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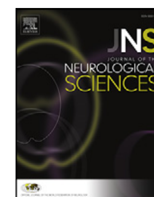

Erythrocytes' antioxidative capacity as a potential marker of oxidative stress intensity in neuroinflammation

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ABSTRACT

The study is designed to assess the oxidative stress intensity in erythrocytes obtained from patients in different clinical phenotypes of neuroinflammation, defined as clinically isolated syndrome (CIS) and relapsing–remitting multiple sclerosis (RRMS). Advanced oxidation protein products (AOPP), malondialdehyde (MDA) and superoxide dismutase (SOD) activity were measured and compared with patients' clinical severity (expanded disability status scale—EDSS), radiological findings (gadolinium enhancement lesion volume—Gd+) and disease duration (DD).

AOPP, MDA values and SOD activity were significantly higher in both study patients than in the control group ($p < 0.05$). While AOPP and MDA approached higher values in RRMS, compared to the CIS group ($p > 0.05$, $p < 0.05$, respectively), SOD activity showed higher values in CIS than in RRMS patients ($p < 0.05$). Both study patients with higher EDSS, higher number of total radiological lesions and longer DD, had higher AOPP and MDA content ($p < 0.05$, $p > 0.05$). SOD activity was lower in both study patients with higher EDSS, higher number of total radiological lesions and longer DD ($p < 0.05$, $p > 0.05$). There were positive correlations between AOPP and DD and EDSS in CIS patients ($p < 0.01$), and MDA levels and DD, EDSS and Gd+ in CIS, as well as with EDSS in RRMS patients ($p < 0.01$). There were negative correlations between SOD activity and DD and EDSS in both study patients ($p < 0.01$), as well as, between SOD activity and Gd+ in CIS patients ($p < 0.01$).

The measured erythrocytes' biomarkers might represent one of the important biomarkers for the evaluation of the oxidative status of neuroinflammation and disease severity, especially in its early phase, defined as CIS.
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1. Introduction

An imbalance in the oxidative/antioxidative status leads to oxidative stress, which is revealed as the main contributor involved in a number of diseases including neuroinflammation and its mediated disease such as multiple sclerosis (MS) [1]. The detrimental effects of oxidative stress are caused by reactive oxygen species (ROS), the inactivation and removal of which depend on the antioxidative defense system, which includes vitamins A, E, and C, beta-carotene, reduced glutathione (GSH), and several antioxidative enzymes, such as superoxide dismutase (SOD) [2]. Erythrocytes, the most abundant cells in blood, are constantly exposed to ROS produced in neuroinflammation during blood circulation. They may be important in regulating oxidant reactions thereby preventing ROS mediated CNS toxicity [3]. Although most studies have shown the different changes of the redox ratio of erythrocytes as a significant parameter for oxidative stress as well as aging [4], and also in some autoimmune diseases [5], rare are those which correlate the named differences with disease pathogenesis i.e. with clinical and paraclinical features by way of different disease stages.

Although MS is defined as an immune mediated disease of CNS, its pathogenesis is not fully understood yet. It has been considered that neuroinflammation is a great contributor of pathogenesis prevailing in the earliest phase of a disease characterized by demyelination, while at a later stage what prevails is mostly oxidative stress which mediates irreversible and neurodegenerative injuries of CNS. The respective roles of overlapping inflammatory and oxidative processes in MS pathogenesis vary over the course of the disease [6]. Most MS patients (85%) experience a relapsing–remitting (RR) course of the disease characterized by a relapse followed by a recovery period. Within years most of them evolve into a secondary progressive (SP) phase characterized by a steady increase in disability. For the most part, MS appears as a clinically isolated syndrome of CNS (CIS) which will be developed in defined MS

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within the time. Only about 15% experience a primary progressive (PP) form defined by the accumulation of disability from the onset of the disease [7].

The present study has been done in order to clarify the possible correlations between imbalances in oxidant and antioxidant defense in erythrocytes, and clinical and paraclinical presentations of neuroinflammatory acute attacks, which were defined as the clinically isolated syndrome of CNS (CIS) and RRMS.

2. Patients and methods

This study was performed as a cross-sectional study. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Nis and informed consent was obtained from each patient prior to entry into the study, according to the Declaration of Helsinki.

2.1. Control patients

Sixty nine (36 male, 33 female) patients, aged 23–55 years and nonsmokers, were involved in the control group (CG). They were admitted at the Clinic for Neurology, Clinical Center Nis, and underwent the complete diagnostic procedure for suspected demyelinating disease, without any objective abnormalities found at laboratory, MRI scan and CSF examination.

2.2. CIS patients

Fifty patients (15 male, 35 female), aged 17–57 years, admitted at the Clinic for Neurology, Clinical Center Nis, presented with an acute or sub-acute attack affecting different brain structures (cerebral hemispheres, brainstem, cerebellum, spinal cord, optic nerve or more than one functional system), which have been suggestive of MS. Neurological findings in details were prescribed in our recent published paper [8]. All clinical, laboratory and MRI investigations were performed less than 3 months after initial neurological presentations. Inclusion in the study was based only on the clinical expression and was not influenced by MRI. Due to exclusion of alternative diagnoses by the appropriate investigations performed, and since they didn't fulfill diagnostic criteria for MS [9], and had no previous history of possible demyelinating events, patients' condition was defined as a CIS.

2.3. RRMS patients

Fifty seven patients (12 male, 45 female), aged 23–58 years, admitted at the Clinic for Neurology, Clinical Center Nis, and presenting with clinically definite MS as two separate attacks disseminated in time and place followed by clinical evidence of two separate lesions [9], were involved in the RRMS group. In all MS patients the disease was verified by clinical, laboratory and neuroimaging approaches. Neurological findings in details were prescribed in our recent published paper [8]. The interval between the previous and the present clinical episode was longer than 6 months. All of the MS defined patients were classified as having a relapsing–remitting (RR) form of MS, according to the Lublin and Reingold criteria [8,10].

The patients with other MS forms or any disease other than MS that would compromise organ function were excluded from the study, as were the patients who had received prior immunosuppressant, interferon or corticosteroid therapy within 6 months of the study entry. All of the patients in both groups, CIS and RRMS, have been non-smokers for at least one year. All CIS and MS patients were categorized according to their age, gender, duration of neurological onset at the admission at the Clinic, disease duration in RRMS patients, frequency of relapses in RRMS patients, affected CNS structure, MRI characteristics and frequency distribution on the clinical scales. Data are presented in Table 1.

Table 1
Demographic data and basic hematologic parameters.

	CG	CIS	RRMS
Number—female/male	69–33/36	50–35/15	57–45/12
Age (years)	37 (23–55)	37.5 (17–57)	40 (23–58)
Disease duration (months)	/	2 (1–3)	84 (1–396)
Er ($\times 10^{12}$/L)	4.79 ± 0.9	4.05 ± 0.75[a]	3.55 ± 0.8[a,b]
Hemoglobin (g/dL)	14 ± 0.85	13.4 ± 1.04[a]	12.7 ± 1.15[a,b]
Hematocrit (%)	42.3 ± 2.1	39.5 ± 4.3	36.5 ± 6.1[a]

Values are presented as medians (range) and means ± SD of healthy subjects, and CIS and RRMS patients. In all statistics calculations Mann Whitney and Kruskal–Wallis tests were done.
[a] $p < 0.05$ CIS and RRMS vs CG.
[b] $p < 0.05$ RRMS vs CIS.

2.4. Clinical assessment

All CIS and RRMS patients' clinical presentations were assessed using Kurtzke's extended disability status scale (EDSS) [11]. Based upon the frequency distribution of EDSS all patients were divided by the median value for EDSS into those which had mild (≤3 for CIS and ≤5 for RRMS patients) and severe (>3 for CIS and >5 for RRMS patients) clinical disability [8].

2.5. MRI assessment

Brain MRI was performed using a 1.5 T system (Avanto, Siemens, Erlangen, Germany). MRI protocol included the following conventional spin echo sequences: axial T1-weighted (repetition time [TR] = 500 ms, echo time [TE] = 78 ms, number of excitations [NEX] = 2) and T2-weighted (TR = 4700 ms, TE = 93 ms, NEX = 2) with 5-mm slice thickness and an intersection gap of 0.5 mm. The pixel size was 0.9 × 0.9 mm. Intravenous gadolinium contrast (Gadovist, Schering, Berlin, Germany) was administered in a dose of 0.1 mmol/kg of body weight. The number of hyperintense lesions seen on T2 images and the lesion load of Gd-enhancing lesions seen on T1-weighted images were calculated. The lesion loads were calculated as volumes. All MRI scans were reported by an experienced neuroradiologist unaware of the clinical findings and the classification of the patients. Considering total T2-weighted lesions (MRItl) all study patients were divided by the median value into those which had mild (≤9 for CIS and ≤40 for RRMS patients) and severe (>9 for CIS and >40 for RRMS patients) MRI changes [8].

2.6. Biochemical assessment

2.6.1. Blood sampling

Venous blood samples were collected into Venoject tubes with EDTA (0.47 mol/L K3-EDTA), from all study subjects after fasting for a duration of at least 12 h. Within 1 h after sampling, the blood samples were centrifuged at 3000 *g* for 10 min at 4 °C to separate plasma and erythrocytes. The buffy coat was removed and the remaining erythrocytes were drawn from the bottom, washed three times in cold saline (9.0 g/L NaCl), and hemolyzed by adding the 9 fold equivalent weight of ice-cold demineralized ultrapure water to yield a 10% hemolysate. The hemolysates were stored in a refrigerator at 4 °C for 15 min and erythrocyte membranes were removed and then hemolysates were frozen in aliquots at −80 °C for later analysis.

2.6.2. Determination of hematological parameters

Erythrocyte count, and hematocrit (Hct), hemoglobin (Hb) and other hematological indexes were analyzed by an electronic hospital auto-analyzer, the automated Cell Counter, MEK-4100, Nihon Kohden, Japan. The hemoglobin concentration in lysates was determined with the aid of Drabkin's reagent [12].

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