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Nitric oxide as an activity marker in multiple sclerosis

Abstract Nitric oxide (NO) molecules have one of the most important roles in the pathogenesis of multiple sclerosis (MS). It has been stated that a continuous and high concentration of NO metabolites in CSF and in the serum of MS patients in relapse may cause toxic damage to myelin and oligodendroglia. The aim of this study was to investigate whether NO is a marker of disease activity and is correlated with other disease activity markers such as active lesions on brain magnetic resonance imaging (MRI) and increased immunoglobulin G (IgG) index.

Cerebrospinal fluid (CSF) and peripheral serum (PS) samples were taken from patients with definite MS (n = 24) during relapse and remission and from control subjects (n = 18). The Griess reaction was used to measure the NO metabolites, nitrite and nitrate in CSF and PS. Cranial MRI was carried out with triple dose (0,3 mmol/kg) gadolinium and the IgG index was determined.

Nitrite and nitrate concentrations (NNCs) of CSF were 11.16 ± 8.60 μmol/ml in relapse and 6.72 ± 3.50 μmol/ml in remission, whereas in PS they were 12.89 ± 7.62 μmol/ml during relapse and 12.35 ± 6.62 μmol/ml during remission. In control subjects NNCs in CSF and PS were 7.42 ± 2.81 μmol/ml and 4.37 ± 1.63 μmol/ml respectively. NNCs in CSF during relapse period were significantly higher than those of both remission phase and control subjects ($p = 0.000$). Although serum NNCs did not differ in relapse and remission, they were still higher than normal controls. Validity analysis revealed that NNC measurement in CSF was 71 % specific and 66 % sensitive to disease activity. The most important result was the significant correlation of increased NNCs with the existence of active lesion in cranial MRI and an increase in IgG index ($p < 0.05$).

In conclusion, these results add background data to assist in further outlining the possible role of NO in the pathogenesis of MS. Together with the other markers it may be used as an activity marker in relapses of MS.

Key words multiple sclerosis · activity marker · nitric oxide

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Introduction

Multiple sclerosis (MS) is the most common chronic demyelinating disease of the central nervous system (CNS) characterized by chronic inflammation of the white matter. Although not a single etiological factor has been identified, there is evidence of an important role of autoimmunity in the pathogenesis of MS [23, 26]. Monocytes and macrophages play key roles during the demyelination process in MS. Following the infiltration of monocytes, macrophages and T lymphocytes into the brain parenchyma via a defective blood-brain barrier, endogenous glia are activated and subsequently myelin

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and oligodendroglia are damaged. Among other freely diffusing molecules produced by activated immune cells and microglia, nitric oxide (NO) is one of the major molecules causing this damage [1, 2, 6, 19].

Nitric oxide is a free radical gas which acts as an important mediator/messenger in neuroprotection, neurotransmission, memory, and synaptic plasticity under physiological conditions [22]. However, it has been reported that high amounts of NO have antiproliferative and/or cytotoxic effects in rat oligodendrocyte cultures leading to damage [4]. Also, in an experimental model the inhibition of inducible nitric oxide synthase (iNOS) expression and NO production prevented development of allergic encephalomyelitis [5]. These reports indicate the neurotoxicity of excessive amounts of NO in MS [22]. In particular lysis of oligodendrocytes by cytotoxic T lymphocytes leading to demyelination can be the result of NO secreted by monocytes, macrophages and microglia. This indicates the modulatory action of NO on T cell function [18].

NO has a short half-life and is rapidly converted to more reactive intermediates such as nitrite and nitrate reflecting *in vivo* production of NO [19]. In a broad range of human diseases and clinical disorders these reactive NO intermediates are elevated [22]. In MS patients in relapse nitrite and nitrate are found to be higher in cerebrospinal fluid (CSF) than healthy controls [7–9, 13, 28]. In none of the previous studies was the validity of NNC measurement reported and its correlation with other disease activity markers was not clear. The correlation between the increase in NNCs during relapse with other activity markers is vitally important in understanding the obscure steps in MS pathogenesis.

In this study the aim was to investigate whether NNCs in the CSF of MS patients are correlated with the activity of the disease and with the other activity markers and also to validate nitrite and nitrate measurements in the CSF of patients having relapsing remitting MS (RRMS).

Materials and methods

Patients

Twenty-four patients who were diagnosed as definite MS according to McDonald's criteria [17] were included in the study. The inclusion criteria were patients having had two or more relapses in the last two years, presenting with relapse to the clinic and admitted to the hospital within 4 weeks after the initial symptom. Also the patients were not receiving any immunosuppressive or immunomodulator drug.

Eighteen age and sex matched control patients were selected among the patients who were awaiting surgery with spinal anesthesia for benign orthopedic conditions who had no history of neurological disease.

All of the subjects were informed about the study protocol and gave their consent.

Clinical evaluation and collection of the material

MS patients were admitted within 2–30 (mean 10.65 ± 8.36) days from the beginning of the symptoms. The neurological examination was scored according to the Expanded Disability Status Scale (EDSS) [15]. After collecting CSF (5 ml) and PS samples (5 ml) by lumbar and venipuncture on the day of admission, the treatment protocol for the presenting relapse was started with 1 g/day i. v. methylprednisolone for five days and the oral steroid dose was tapered. Three months later when the patients were in remission neurological evaluation and lumbar and venous punctures were repeated.

In control subjects a detailed history was taken and neurological examination was performed in order to eliminate any patient with neurological dysfunction. In the operation room CSF (5 ml) and PS samples (5 ml) were collected before spinal anesthesia.

Biochemical analysis

Determination of nitrite and nitrate concentrations

Since NO has a very short half-life, in solution it is rapidly oxidized by O_2 forming reactive nitric oxide derivatives such as nitrite and nitrate. Therefore nitrite and nitrate concentrations were measured which provided an indirect and useful estimation of NO concentrations in CSF and PS samples [27]. The Griess reaction was used to determine the concentrations of these two derivatives. The total amount of nitrite present in the experimental milieu is measured spectrophotometrically and expressed in μmol/ml.

Immunglobulin G index

Albumin and IgG were measured by automated immunoprecipitation nephelometry. The IgG index was calculated according to the standard formula and the values greater than or equal to 0.7 were considered to reflect abnormal intrathecal IgG synthesis which is a marker of disease activity in MS.

Magnetic resonance imaging (MRI)

Brain MRI was performed on MS patients on the day of admission during relapse period and 3 months later during remission. A Siemens Magnetom 42SP with 1 Tesla magnetic field was used to perform T2 weighted axial and sagittal sections and T1 weighted axial and sagittal sections after i. v. gadolinium (Gd) injection. In order to increase the sensitivity of MRI in imaging the demyelinated plaques triple dose (0.3 mmol/kg) of Gd was administered. The hyperintense lesions in T2 weighted images and contrast enhancing (active) lesions in T1 images following a triple dose Gd injection were counted for each patient during relapse and remission phases. The stable active lesions which were also active in relapse and newly formed active lesions were taken into consideration when counting during the remission.

Statistical analysis

In the healthy controls the mean value of NNC was calculated for CSF and serum. Normal values were obtained for both. For the sake of the reliability of the analysis 2 standard deviations (SD) were added to the mean NNC values of both normal CSF and serum. In this way two threshold values were obtained for both CSF and serum measurements. The concentrations above these values were accepted as abnormal values.

Non-parametric Wilcoxon, Mann-Whitney U, validity analysis and non-parametric correlation tests were applied in the analysis of the data.

Results

Patients

The mean age of the 24 patients (9 male, 15 female) was 30.2 ± 8.3 years (range 18–46) and median disease duration was 5 years (range 1–27). All of the patients were admitted with relapse and mean EDSS score was 2.8 ± 0.8 at admission. Three months later the patients were evaluated and mean EDSS score was 1 ± 0.9 during remission. The mean age of the 18 (8 males, 10 females) control patients was 32 ± 2.34 years.

Biochemical analysis

Nitrite and nitrate concentrations in CSF and PS

The threshold value for NNCs in CSF was 7.4 µmol/ml and the measurements above this value (15/24 patients) were accepted as abnormal. During relapse NNCs in CSF of MS patients were significantly higher than those of control patients ($p = 0.000$, Table 1). The NNCs were significantly decreased in remission, though they did not reach the control levels ($p = 0.000$, Table 1). In addition to this, NNC measurement in CSF was 71% sensitive, while 66% specific in the reflection of disease activity.

The threshold value for NNCs in PS was 13 µmol/ml and 11/24 patients were above this value. Although the NNCs in PS of patients with MS did not show any difference in both relapse and remission periods ($p = 0.53$), it was still significantly higher when compared with the concentrations in control subjects ($p = 0.000$, Table 1). The calculated sensitivity was 57% and the specificity was 41%.

CSF/PS NO ratio was 0.82 in relapse, 0.57 in remission and 0.59 in healthy controls. The statistical significance between relapse-remission and relapse-healthy controls was also preserved when the ratios are taken into consideration ($p < 0.001$).

Table 1 NNCs of the healthy controls and MS patients during relapse and remission

	n	Mean NNC (µmol/ml)
Relapse CSF	24	11.16 ± 8.60*
Remission CSF	24	6.72 ± 3.50*
Relapse Serum	24	12.89 ± 7.62*
Remission Serum	24	12.35 ± 6.62*
Control CSF	18	4.32 ± 1.63
Control Serum	18	7.42 ± 2.81

* higher than control subjects $p = 0.000$

Immunglobulin G index

The mean value of IgG index in relapse and remission was 0.88 ± 0.37 and 0.67 ± 0.28, respectively. The difference between relapse and remission values was significant ($p < 0.005$). There was also a significant correlation between high IgG index and high NNCs during relapse period (Pearson correlation = 0.44, $p < 0.05$). In 15 of the 24 MS patients both IgG index and NNCs were increased in relapse.

Magnetic resonance imaging

Table 2 shows the number of the total lesions in T2 weighted images and contrast enhanced lesions in T1 weighted images in relapse and remission. There was no significant difference between relapse and remission periods regarding the number of non-contrast enhancing lesions. The number of contrast enhancing lesions was significantly higher in relapse than in remission period ($p = 0.000$). The 14 of 19 patients who demonstrated active lesions in brain MRI had NNCs above 7.5 µmol/ml during the relapse period (Table 3). Analysis of these results led to a significant correlation between high NNCs and contrast enhancement in the lesions (Pearson correlation = 0.45, $p < 0.05$). Eleven of these 14 patients who had both active lesions and high NNCs were admitted within 10 days after the initial symptom. The rest of the patients were admitted 10–30 days after the initial symptom.

Stable active and newly formed active lesions were detected in three (3/24) patients who had increased NNCs both in relapse and remission. There was a positive and mild correlation between increased NNCs and active lesion existence in brain MRI during remission, but it was insignificant (Pearson correlation = 0.33, $p = 0.16$).

Table 2 The mean value of the brain MRI lesions during relapse and remission

	Number of total lesions (T2-weighted images)	Number of Gd (+) lesions (T1-weighted images)
Relapse	19.66 ± 16.63	14.12 ± 4.47*
Remission	19.58 ± 16.53	0.54 ± 1.31

Gd (–): non-contrast enhancing; Gd (+): contrast enhancing (significant from remission $p = 0.000$)

Table 3 In this table the Gd enhancement and the state of NNCs are presented for the patients who were in relapse period

	High NNC	Low NNC
Gd enhancement (+) (n)	14	5
Gd enhancement (–) (n)	1	4

Discussion

In this study, we found very high NNCs in CSF and PS of MS patients during both relapse and remission periods. This implies a continuous immunological activity leading to high NO production which increases during relapse and subsides in remission periods. The immunological activation causing excessive NO production can either take place in the periphery or in the central nervous system and may be due to a diffuse or local stimulation of iNOS [22]. The high NNCs of serum both in relapse and remission suggests the continuous immunological activation in the periphery; however, it can be suggested that this activation is reflected in the central nervous system during the relapse period due to blood brain barrier damage. Giovannoni et al. have also reported compatible results in relapsing-remitting patients displaying elevated serum NNCs during remission [7]. The systemic monocyte/macrophage activation could be a logical explanation for this, because increased iNOS expression has been reported in these cells during immunological activation [20, 24]. Therefore, this study is important in providing additional data to show that the triggering mechanism in MS is not restricted only to the central nervous system.

The correlation between NO metabolites and other activity markers has been little emphasized in previous studies [7–9, 13, 28]. Therefore, the significant correlation between the high levels of IgG index and NNCs is an important finding, suggesting that NNC measurements could be a valuable marker of disease activity in MS. Moreover, administration of the triple dose Gd MRI technique which provides more sensitive determination of the active lesions gives this study unique features. The positive significant correlation of active lesion numbers in brain MRI and high concentrations of NO metabolites in CSF supports the suggestion that NO metabolites are a disease activity marker. Moreover, the specificity and sensitivity (71% and 66% respectively) of high NO metabolites in CSF during relapse supports this suggestion. In addition, the positive but statistically insignificant correlation of the high NNCs and active lesion existence during remission may also be considered an important finding to show that there is disease activity in remission phase.

Among several studies to clarify the pathogenesis of MS, the reports which focus on the elevated concentration of NO metabolites during the relapse periods are the most striking ones [5, 7–9, 25, 28]. However, not all of the findings agree with each other.

For instance, it has been reported that the serum NNCs were increased in three groups of patients with demyelinating disease, AIDS and inflammatory neurological disease, when compared with normal control subjects and patients with non-inflammatory neurological diseases. NO metabolites are also found to be increased in all subtypes of MS including isolated syndromes, but this was neither associated with disease progression nor disability [7]. In another study by the same group, serum nitrate levels are found to be correlated with relapses in 24 RRMS and secondary progressive MS (SPMS) patients and there was no relation with either the activity of lesions found in brain MRI and disease progression or cerebral atrophy development [9]. In this study evaluation was made on the patients who had high CSF NNCs and active MRI lesions according to their time of admittance. There seems to be no correlation between high NNCs and early admission, thus we can suggest that excessive NO production is independent of the progression in the relapse period.

In accordance with our results Giovannoni et al. [9] reported that there is significant correlation between NNCs and albumin in CSF and it is suggested that NO may have a possible role in the damage to the blood-brain barrier. However, de Bustos and colleagues [5] did not find any difference in CSF nitrate concentrations between control subjects and MS patients. Furthermore, serum nitrate concentrations were found to be lower than normal controls. Ikeda et al. [12] found similar results in CSF nitrate concentrations. The most important difference between those two studies and our study is that only nitrate was measured in CSF instead of nitrite and nitrate.

The substances and molecules that are produced in different steps of NO metabolism have been investigated in MS patients. For instance, it has been reported that iNOS mRNA is significantly increased in the brain of MS patients [1, 2]. In addition to this, NADPH-diaphorase (NOS activity marker) positive astrocytes are found in the outermost margin of chronic active demyelinating MS lesions [2, 3]. Together with the reports mentioned above, the prevention of clinical symptoms in experimental allergic encephalitis by inhibition of iNOS [11] suggests a key role for NO in relapses. Especially during this period, infiltration of both monocyte/macrophages and activated T lymphocytes into the lesions result in the release of proinflammatory cytokines like IL-2 and IFN-γ. Interferon-γ leads to increased synthesis and release of NO by stimulating iNOS located in the monocyte and macrophage [10].

In conclusion, the results of this study provide evidence that NO is importantly involved in the pathogenesis of MS. Determination of elevated levels of reactive NO derivatives such as nitrite and nitrate in CSF of MS patients can be used as a disease activity marker besides an increased IgG index and active lesions on brain MRI. Similar studies including patients with other systemic inflammatory diseases without CNS involvement will help in understanding the role of NO in the autoimmune process. Moreover novel therapeutic approaches targeting the prevention of high amounts of NO production in CNS for the treatment of MS should be seriously considered.

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