant enzymes and clinical parameters (age of patients, disease duration, age of disease onset), clinical MIRS scores, CK levels, and CTK number, except for GR activity [it was significantly positively correlated with duration of the disease ($\sigma = 0.42$, $P < 0.05$). In contrast, significant positive correlations were found between antioxidant enzyme activities in DM1 patient erythrocytes: CAT versus GSH-Px ($P < 0.001$), CAT versus GR ($P < 0.05$), and GSH-Px versus GR ($P < 0.05$).

As antioxidant enzymes form complex inter-connecting physiological functions, we performed canonical discriminant analysis between different groups of patients and HCs. Canonical discriminant analysis significantly separated DM1 patients with MIRS III ($P < 0.001$) and MIRS IV ($P < 0.01$) scores from HCs. The composition of antioxidant enzymes in MIRS II and MIRS V groups was not significantly different from that in HCs (Fig. 3A).

Furthermore, there were no differences in the composition of antioxidant enzymes in patients with different CK levels (CK normal and CK increased), but both groups of patients (CK normal and CK increased) had a different composition compared to HCs (Fig. 3B). For all comparisons SOD1 activity was most dominant ($P < 0.001$).

Discussion

Our results clearly showed decreased SOD1 activity in DM1 patient erythrocytes compared to HCs. The decrease in SOD1 activity may have been caused by H_2O_2-mediated inhibition,[21] as demonstrated by native PAGE (Fig. 2). After partial inhibition with H_2O_2, SOD1 activity was more reduced in DM1 patients than in HCs. It is well known that H_2O_2 inhibits SOD1 activity. In addition, H_2O_2 directly degrades the enzyme itself.[20] It appears that SOD1 in DM1 patients is more susceptible to this process (Fig. 2). Previous studies have shown increased apoptosis in myogenic cell lines possessing increased number of CTG repeats after administration of methylmercury, a known ROS producer.[22] Advanced oxidation protein products are significantly higher in sera of DM1 patients compared to HCs.[10] These findings are in line with our own results, which suggest that

Table 3 Correlation analysis of different clinical parameters with activity of antioxidant enzymes in DM1 patients ($n = 30$)

	Age of onset	MIRS	CK	Disease duration	Age	Duration/age	CTG number	SOD1	CAT	GSH-Px	GR
Age of onset											
MIRS	−0.06										
CK	−0.30	−0.10									
Disease duration	−0.32	0.44*	0.09								
Age	0.60**	0.32	−0.18	0.57**							
Duration/age	−0.64***	0.36	0.14	0.88***	0.18						
CTG number	−0.42	0.06	0.17	−0.07	−0.40	0.21					
SOD1	0.15	−0.07	0.18	0.15	0.25	−0.01	−0.31				
CAT	−0.18	−0.04	0.12	0.25	0.07	0.21	0.36	0.19			
GSH-Px	0.18	0.05	0.17	0.14	0.27	−0.05	0.27	0.34	0.71***		
GR	−0.04	0.08	−0.16	0.42*	0.01	0.31	0.17	−0.01	0.39*	0.47*	

Pearson correlation coefficients are shown.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

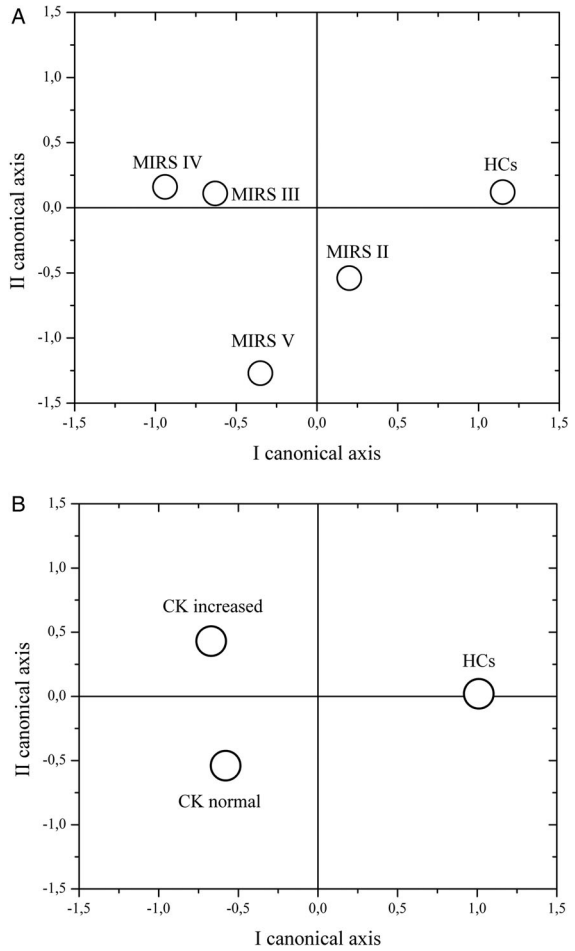

Figure 3. Canonical discriminant analysis of the activity of antioxidant enzymes in different forms of DM1 according to (A) different severity of MIRS and (B) CK levels, presented as two-dimensional canonical space. Canonical analysis significantly separated DM1 patients with MIRS III and IV stages from HCs and the other two stages ($P < 0.001$). DM1 patients were significantly separated from HCs regardless if CK levels were increased ($P < 0.001$).

there is a certain degree of disturbance in the activity of antioxidant enzymes in DM1 patients. Decreased levels of antioxidant enzymes in blood samples from DM1 patients, including decreased SOD, have also been reported.[23] In contrast, earlier reports showed increased SOD1 activity.[5] These latter activities were measured in blood serum and could have been a reflection of erythrocyte lysis, or other interfering substances. In our current study, decreased SOD1 activity was measured in erythrocytes. This finding could represent a suppressive reaction to increased oxidative stress during a particular phase of the disease, since SOD1 activity from DM1 patients are more susceptible to H_2O_2-mediated inhibition. Additionally, in our cohort of patients, decreased SOD1 activity was particularly pronounced in patients with moderate muscular impairment severity (MIRS grades III and IV). This was confirmed by canonical discriminant analysis, as DM1 patients with MIRS III and IV scores were significantly separated from other groups when taking into account the composition of antioxidant enzymes in erythrocytes. These results also demonstrated that oxidative stress in DM1 patients is dynamic and changeable during the disease stages. Furthermore, the activity of antioxidant enzymes in erythrocytes did not correlate with MIRS score suggesting that oxidative stress and involvement of particular ROS is stage specific and does not linearly follow disease progression. Moreover, the activity of antioxidant enzymes in erythrocytes was not correlated with either CK level nor CTG number, although SOD1 and CAT activities were lower in DM1 patients regardless if CK levels were elevated (our results showed that CAT activity was significantly decreased only in patients with increased CK, but the significance was slightly below the confidence limit, $P < 0.05$). It has been shown that CK concentration is often, but not always, elevated in muscle disease.[24] Our results suggest that there is an association between muscle deterioration, increased CK levels and CAT inhibition in erythrocytes.

The effect of oxidative stress on muscle damage has been shown to be present in muscular diseases such as dystrophinopathy,[11] mitochondrial disorders[25] and in sarcopenia, which is age-related muscle loss.[5] Normal muscle tissue responds to the stress of contractions by up-regulating protective enzymes (including SOD1 and CAT) and stress proteins.[5,26] The primary reactive species generated by muscle, both at rest and during contraction, are $NO^{\cdot}$ and $O_2^{\cdot -}$.[27] Small increases in reactive species are favorable and lead to increased force production. Greater production of reactive species contributes to the development of muscle fatigue.[28] The predominant effects of ROS and $NO\cdot$ on muscle force production are due to changes in calcium metabolism influenced by the ryanodine receptor and sarcoplasmic reticulum Ca^{2+} ATPase-SERCA.[29] Interestingly, both mRNAs of these proteins are mis-spliced in DM.[30]

Since the above-mentioned mechanisms lead to aging in normal muscle tissue, they are also probably related to progeroid processes in DM1.[4] In line with this, there is evidence that increased cellular $O_2^{\cdot -}$ can cause muscle ageing since mice lacking SOD1 exhibited accelerated age-related loss of skeletal muscle mass and function.[31] At present treatment for DM1 is limited to symptomatic intervention and there is no therapeutic approach to prevent or reverse disease progression.[32] As oxidative stress plays a role in the progression of neurodegeneration and muscle wasting,[33] there is a possibility that supplemented antioxidant therapy could be beneficial, particularly at intermediate stages of DM1, when decreased SOD 1 is present.

In physiological settings erythrocytes exhibit a self-sustaining activity of antioxidant defense enzymes

and their coordinated actions protect the erythrocytes' bio-macromolecules from free radical-mediated damage. This is the case in this current study, as positive correlation was found between CAT, GSH-Px, and GR activities in erythrocytes suggesting that erythrocytes adjust their antioxidant potential according to the presence of oxidative pressure especially by H_2O_2. However, we found that there was no significant correlation between the activity of the antioxidant enzymes and the clinical outcome. Therefore, the measurement of the activity of these enzymes cannot be exploited for diagnostics, but instead suggests that oxidative processes occur in DM1 patients providing new insight into the disease.

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Disclaimer statements

Contributors All authors contributed equally.

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Conflicts of interest The authors state that there are no conflicts of interest regarding the publication of this article.

Ethics approval The study was approved by the Ethical Board of the Neurology Clinic, University of Medical Sciences, Belgrade, No29/X-5.

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