Assessment of nitrosative oxidative stress in patients with middle cerebral artery occlusion

Marwan S. Al-Nimer, *MD, PhD,* ***Akram M. Al-Mahdawi,*** *MD, CABM,* ***Riyadh A. Sakeni,*** *MBChB, MSc.*

ABSTRACT

Objective: To assess serum levels of nitric oxide and peroxynitrite in patients presenting with cerebral infarction resulting from middle cerebral artery occlusion, at 48 hours from stroke onset.

Methods: We conducted the study in the Department of Pharmacology and in cooperation with Al-Yarmouk Teaching Hospital and the Department of Medicine, College of Medicine, Al-Mustansiriya University, Baghdad, Iraq from October 2003 to May 2004. Twenty patients presented with neurological deficits of middle cerebral artery occlusion, and we also enrolled 20 healthy individuals to serve as a control group. We obtained venous blood samples from each patient after 48 hours of stroke onset and each healthy individual. We determined the serum level of nitric oxide as well as peroxynitrite.

Results: Serum nitric oxide and peroxynitrite were significantly (p<0.001) higher in patients (103.9 ± 40.2 µmol and 2.7 ± 0.6 µmol) than in healthy individuals (53.3 ± 20.7 µmol and 2.3 ± 0.2 µmol). The formation of peroxynitrite directly correlated with nitric oxide in healthy individuals (r=0.84), and patients (r=0.514).

Conclusions: Serum intermediate nitrogen species; nitric oxide and peroxynitrite were significantly increased after 48 hours of stroke onset in patients with middle cerebral artery occlusion. The rate of peroxynitrite formation from nitric oxide was slightly increased.

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From the Departments of Pharmacology (Al-Nimer), and Medicine (Al-Mahdawi, Sakeni), College of Medicine, Al-Mustansiriya University, Baghdad, Iraq.

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Address correspondence and reprint request to: Dr. Marwan S. M. Al-Nimer, Professor of Pharmacology, Department of Pharmacology, College of Medicine, Al-Mustansiriya University, PO Box 14132, Baghdad, Iraq. E-mail: marwansalih2003@yahoo.com

Nitric oxide (NO) is an organic gas that plays a part in the control of cerebral blood flow, thrombogenesis, and modulation of neuronal activity.[1,2] The endothelial cells, neurons, glia, and macrophages produce nitric oxide by 3 different isoforms of the enzyme nitric oxide synthase (NOS).[3,4] The production of nitric oxide may be protective by virtue of its ability to induce cerebral vasodilatation,[5] but overproduction of nitric oxide may result in a greater infarct area.[6] In cerebral ischemia produced by occlusion of the rat middle cerebral artery (MCA), inducible nitric oxide synthase (iNOS) expression begins 12 hours after induction of ischemia, peaks at 48 hours, and subsides at 7 days.[7] Iadecola et al[8] reported that iNOS did not participate in the initiation of ischemic brain damage. Its expression begins more than 24 hours after induction of ischemia and peaks at 96 hours. In addition, they observed improvement in neurological deficits at 96 hours but not at 24 hours after MCA occlusion. Nitric oxide is thought to be produced shortly after induction of ischemia, in response to activation of glutamate receptors, which in turn leads to sustained activation of neuronal nitric oxide synthase.[9,10] In humans, NOS activity and NO release are greatly increased in the acutely ischemic brain.[11] Add to this, the nitric oxide metabolite (NO-m) concentrations in cerebrospinal fluid were significantly higher in patients with cerebral infarction of whatever origin.[12] The other intermediate nitrogen species involved in cerebral infarction, as a result of MCA occlusion, is peroxynitrite ($ONOO^-$). Experimentally, nitrotyrosine, a marker of peroxynitrite, appeared 4 hours after reperfusion in the cortex, and increased substantially at 22-46 hours in the vascular wall.[13] Also, Tabuchi et al[14] demonstrated nitrotyrosine, a peroxynitrite marker, around vessels, neuronal cells, and parenchyma in cerebral lesions in malignant stroke-prone spontaneously hypertensive rats (M-SHRSP).[14] One hour transient occlusion of MCA of wild-type mice resulted in demonstration of 3-nitro-L-tyrosine in the neurons and microvasculature 24 hours after transient MCA occlusion.[15] Our aim is to assess the serum levels of nitric oxide and peroxynitrite in patients with cerebral infarction after 48 hours of MCA occlusion.

Methods. We conducted this study in the Department of Pharmacology in cooperation with Al-Yarmouk Teaching

hospital and the Department of Medicine, College of Medicine, Al-Mustansiriya University in Baghdad, Iraq from October 2003 to May 2004. After obtaining permission from the local ethics committee and informed patient consent, we enrolled patients with neurological deficits from the medical ward at Al-Yarmouk Teaching Hospital. Consultant Neurologists provisionally diagnosed them with ischemic stroke resulting from MCA occlusion, proven by radiological investigations including CT and MRI of brain. We excluded cases with hemorrhagic stroke or occlusion of other cerebral vessels. Following selection, we studied 20 patients (15 males and 5 females) with a definite diagnosis of MCA occlusion. We also randomly selected 20 subjects, well matched to the patient group with regard to age and gender, from the outpatient clinic at Al-Yarmouk Teaching Hospital. We clinically examined all subjects, and investigated them by routine laboratory tests, and they appeared healthy. We obtained venous blood sampling from each patient 48 hours after stroke onset as well as from the healthy subjects. We separated the sera by centrifugation (3000 rpm for 3 minutes), and immediately processed this for nitric oxide and peroxynitrite assay. We determined the nitric oxide in the serum according to the method of Navarro-Gonzalvez et al,[16] by measuring the concentration of nitrates. Reduction of nitrate to nitrite by cadmium is the basis for this method. We used the Griess reaction to determine the nitrite, which reacts with a Griess reagent to form Griess chromophore. In brief, we deproteinized 150 µL of serum by adding 250 µL of 75 mM $ZnSO_4$ solution, stirring and centrifuging at 10000g for 5 minutes at 25°C. Then added 350 µL of 55 mM NaOH and stirred and centrifuged the solution at 10000 g for 5 minutes at 25°C. We recovered the supernatant, which was free from turbidity, and diluted 750 µL of this with 250 µL of glycine buffer (45 g/L, pH 9.7). We then added 2-2.5 gm of freshly activated cadmium granules to 1 mL of pretreated deproteinized serum and stirred this continuously for 10 minutes. After that, we transferred 200 µL of the treated serum into another tube and added Griess reagent (750 µL of 25 mg N-naphthylenediamine in 250 mL distilled water and 800 µL of 5 g sulfanilic acid in 500 mL 3M HCl). We recorded the absorbance of the sample at 340 nm by SpeCol spectrophotometer. The concentration of serum nitric oxide (µmol) is calculated in respect to standard lithium nitrate absorbance-concentration curve with a best fit line equation of: Absorbance (O.D) = 0.0018 ± 0.0002 x concentration (r = 0.997) .

We determined the peroxynitrite level in serum according to the method described by Beckman et al,[17] cited by VanUffelen et al 1998.[18] Peroxynitrite mediated nitration of phenol, resulting in nitrophenol formation, formed the basis of the peroxynitrite assay. In brief, we placed 10 µL of serum in a glass test tube and added 5mM phenol in 50 mM sodium phosphate buffer to a final volume of 2 mL and mixed well. This was then incubated for 2 hours at 37°C, followed with addition 15 µL of 0.1 NaOH and mixed. We then recorded the absorbance of the sample at 412 nm by SpeCol spectrophotometer (PGH, Radi fernesehen Electro, DDR). We calculated the yield of nitrophenol from $\varepsilon = 4400\ M^{-1}.cm^{-1}$.

All the chemicals used in this work were of analar grade, dissolved in distilled water and prepared freshly at the time of assay. We present the data as mean ± standard deviation of number of observations. We used Student's "t" test (paired and unpaired, two tailed), and simple correlation test with $p \leq 0.05$ as the lowest limit of significance for the data analysis.

Results. **Table 1** shows the characteristics of the study. There was insignificant difference between patients with ischemic stroke and healthy individuals. We found risk factors in 17 patients in terms of hypertension (13 cases), diabetes mellitus (3 cases), and atrial fibrillation (1 case). Eight patients had had more than one risk factor. Fourteen cases were active smokers. We found recurrent stroke reported in 9 patients. The arrival time to hospital was more than 24 hours for all cases. The clinical presentations were; disturbances in conscious state (5 cases), speech defects (14 cases), right (15 cases) and left (5 cases) hemiparesis, and facial nerve palsy (5 cases). Radiological investigations showed all cases had ischemic cerebral infarction as a result of thrombo-embolic occlusion of MCA. The serum nitric oxide was significantly ($p<0.001$) higher in patients with MCA occlusion (103.9 ± 40.2 µmol) in comparison with apparently healthy individuals (53.3 ± 20.7 µmol). We also observed a higher significant difference ($p<0.001$) in serum peroxynitrite levels between patients (2.7 ± 0.6 µmol) and healthy individuals (2.3 ± 0.2 µmol). From

Table 1 - Study characteristics.

Characteristic	Controls	Patients
Number	20	20
Gender		
Male	15	5
Female	15	5
Age (years), mean ± SD	52.7±7.4	57.5±8.2
Residency		
Rural	7	12
Urban	13	8
Social history		
Alcohol intake	0	0
Active smoking	14	16

the biochemical point of view, we found that for each 1 µmol of nitric oxide, there was a generation of 1.798 µmol ($r = 0.84$, $a = 1.79$, $b = 8.96 \times 10^{-3}$) of peroxynitrite in healthy individuals and 1.861 µmol ($r = 0.514$, $a = 1.853$, $b = 8.079 \times 10^{-3}$) in patients.

Discussion. We found a linkage between an increase in serum reactive nitrogen species, after 48 hours of stroke onset, and the occlusion of middle cerebral artery in this study. Previous studies reported conflicting results on the role of intermediate nitrogen species in stroke, with significant increase in plasma level of nitric oxide observed in patients with thrombotic cerebrovascular stroke after 2 days of onset.[19] Experimentally, nitric oxide is released in the brain after occlusion of the MCA,[20] or after heat stroke.[21] Plotkine and Margaill[22] showed that in the early phase after ischemia, nitric oxide is produced by the constitutive endothelial and neuronal isoforms of nitric oxide synthases (NOS3 and NOS1), while in the late phase, the inducible nitric oxide synthase (NOS2) is responsible for the delayed production of nitric oxide. Willmot et al[23] highlighted the importance of selective neuronal NOS (nNOS) and inducible NOS (iNOS) inhibitors in the treatment of transient acute stroke by the evidence of their experimental study that showed significant reduction in infarct size by NOS inhibitors. Therefore, the significant higher level of nitric oxide in this study is possibly related to the activation of inducible rather than constitutive nitric oxide synthases.

One may ask the following question; is the significant higher level of nitric oxide offering cerebral tissue damage or protection? Evidence shows that overproduction of nitric oxide is harmful. It directly correlated with cerebral infarct volume and with stroke severity,[20,24] and several therapeutic measures aiming to attenuate the higher nitric oxide level are useful in management of stroke, for example, hexasulfobutylated C60,[25] N-acetylcysteine,[26] 7-nitroindazole,[27] and ebseten.[28] Unfortunately, we did not estimate the cerebral-infarct volume in this work and therefore, missed the relationship between the cerebral-infarct volume and intermediate nitrogen species.

Conversely, some authors believe that nitric oxide protects the cerebral tissue from ischemic stroke. Salom et al[29] showed that nitric oxide donors like sodium nitroprusside, or NONOate spermin reduced the cerebral-infarcted size in an experimental animal model of transient focal cerebral ischemia. Also, the use of nitric oxide substrate, for example, arginine, may be of value in limiting the cerebral-infracted volume.[30]Recently, Bath et al[31] reviewed 2 completed trials that assessed the effects of nitric oxide donors and nitric oxide synthase inhibitors in people with acute stroke and concluded insignificant effects of such treatment on the outcome of stroke.[31]

Nitric oxide is not the only intermediate nitrogen species involved in the neuronal damage following stroke, there are other species such as peroxynitrite. Peroxynitrite is a far stronger oxidant and much more toxic than nitric oxide, and possibly related to the reperfusion injury in stroke.[32] The higher significant level of serum peroxynitrite after 48 hours of stroke onset in this work supports others who suggest the role of peroxynitrite in reperfusion injury. The concomitant estimation of serum nitric oxide and peroxynitrite lead us to observe that not only these intermediate nitrogen species are proportionally increased, but also the rate of peroxynitrite formation is retarded. We observed that for each 1 µmol of nitric oxide, there is 1.798 µmol peroxynitrite production in healthy individuals, and it increased to 1.861 µmol in patients. Although we measured both serum nitric oxide and peroxynitrite at the same time in patients with stroke, there are certain limitations to our study, namely, the small number of patients and the lack of serum nitrogen species levels at the onset of stroke.

To conclude, we found a significant level of intermediate nitrogen species generation, and a slight increase in the rate of peroxynitrite formation from nitric oxide 48 hours after stroke onset in patients with MCA occlusion. We therefore recommend that serum nitric oxide and peroxynitrite levels are of value in assessment of ischemic-reperfusion injury in patients with stroke.

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