Full Length Research Paper

The concentration of cGMP in serum and clinical activity of multiple sclerosis

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Multiple sclerosis (MS) is a demyelinating disease of the central nervous system of unknown etiology. The processes of demyelination accompanied by a local inflammatory process, which is the result of an autoimmune response to myelin antigens. The aim of this study was to analyze the levels of nitric oxide and cGMP in the serum of patients with multiple sclerosis (MS) during the relapse and remission. The study involved 27persons, including 11 patients (K7 / M4) during the MS relapse and 11 (K7 / M4) patients with MS in remission, and 7 normal subjects (5K / 2M). The concentration of cGMP and immunochemical method (ELISA) and the concentration of nitrite by Griess. The results showed higher levels of cGMP and nitrite in the serum of MS patients at relapse when compared to a control group and a group of MS patients in remission. Significant deterioration of neurological condition is a plan view of the disease evaluated by using the EDSS in patients with MS. The higher concentration of NO and cGMP levels in plan correlated with worse neurological status of patients with MS. The results suggest that nitric oxide and cGMP mediated MS causing neurologic deterioration of MS patients but not the main factors responsible for the changes taking place in the brain of MS.

Keywords: multiple sclerosis, cGMP, nitrogen oxide, EDSS

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease that is characterized by the loss of myelin, mainly in the central nervous system. Demyelination is accompanied by a chronic inflammatory process, which is based on the infiltration of T lymphocytes, blood macrophage recruitment, and local microglia activation (Ranshoff et al., 1991). As a consequence, an impairment of axonal conduction occurs, resulting in neurological deficits and progression of disability (Bjartmar et al., 2003; Fassas et al., 2004). The disease most commonly occurs in people aged 20-40 and has a relapsing-remitting form. The severity of demyelination and inflammation depends, inter alia, on the balance between Th1 and Th2 cells, which produce pro-inflammatory and anti-inflammatory cytokines (Conti-Fine et al. 2006; Ekholm et al. 1997; Skapenko et al., 2005; Weiner et al., 2009) and antioxidative system disorders (Ljubisavljevic et al., 2012). Activation of microglia and astrocytes then occurs, leading to an increased concentration of pro-inflammatory cytokines in the brain, which has been observed in a rat MS model with autoimmune

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encephalomyelitis (EAE) (Sulkowski et al., 2013). An increase of pro-inflammatory cytokines causes nitric oxide synthase (iNOS) induction and increases the levels of nitric oxide (NO) in macrophages, microglia cells, and astrocytes (Giulivi et al., 2003). Studies carried out by Shen, *et al.* have shown that the INF-γ, IL-1β, and TNF-α cause induction of iNOS expression in astrocytes (Shen et al. 2005). Excess NO may react with the superoxide radical ($O_2^{\bullet-}$) to form the peroxynitrate anion (ONOO-), which quickly disintegrates into a noxious, highly reactive hydroxyl radical (Beckman et al., 1994). Oxygen radicals and NO can have a strong cytotoxic effect on nerve cells through the inhibition of mitochondrial respiration (Kozubowski et al., 2004). It seems that NO may play a role in the pathogenesis of MS through its impact on demyelination and the formation of glial scars (Prinz et al., 2012). The direct impact of NO on increased permeability of the blood-brain barrier and the excitation and control of the inflammatory process (Giulivi et al., 2003) has been shown.

NO may participate in neurodegeneration of oligodendrocytes as well as causing functional and structural damage to the axons (Tiberio et al., 2005; Acar et al., 2003). However, Bo, *et al.* (1994) have shown that cGMP, which is synthesized by guanylyl cyclase (CG) activated by NO, accelerates the remyelination process (Bo et al., 1994). The administration of sildenafil, a phosphodiesterase type 5 inhibitor (PDE5 hydrolyzing cGMP), sidnafil, to rats improved their neurological condition and accelerated the process of remyelination (Pifarre et al. 2011, Polman et al. 2011). It was found that cGMP, through the activation of kinase cGMP-dependent (PKG), may participate in the rebuilding process of the myelin, but the mechanism is still unclear. Unfortunately, the literature shows controversies relating both to the role of NO and cGMP in the process of demyelination – remyelination (Bo et al., 1994; Tran et al., 1997; Nazliel et al., 2002, Pifarre et al. 2011). As has been previously demonstrated, NO and cGMP may show opposite effects (protective or neurotoxic) depending on their concentration. Maintaining physiological concentrations of cGMP is very important, since cGMP regulates many biochemical processes directly or indirectly through cGMP-dependent kinase. The aim of this study was to investigate the concentration of nitric oxide and cGMP in the serum of patients with multiple sclerosis (MS) at the time of relapse and remission.

MATERIALS AND METHODS

Patients with MS and controls

This study was conducted with a group of 29 people, including 22 MS patients and 7 healthy individuals. In the MS group, there were 14 women and 8 men. The diagnosis was established on the basis of the McDonald criteria of 2011 (Polman et al., 2011) and the exclusion criteria. Eleven patients were in disease relapse and 11 were in remission. This study did not include persons who were, in addition to the suspected MS, affected by other chronic diseases such as depression, liver, kidney, thyroid diseases, and abnormalities in blood morphology and biochemical tests (exclusion criteria). The group was homogeneous in terms of age, duration of disease, incidence of relapse, and remission and disability as assessed on the EDSS scale (Kurtzke's Explanded Disabilling Status Scale, EDSS). Those affected by the disease and the control group was of similar age (table 1). The age of patients during the disease remission phase averaged 31 ± 3.8, and those in the phase of relapse averaged 32 ± 4.6. In the control group, there were 7 healthy individuals (5 women and 2 men) with an average age of 23 ± 1.9. The relapsed patients had suffered from multiple sclerosis for an average of 3.8 years ± 1.8, and those in the remission phase, for 3.5 years ± 1.7 years (tab. 1). All patients with MS had undergone immunomodulation therapy with beta interferon. Patients with MS were administered, subcutaneously, every second day, IFN-â1b (Betaferon®, Bayer Schering Pharma, Berlin, Germany) at a dose of 250 µg. The relapsed patients came to follow-up appointments in order to be administered Solu-Medrolem and blood collection. All relapses were treated intravenously with Solu-Medrolem at a dose of 1 g/day for 3-5 days (Solu-Medrol, Pfizer, Switzerland). The participants of the study expressed their written consent to participate in the project. The study protocol was approved by the Bioethical Commission of the Military Medical Institute (Approval No. 34/WIM/2009).

The results are presented as mean values ± SEM, n = 11 for the MS relapse group, n = 11 for the MS remission group, n = 7 for the control group.

Results are presented as mean ± SEM of n = 11 for group MS relapse, n = 11 for MS remission group, n = 7 for the control group.

Table 1. Characteristics of patients with MS and the control group

	Age [years]	Years of illness	Number of persons (sex m/f)
Control	23 ± 1.9	-	7 (2/5)
MS relapse	32 ± 4.6	3.8 ± 1.8	11 (4/7)
MS remission	31 ± 3.8	3.5 ± 1.7	11 (4/7)

Serum collection

Blood for testing was collected while fasting between 8:00 and 10:00 in the morning. Patients with MS took the last dose of medication at 20:00 on the previous day. Serum was obtained from the blood. After clotting at room temperature (30 minutes), the blood samples were

vortexed at 1500g for 15 min. The serum was stored at -70ºC until the measurement of cGMP and nitrate concentration could be performed.

Determination of cGMP concentration

The concentration of cGMP was determined using an immunochemical method (ELISA) based on the competition between unmarked cGMP and cGMP marked with acetylcholinesterase to a certain number of specific spaces in the antibody against cGMP. To determine the concentration of cGMP, a commercial ELISA kit by Cayman Chemical Company was used (cat. no. 581 021, Ann Arbor, MI, USA) according to manufacturer's instructions.

Determination of nitrate concentration

The nitrate concentration (nitrite, NO_2^-), measured as the exponent of the concentration of serum nitric oxide (NO), was marked using the Griess spectrophotometric method (Li et al., 1999).

Statistical analysis

The results were presented as an average value ± standard error of the mean (SEM) calculated from individual measurements assumed as the final result. The statistical analysis of the results was performed in Statistica 7.0. To determine the differences in values of clinical data: EDSS scale, duration of disease, age, concentration of cGMP, and nitrate in serum, the Krus-Willis non-parametric test was used. Dependencies between the groups were tested using the U Mann-Whitney test. The results were considered statistically significant at the significance level $p < 0.05$. The correlation of phenomena was developed by calculating the "r" Person's correlation coefficients, which are an expression of the strength of linearity in the correlations between the two variables. To be considered statistically significant, the values at $p < 0.05$ were assumed.

RESULTS

The concentration of cGMP was determined at the time of relapse and remission in patients with MS and healthy individuals (control group; figure 1). The concentration of cGMP in the serum was 39.1 ± 6.6 pm/ml during relapse, 3.8 ± 1.0 pmol/ml during Re mission, and 14 ± 2.4 pmol/ml in healthy individuals (figure 1). Statistical analysis showed that the concentration of cGMP levels was significantly higher in patients during relapse compared to either those in the control group or patients with MS in remission (MS group - remission) ($p < 0.001$). In addition, the concentration of cGMP in the serum of patients during remission was significantly lower
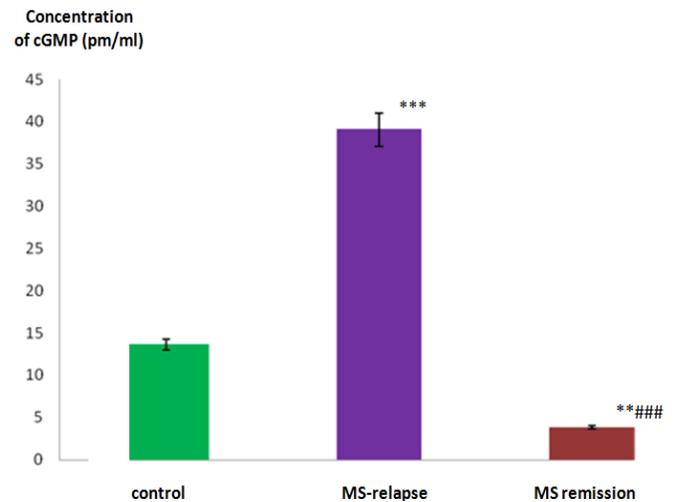
Figure 1. The concentration of cGMP in the serum of MS patients during the relapse, remission and healthy volunteers (control group)
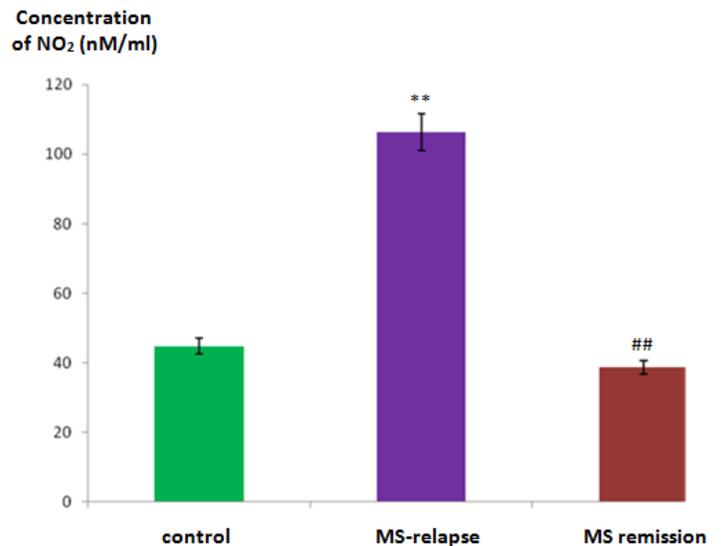

Figure 2. The NO2- concentration in the serum of MS patients with relapse and remission and in healthy (control group)

compared to the control group ($p < 0.01$; figure 1).

The level of cGMP were determined by ELISA. Results are presented as mean ± SEM of n = 11 for group SM a, n = 11 for MS remission group, n = 7 for the control group independent measurements. ** $p < 0.01$, *** $p < 0.001$ compared to control, ### $p < 0.001$ compared to group the SM-remission.

The value of NO_2^- concentration in the serum was 106.3 ± 19.4 nmol/ml during relapse, 38.8 ± 4.4 nmol/ml during remission, and 44.8 ± 2.8 nmol/ml in the control group,(figure 2). Statistical analysis showed that the serum concentration of NO_2^- was higher in patients with MS during relapse compared to the control and also to the MS group in remission ($p < 0.001$, figure 2).

Evaluation of neurological status (EDSS scale)
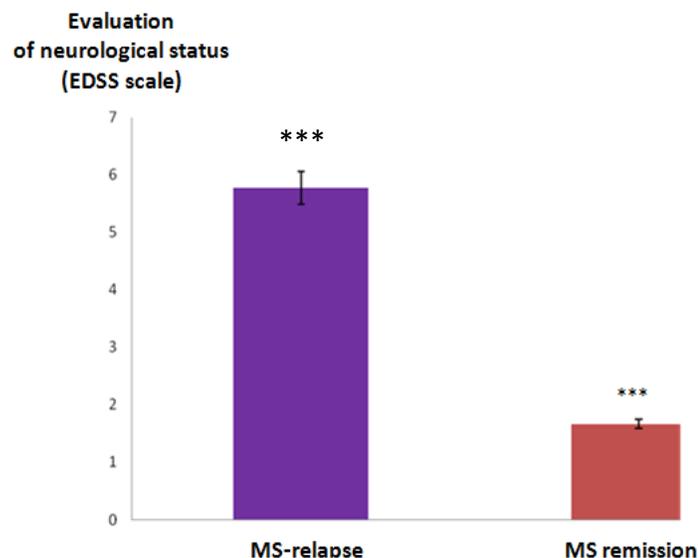
Figure 3. Evaluation of neurological status (EDSS scale) patients with relapse and remission of multiple sclerosis

The concentration of NO2- were determined by the Griess. Results are presented as mean ± SEM of n = 11 for group SM a, n = 11 for MS remission group, n = 7 for the control group independent measurements. ** p <0.01w compared to the control, ## p <0.01 in comparison to MS-relapse.

In both studied groups, MS patients showed an impaired neurological condition, both at the time of relapse and remission. During relapse, significant deterioration in the neurological condition was observed (figure 3). During disease relapse, a deterioration of the neurological condition was observed, which was manifested by a higher degree of disability as was assessed according to the EDSS scale at 5.8 ± 1.4 points than in patients who were in the remission phase. However, in patients undergoing remission, an improvement of the neurological condition was observed compared to the MS-relapse group (p<0.001; Figure 3), but no total improvement was observed. Neurological condition was assessed in these patients using the EDSS scale at 1.7 ± 0.7 points (Figure 3).

The assessment of the neurological condition is described using the EDSS (Ch. I.1.4 and annex 1). Results are presented as mean ± SEM of n = 11 for group SM a, n = 11 for group SM remission. *** p <0.001 for relaspe and *** P <0.001 for remission

The results showed a correlation between the neurological state as was evaluated using the EDSS scale and the concentration of cGMP (r=0.790, p<0.001) or nitrite (r=0.682, p<0.001) in the serum of MS patients during the relapse and remission phases. As the concentration of cGMP increased in MS patients, a deterioration of the neurological condition was observed.

DISCUSSION

The results of the conducted studies indicated the presence of higher concentrations of cGMP in the serum of MS patients during the relapse phase compared to healthy individuals and patients during remission. The changes in cGMP concentration were accompanied by an increase in the concentration of nitrites (the exponent of the NO concentration) in the serum of relapsed patients. Higher concentrations of nitrite and cGMP in the serum during the relapse correlated with aggravated disability as evaluated by the EDSS scale.

The demyelination processes in MS are accompanied by inflammation, resulting from the activation of CNS cells (astrocytes and microglia) as well as macrophages, monocytes, and lymphocytes. At the time of activation, the cells release various pro-inflammatory factors such as cytokines, and free radicals, such as ROS, both in the brain, blood, and cerebrospinal fluid (Brosnan et al. 1996; Gołąb et al., 2012). The released pro-inflammatory factors may cause the activation of pro-inflammatory enzymes such as induced nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), and thereby activate the demyelination process (Raine et al., 1997). This leads to a deceleration in neural conductivity and a degradation of the myelin sheaths [(Polman et al., 2011). Numerous studies suggest that NO plays an important role in MS (Nazliel et al., 2002; Miljkovic et al., 2002). Maciejek, *et al.* (1985) demonstrated that, in the initial stage of the disease, cGMP concentrations increased compared to the control group.. However, in the later course of the disease, during the development of the various clinical forms of MS, cGMP concentrations returned to the control values (Maciejek et al., 1985). One of the main receptors of NO is soluble guanylyl cyclase (sGC), and its activation causes an increase in cGMP in neurons.

Cyclic GMP can be synthesized by the endothelial cells of blood vessels, blood cells, and other peripheral tissues, or it can be removed from the CNS completely (Sager et al., 2004). Throughout these processes, cGMP is released into to blood. It is known that an increase in the concentration of cGMP in the cortex of the brain occurs during the relapse of EAE. An increase of cGMP in the cortex was caused by both the activation of the soluble and mostly membrane CG. Studies have shown that iNOS expression occurs in the cerebral cortex of rats with EAE (Sulkowski et al., 2013). Previous studies have demonstrated an increased reactivity of iNOS protein and the level of mRNA in the areas of demyelination (deposits) in the cerebral tissue in patients (Bagasra et al., 1995). The expression of iNOS in the areas of demyelinating lesions in the brain in patients with MS may be caused by an increase in the release of pro-inflammatory cytokines: TNF-α, IFN-γ, and IL-□□ in astrocytes and microglia (Gołąb et al., 2002). *In vitro* studies have indicated that the joint effect of pro-inflammatory cytokines TNF-α, IL-1□, and IFN-γ induces

iNOS (Xu et al., 2003, Shen et al., 2005). Pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) cause the activation of the NF-kB factor (nuclear factor kappa-light-chain-enhancer of activated B cells) and translocates to the nucleus, resulting in the expression of iNOS in macrophages, microglia, and astrocytes (Shen et al., 2005).

However, controversies are present in the literature relating both to the role of NO and cGMP in the process of demyelination and remyelination (Bo et al., 1994; Tran et al., 1997; Nazliel et al., 2002). In their research, Nazliel, *et al.* (2002) have shown that the concentration of NO_2^- and NO_3^- in the serum was higher in the MS group compared to the control group, which correlated strongly with the disease activity irrespective of the sex of the patient (Nazliel et al., 2002). Similarly, Svenningsson, *et al.* (1999) demonstrated changes in the concentration of nitric oxide metabolites in the cerebrospinal fluid in MS patients depending on the clinical form of the disease (Svenningsson et al., 1999). In relapsed patients, increased levels of nitrites in the cerebrospinal fluid were seen when compared to patients during remission and in healthy individuals (the control group) (Svenningsson et al., 1999). Markianos and Sfagos (1988) demonstrated a negative correlation between the concentration of cGMP and the degree of disability according to the EDSS scale (Markianoset al., 1988). Nazliel, *et al.* (2002) did not observe a correlation between the degree of disability according to the EDSS scale and the average duration of the disease, as well as the level of NO_2^- i NO_3^- (Nazliel et al., 2002).

A positive correlation between NO and cGMP concentrations in the serum of MS patients indicates that cGMP may not be a good marker for the measurement of NO. The measurement of nitrites in the blood may be flawed due to the impact of a diet rich in nitrates. Our results indicate that there is a positive correlation between the concentration of cGMP and the concentration of nitrites in the serum of MS patients. Changes in the NO (nitrites) concentration reflect the changes in the concentration of cGMP in the serum, which suggests that the increase in cGMP is dependent on NO and the activation of NOS isoforms in MS.

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