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Serum nitric oxide products in patients with multiple sclerosis: relationship with clinical activity

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Abstract: We have assessed nitric oxide (NO) products, nitrites and nitrates in the sera of 80 controls and 14 patients with multiple sclerosis (MS) in different stages of clinical activity: seven with clinical remission (RR) (clinically inactive) and seven with chronic progressive (CP) disease (four active and three inactive). Overall, the levels of nitrites, nitrates and total NO products were significantly lower ($p < 0.001$) in MS patients than in controls. However, this decrease was significant only in patients with RR ($P < 0.0001$) and inactive CP ($P < 0.0005$). Likewise, patients with RR and inactive CP patients had significantly ($P < 0.001$ and $P < 0.05$ respectively) lower levels of NO products (16.92 ± 3.1 μmol/l and 18 ± 3.17 μmol/l respectively) as compared to active CP patients (24.1 ± 2.81 μmol/l). Serum NO levels may vary with the stage and activity of the disease. Future studies should ascertain the importance of serum NO in MS.

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Introduction: nitric oxide (NO), initially identified as an endothelial factor responsible for the relaxation of smooth muscle cells, is generated by the conversion of arginine to citruline by nitric oxide synthase using NADPH as a cofactor. The end-product is quickly transformed into a radical which is readily active, then it can be transformed into nitrites and subsequently nitrates [1, 2].

Three enzymes are responsible for nitric oxide production: one endothelial (eNOS), one neuronal (nNOS) and one inducible (iNOS). The iNOS is produced by immune cells and consequently its transcription is enhanced in inflammation. This induction may be reverted by steroids and corticosteroids [2]. On the other hand, proinflammatory cytokines can down-regulate eNOS expression and activity [3], suggesting that NO levels *in vivo* may be maintained by a fine regulation of enzyme levels and activity.

There is increasing evidence that NO *in vivo* plays an important role in CNS inflammation [4]. Raised levels of NO metabolites have been found in the cerebrospinal fluid and serum samples of multiple sclerosis patients (MS) [4, 5]. However, there was no relationship between the clinical activity of these patients and NO metabolites.

The aim of the present study was to ascertain the possible importance of serum NO products in different clinical stages of MS.

Patients and methods: 14 patients (seven female and seven male, age 40 ± 11 years old) with MS confirmed by clinical and laboratory findings [6] were enrolled from the department of neurology of the Vargas Hospital (Caracas, Venezuela) or the Clinical University Hospital (Caracas, Venezuela). Written consent was obtained from each patient. The study was approved by the hospital's Ethical Committee.

The patients had not received any immunosuppressor for three months prior to the study or any other drug for a period no less than 72 h. Clinically, five patients were classified as having disease activity and 10 were stable or inactive. The subjects were divided into three groups: relapsing remitting (RR), which were clinically inactive, and chronic progressive (CP), either clinically inactive (CP I) or active (CP A).

The controls were 80 normal blood donors and laboratory personnel.

Blood samples were taken after the subjects had fasted for no less than 4 h and no longer than 24 h. The normal food intake was similar. All the patients and controls received a diet with low contents of nitrite and nitrate following guidelines set out elsewhere [7].

Table 1. Characteristics of the different groups of MS patients and their serum nitric oxide levels (means ± SD)

	C	RR	CP I	CP A
Number	80	7	3	4
Sex (F:M)	50:30	5:2	1:2	2:2
Age (yr)	35 ± 11	33 ± 9	50 ± 1*	46 ± 6
Disease onset (yr)		6 ± 3	17 ± 3##	9 ± 2
Nitrites (μmol/l)	7.00 ± 1.97	3.54 ± 2.80****	3.90 ± 3.00**	5.15 ± 1.20
Nitrates (μmol/l)	18.87 ± 2.8	13.38 ± 3.37****	14.10 ± 3.20**	19.95 ± 3.11
Total products (μmol/l)	25.97 ± 4.6	16.92 ± 3.10****	18.00 ± 3.17***	24.1 ± 2.81

* *As compared with controls, P < 0.05 (ANOVA).*
** *As compared with controls, P < 0.005 (ANOVA).*
*** *As compared with controls, P < 0.0005 (ANOVA).*
**** *As compared with controls, P < 0.0001 (ANOVA).*
As compared with RR and CP A patients, P = 0.0003 (ANOVA)

NO levels were determined indirectly by quantification of their oxidized products of degradation, nitrates and nitrites, using nitrate reductase and the Greiss reagent as described previously [7]. Briefly, serum samples were centrifuged at 3000*g*, diluted four-fold with distilled water and incubated with nitrate reductase from *Aspergillus* spp. to quantify the total amount of nitric oxide products (nitrites + nitrates). In the absence of the enzyme only nitrite concentrations are determined.

The samples were incubated for 30 min at 37° C in the presence of enzyme and its cofactors NADPH and FAD, and for a further 10 min with sodium pyruvate and lactic dehydrogenase to degrade excess NADPH. They were then deproteinized with zinc sulfate, and 100 µl of the supernatant was mixed with 100 µl of Greiss reagent. A standard curve was obtained using sodium nitrate dissolved in water or in a pool of 20 normal human sera.

Nitrite concentration was determined at 540 nm using an ELISA plate reader (Labsystems Multiscan MCC/340, Helsinki, Finland). The interassay and intraassay coefficient of variation for the Greiss reaction were 5.6% and 3.6% respectively.

Results: Table 1 illustrates the characteristics of the patients and their serum levels of nitrite and nitrate. The levels of total NO product were significantly lower in MS patients as compared to controls ($P < 0.001$). However, if the patients were divided according to the classification specified in the table, marked differences became apparent. There were significant differences in the age and years of the clinical onset of the disease in the different groups.

As compared to controls, patients classified as RR had significantly lower ($P < 0.0001$) levels of nitrite, nitrate and total NO products. Similarly, patients with inactive CP (CP I) had lower levels of nitrites and nitrate ($P < 0.005$) and total NO products ($P < 0.0005$). Those with active CP (CP A) had similar levels of nitrate and NO products as the controls and lower, non-significant, levels of nitrite.

Moreover, patients with CP A had significantly higher levels of nitrite ($P < 0.001$), nitrate ($P < 0.05$) and total NO products ($P < 0.05$) as compared with patients RR or with CP I. These differences were due mainly to the higher levels of nitrates in the CP A than in the other groups. The levels of nitrite were lower than those of controls in the three groups. There was no correlation between age and any of the NO products or between the time of the onset of the disease and NO products.

Discussion: Several lines of evidence indicate that NO plays an important role in MS [4, 5]. Elevated levels have been observed in the cerebrospinal fluid of MS patients, and a mal models of this disease (allergic encephalomyelitis) ha confirmed the importance of NO in the lesions [4]. Moreov raised amounts of serum nitric oxide products have be reported in MS patients as compared to patients with oth clinical syndromes compatible with demyelinization [5]. has been proposed [4, 5] that the increased NO leve observed in MS patients are mainly due to the enhanc activity of iNOS, modulated by proinflammatory cytokine

The results presented in this report do not seem to suppo the aforementioned hypothesis. They differ from tho reported by Giovannoni *et al.* [5], although several possib explanations may account for the differences. The metho used by these authors to determine the levels of nitrite an nitrate in the serum samples was not followed by protei precipitation. Also, they did not consider the effect of sever parameters which may affect NO determination (die vasoactive drugs, fasting) [7]. In concordance with ou results, however, the levels of NO products in chronic pro gressors (probably clinically active) did not differ signifi cantly from those reported for the controls.

One may propose, then, that serum nitrite and nitrate lev els provide only partial evidence of a general inflammator phenomenon. Other radical products, such as those produce by peroxinitrates, should also be considered. In addition, the origin of serum NO needs to be carefully assessed since inflammatory cytokines may modulate iNOS and eNOS dif ferently [2, 3]. Consequently, the levels of NO product detected in serum may not differ from those in controls Future studies should ascertain the roles of these enzymes in MS.

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