Determination of Nitrite and Nitrate in Blood Specimens by Flow Injection Analysis

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Abstract

Although many methods for the determination of NOx have been reported, there are few that examined the caution of pretreatment and stability in blood. In this study, we attempted to investigate the stability of NOx in blood specimens and the pollution of NOx in instruments used. We measured nitrate and nitrite (NOx) n blood specimens by flow injection analysis. There was contamination of NOx in test tubes and tips left in air, but careful washing with deionized distilled water removed it. After being washed and dried, the test tubes must be kept in a sealed case to avoid re-pollution. Though EDTA was a suitable anticoagulant, a specific maker of the vacuum tube containing EDTA must be chosen because one maker's product contained NOx. The concentration of NOx was not affected by hemolysis and was stable for at least 4 hours at room temperature or 7 days at 4℃. The method exhibited linearity over the range of 1.0 to 100 μM nitrate and an upper sensitivity of 0.5 μM (99% confidence limit). The inter-assay and intra-assay of CV values, performed at 2 concentration levels, ranged between 0.6 and 4.0%, and the sampling frequency was 25 per hour.

We suggest that NOx concentration in blood be determined with consideration of these findings and that the NOx auto analyzer with automated flow injection analysis be applied clinically.

Key Words: blood, plasma, nitric oxide, nitrite, nitrate, flow injection analysis

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Introduction

Nitric oxide (NO) has been shown to be produced endogenously and play an important role in organisms[1-6]. It is implicated as a mediator, messenger, or regulator of cell function in variety of physiologic states that include vascular tone, platelet function, short- and long-term memory, hepatocyte respiratory function, septic shock, and penile erectile function[2-6]. NO is synthesized by the enzyme NO synthase (NOS)[5].

Several isoforms of NOS have been identified. They are the constitutive NOS present in the endothelium (eNOS)[7] and neural (nNOS)[8] tissue and the inducible enzyme (iNOS)[9] formed in activated immune cells. eNOS and nNOS are Ca^{2+} dependent and synthesize small amount of NO on demand, but iNOS is functionally Ca^{2+} independent and releases large amount of NO (more than 100-fold greater than that produced by eNOS and cNOS) after induction by stimulation with endotoxin or cytokines[5]. In organisms, NO undergoes rapid oxidation to NO_2^- and NO_3^- (NOx) and has an estimated half-life of less than 4 seconds[4,5]. Therefore, elevation of NOx concentration in the blood may indicate the occurrence of diseases, especially inflammations in body. Although many methods for the determination of NOx have been reported[10-12], there are few that examined the caution of pretreatment and stability in blood. In this study, we attempted to investigate the stability of NOx in blood specimens by flow injection analysis used for the measurement of brain NOx levels described by Habu et al.[12]. Moreover, we investigated the pollution of NOx in instruments used in this examination.

Materials and methods

NOx pollution in the instruments used for measurement of NOx concentrations in blood specimens

NOx pollution was examined in disposable glass test tubes, plastic test tubes, pipette tips, a syringe and vacuum tubes for blood sample.

Glass test tubes, plastic test tubes and pipette tips were divided into two groups. One group was tested without washing. Another group was washed with water, rinsed 4 times with deionized distilled water, and dried in a drying room with the tubes opening turned down. They were tested immediately, one day, 3 days and 7 days after drying. After loading a half ml of deionized distilled water and agitating using a mixer for 1 minute, the NOx concentration in the solution was measured.

The disposable syringe was tested as follows. Two ml of deionized distilled water was drawn into the syringe attached with a needle and agitated. After one minute of agitation, NOx concentration in the solution was measured.

Seven kinds of vacuum tubes from three different companies were tested. Five of them contained ethylenediaminetetraacetic acid (EDTA), and two contained heparin as the anticoagulant. One ml of deionized distilled water was injected into the tube using a NO-free syringe, and the tube was shaken well. NOx concentration in the water in EDTA-containing tubes was measured directly, and that in heparin-containing tubes was measured after deproteinization.

Stability of NOx in the blood

Blood for the examination of stability was supplied from five volunteers (25-39 years old, male: 2 and female: 3) at 9:00 AM. Each blood was injected into NOx-free plastic tubes and EDTA-containing vacuum tubes. Blood samples in the plastic tubes were immediately centrifuged at 2,000 x g for 10 minutes. After deproteinization, NOx concentrations in the blood were measured. Part of the samples in EDTA-tubes were centrifuged at 15,000 x g for 10 minutes after 0, 2 and 4 hours stay at room temperature. Another part was centrifuged at 15,000 x g for 10 minutes after 1, 3 and 7 days stay at 4 ℃. After deproteinization, NOx concentrations in the blood of each sample were measured at each time. Part of the samples in EDTA-tubes were stored at −80 ℃ for 3 days

to be hemolysed. After deproteinization, NOx concentrations in the samples were measured.

Deproteinization

We used the method described by Habu et al.[12] for deproteinizing heparin, serum, plasma and blood. Each sample (30μl) was added to 180 μl of 0.3 N NaOH solution. After incubation for 5 minutes at room temperature, 180 μl of 5% (w/v) $ZnSO_4$ was added. The mixture was then allowed to stand for 5 minutes and was centrifuged at 2,800 x g for 20 minutes. Nitrate and nitrite levels in 100μl of the supernatant were determined using the automated NOx analyzer.

Recovery of NOx in blood specimens

Sodium nitrate (25, 50, and 100μM) was added to the plasma supernatant of the blood in the EDTA-containing vacuum tubes after centrifugation. Each sample was deproteinized and analyzed for NOx concentration to observe the analytical recovery of NOx in the plasma.

Measurement of NOx

NOx was determined using an autoanalyser (Model TIC－NOX 1000; Tokyo Kasei Kogyo, Tokyo, Japan), which employs the technique of automated flow injection analysis[12]. Nitrite reacted with the Griess reagent to form a purple azo compound; the absorbance at 540 nm[13] was measured with a spectrophotometer (Model S/3250; Somakogaku, Tokyo, Japan) connected to a chart recorder (Phonix; TOA Electronics Ltd, Tokyo, Japan). Nitrate was determined by reducing it to nitrite using an A7200 Cd-Cu reduction column (Tokyo Kasei Kogyo, Tokyo, Japan). The reaction was as follows:

$$NO_3^- + Cd + 2H^+ \rightarrow NO_2^- + Cd^{2+} + H_2O$$

Then, nitrite was measured as above. Samples were loaded on the sample injector (Model SVI-6U7; Tokyo Kasei Kogyo, Tokyo, Japan). Both the carrier solution and the Griess reagent were prepared daily, warmed to 30℃ and made to flow at a rate of 1.5 ml/minute. Sodium nitrate solution (NOx standard solution) was used as the standard. The results obtained with this method exhibited linearity over a range of 1.0 to 100μM nitrate (99% confidence limit). The autoanalyser could analyze 25 samples per hour.

Chemicals

The carrier solution (Ethylenediaminetetraacetic acid disodium salt dihydrate (0.7g) and ammonium chloride (3 g) in 1 L of water, then adjusted to the pH of 8.0－8.5 with NaOH), the Griess reagent (Sulfanilamide (5 g) and N-(1-naphthyl) ethylenediamine dihydrochloride (0.5 g) in 1 L of HCl (0.6N)) and the NOx standard solution were obtained from Tokyo Kasei Kogyo Co., LTD. (Tokyo, Japan). The other chemicals were of the highest quality available and were obtained from Katayama Chemical Industries Co., LTD. (Osaka, Japan).

Statistical analysis

The data obtained were analyzed using the paired t-test. The level of significance was set at $p<0.05$.

Results

NOx pollution was measured in disposable glass test tubes, plastic test tubes, pipette tips, a syringe and vacuum tubes for blood sample.

There was contamination of NOx in disposable glass test tubes, plastic test tubes and pipette tips left in air even with first-use. A careful washing with deionized distilled water removed it (Fig. 1). Thus, we did not detect NOx in the tubes and tips immediately after drying. However, NOx contamination increased gradually when the tubes and tips were left in air (Fig. 1). When they were put in a sealed case immediately after drying, we did not detect NOx even after 7 days (data are not shown).

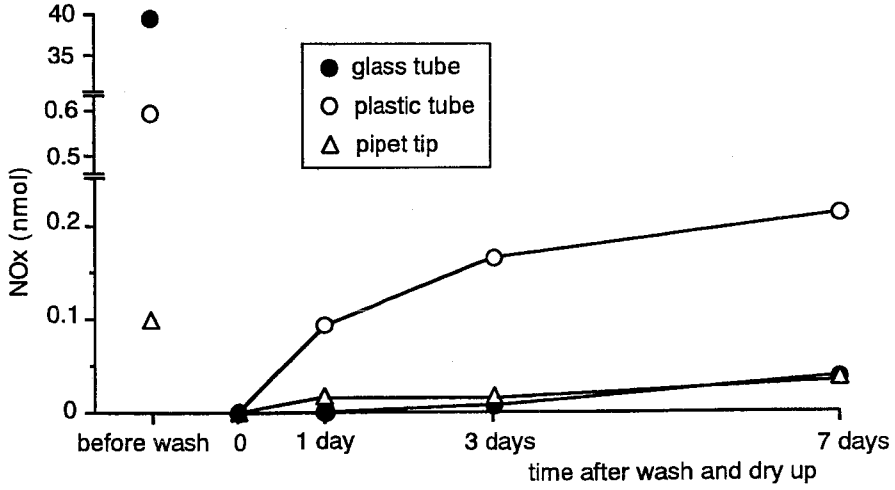

Fig. 1.
NOx contaminations in test tubes and pipette tips. NOx adhered to the insides of test tubes and pipet tips left in air. Though careful washing with deionized distilled water removed it, re—pollution occurred when they were left in the air (n= 3).

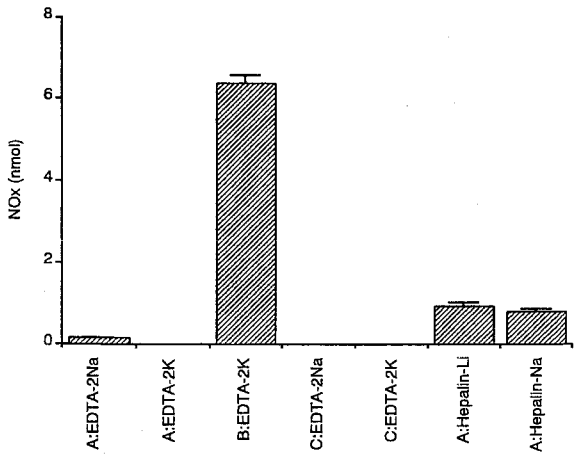

Fig. 2.
NOx contaminations in the vacuum tubes for obtaining blood specimens. The tubes of 3 different makers (A, B, C) and 4 different anticoagulants (EDTA- 2 Na, EDTA- 2 K, hepalin-Li, hepalin-Na) were tested (mean±SEM, n= 8).

Vacuum tubes with heparin contained NOx contamination at about one nmol/tube (Fig. 2). We did not detect NOx in 3 types of vacuum tubes with EDTA but detected over 6 nmol/tube of NOx in one type which was a product of a different company from the other vacuum tubes (Fig. 2).

Stability of NOx in the blood specimen

First, we examined the effect of EDTA on the levels of NOx in blood. The concentration of NOx in the plasma and serum obtained from the same blood were compared. Serum was obtained from blood in NOx-free plastic tubes, and plasma was obtained from blood in EDTA-containing vacuum tubes in which NOx was not detected. There was no significant difference between the concentrations of NOx in the plasma and serum (Fig. 3).

Second, we examined the effect of hemolysis on the levels of NOx in blood. Hemolysis was caused by freezing and thawing. NOx concentrations in blood with hemolysis and without hemolysis obtained from the same blood were compared after deproteinization. No significant difference was observed between the blood with or without hemolysis (Fig. 4).

We then examined the stability of NOx with time. There were no changes in the concentrations of NOx in the blood when it was kept for 4 hours at room temperature or for 7 days at 4 ℃ (Fig. 5).

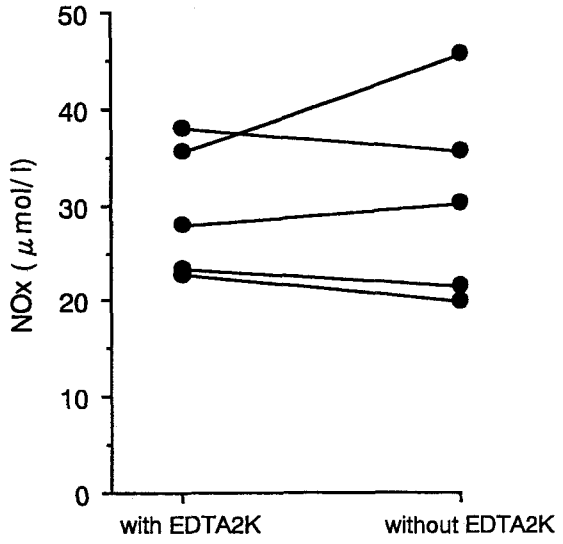

Fig. 3.
Effect of EDTA-2K on the levels of NOx in blood. Right circles and left circles connected with a line represent the same sample.There was no significant difference between those with and without EDTA-2K (Paired t-test, $p<0.05$).

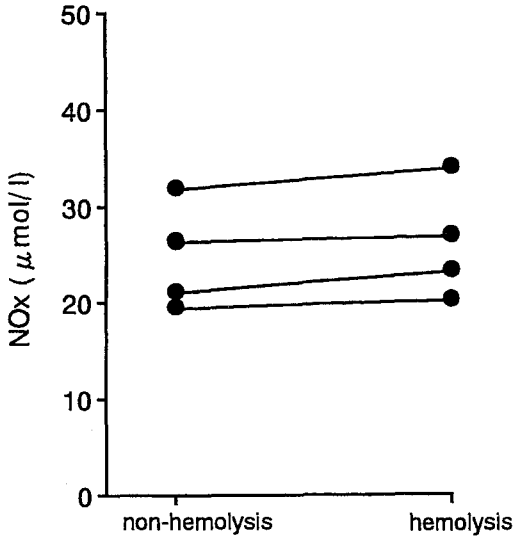

Fig. 4.
Effect of hemolysis on the levels of NOx in blood. Right circles and left circles connected with a line represent the same sample. There was no significant difference (Paired t-test, $p<0.05$).

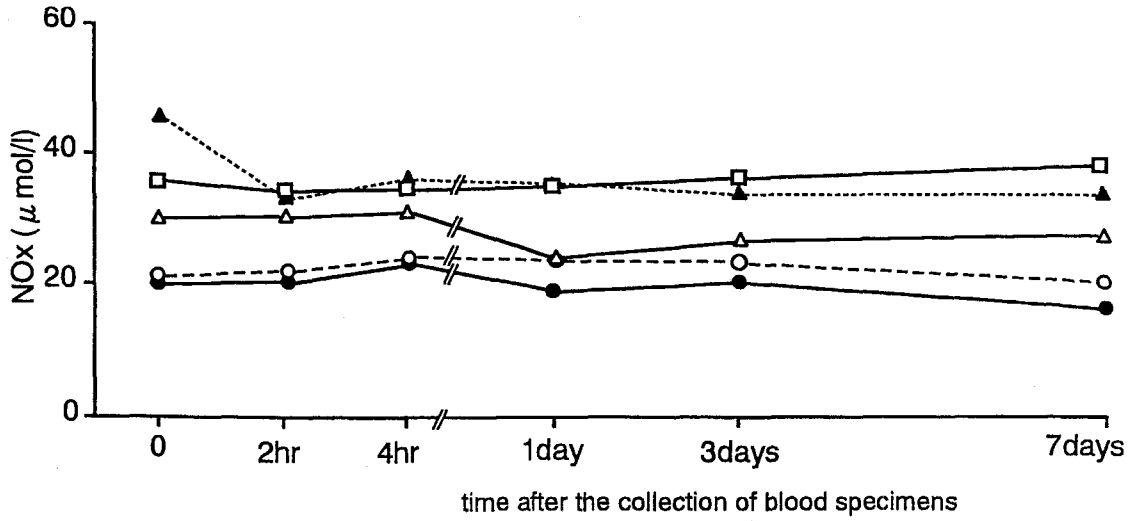

Fig. 5.
Changes of NOx concentrations in blood. Each sample was stored at room temperature and at 4℃ (after 4 hours). The same symbol represents the same sample. The NOx levels had no significant difference between 0 and other times (Paired t—test, $p<0.05$).

Recovery of NOx in plasma

Fig. 6 shows the analytical curves generated by adding nitrate to plasma, indicating a linear relationship. The overall recovery of nitrate added to the plasma was 98.49%.

Coefficient of variation (CV) was measured by using plasma (low dose) and the same volume of sodium nitrate solution (100μM) with added plasma (high dose). The inter-assay and intra-assay of CV values obtained from 10 measurements were 4.0 and 1.0% (low dose) and 2.7

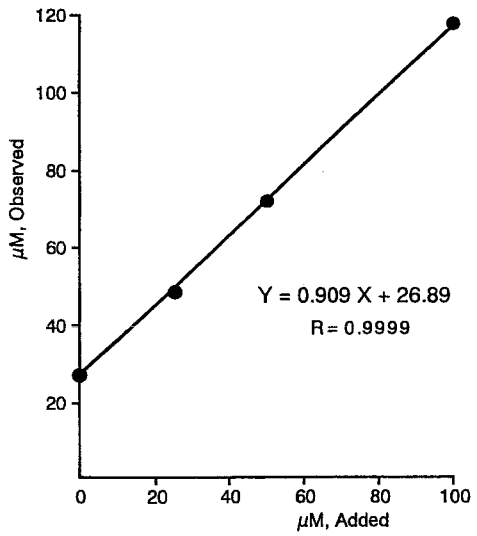

Fig. 6.
Analytical recovery of nitrate from blood. R=0.9999, Y=0.909X+2.689. Abscissa : nitrate in blood, added. Ordinate : nitrate in blood, observed.

and 0.6% (high dose), respectively.

Discussion

NOx is abundant in the air as noxious gases from the exhaust of motorcars and jet planes, and industrial fumes[3,4]. Certain bacteria can also generate NOx. NOx dissolves in water and causes acid rain. Almost all the water which we used contains NOx. For example, our tap water contains over 40 μM NOx. Therefore, NOx contamination in the instruments like tubes and tips used for measuring NOx in the blood was speculated. We tested the NOx contamination in different instruments. We poured deionized distilled water into every instrument and agitated to dissolve the NOx stains. Then, we measured NOx in the solution. The tubes and tips which were not sealed were polluted with NOx, although the disposable syringes and needles which were sealed soon after production did not contain NOx. We are able to remove this NOx pollution by washing with water and deionized distilled water. NOx pollution re-occurred if they were kept in air but was not detected if kept in a sealed box. Therefore, after being washed and dried, the test tubes must be kept in a sealed case to avoid re-pollution.

Heparin tubes contained a little NOx. This was expected because the heparin was extracted from organisms. Among five types of EDTA-containing vacuum tubes, one contained a lot of NOx, though others contained little or none. The NOx-contaminated vacuum tube was a product of a company which was in a different country from the other two companies. NOx contamination seems to be influenced by the factory surroundings. Therefore, though EDTA was a suitable anticoagulant, the makers and lot numbers of the vacuum tube containing EDTA must be specifically chosen, and it is necessary to check NOx contamination before using.

We obtained reproducible results of NOx levels in human blood by the method for mouse brain described by Habu et al.[12]. In this method, deproteinization proceeded under alkaline conditions, and NOx was measured by automated flow injection analysis. If deproteinization proceeded under acidic condition, the NOx values obtained varied widely[12] because some of the NOx was emited as a gas from the solution.

There was no significant difference between the concentrations of NOx in the plasma (EDTA-treated blood specimen) and serum (EDTA-untreated blood specimen). Hemolysis also had no effect on the concentration of NOx in blood specimen. These findings suggest that NOx is distributed equally in plasma and corpuscles. The concentration of NOx was stable for at least 4 hours (room temperature) or 7 days (4℃) if they were sealed, suggesting that NOx is stable in blood and that the blood can be stored in a refrigerator for at least one week after drawing.

The values obtained were reproducible, linear and did not show a wide distribution with repetitive determinations. After the addition of a standard sample, the data indicated a linear relationship with a slope of almost 1. This finding suggests that there are no substances in the plasma which interfered with or enhanced the quantitative reduction of NOx.

There are many reports that NOx concentra-

tion is increased in the blood of patients with rheumatic diseases[14,15], in the blood and urine of diabetic renal hyperfiltration model[6,16], in the urine of nephritic nephritis model rats[17], in the cerebrospinal fluid of neurological diseases in human[18], in the cerebrospinal fluid and blood of patients with head injury[19,20], and in the cortex of stress model rat[21]. These observations suggest that NOx increases in body fluid indicate the occurrence of inflammations[22-24]. Wong et al. proposed that NOx concentration was a clinically useful diagnostic tool and therapeutic monitor in evaluating children at risk for sepsis syndrome or acute allograft rejection[25]. Our method can be applied to measure NOx in urine and cerebrospinal fluid. We can measure NOx in many samples (25 samples/hr), and the auto-analyzer is simple to operate and highly sensitive (limit of detection 0.5μM) with a low rate of mechanical failure, and the operating cost is quite low.

References

1) McCann, S.M., Licinio, J., Wong, M.L., Yu, W.H., Karanth, S. and Rettorri, V. (1998) The nitric oxide hypothesis of aging. Exp. Gerontol. 33：813－826.

2) Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 43：109－142.

3) Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. FASEB. J. 6：3051－3064.

4) Anggard, E. (1994) Nitric oxide: mediator, murderer, and medicine. Lancet. 343：1199－1206.

5) Kuo, P.C. and Schroeder, R.A. (1995) The emerging multifaceted roles of nitric oxide. Ann. Surg. 221：220－235.

6) el-Sakka, A.I., Lin, C.S., Chui, R.M., Dahiya, R. and Lue, T.F. (1999) Effects of diabetes on nitric oxide synthase and growth factor genes and protein expression in an animal model. Int. J. Impot. Res. 11：123－132.

7) Lamas, S., Marsden, P.A., Li, G.K., Tempst, P. and Michel, T. (1992) Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc. Natl. Acad. Sci. USA. 89：6348－6352.

8) Garthwaite, J. (1991) Glutamate, nitriic oxide and cell-cell signalling in the nervous system. TINS. 14：60－67.

9) Xie, Q.W., Cho, H.J., Calaycay, J., Munford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T. and Nathan, C. (1992) Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 256：225－228.

10) Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P. L., Wishnok, J.S. and Tannenbaum, R. (1982) Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal. Biochem. 126：131－138.

11) Garside, C. (1982) A chemiluminescent technique for the determination of nanomolar concentrations of nitrate and nitrite in seawater. Marine Chemistry 11：159－167.

12) Habu, H., Yokoi, I., Kabuto, H. and Mori, A. (1994) Application of automated flow injection analysis to determine nitrite and nitrate in mouse brain. Neuro. Report. 5：1571－1573.

13) Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal. Biochem. 126：131－138.

14) Farrell, A.J., Blake, D.R., Palmer, R.M. and Moncada S. (1992) Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. Ann. Rheum Dis. 51：1219－1222.

15) Borderie, D., Hilliquin, P., Hernvann, A., Kahan, A., Menkes, C.J. and Ekindjian, O.G. (1999) Nitric oxide synthase is expressed in the lymphomononuclear cells of synovial fluid in patients with rheumatoid arthritis. J. Rheumatol. 26：2083－2088.

16) Bank, N. and Aynedjian, H.S. (1993) Role of EDRF in diabetic renal hyperfiltration. Kidney Int. 43：1306－1312.

17) Cattel, V., Cook, T. and Moncada, S. (1990) Glomeruli synthesize nitrite in experimental nephrotic nephritis. Kidney Int. 38：1056－1060.

18) Milstien, S., Sakai, N., Brew, B.J., Krieger, C., Vickers, J.H., Saito, K. and Heyes, M.P. (1994) Cerebrospinal fluid nitrite/nitrate levels in neurologic diseases. J. Neurochem. 63：1178－1180.

19) Clark, R.S., Kochanek, P.M., Obrist, W.D., Wong, H.R.,

Billiar, T.R., Wisniewski, S.R. and Marion, D.W. (1996) Cerebrospinal fluid and plasma nitrite and nitrate concentrations after head injury in humans. Crit. Care. Med. 24 : 1243－1251.

20) Navarro-Gonzalvez, J.A., Arenas, J., Diez－Lobato, R., Gomez, P. and Rodrigeuz－Arias, C.A. (2000) Levels of nitric oxide are markedly increased in cerebrospinal fluid from patients with severe head injury. Clin. Chim. Acta 290 : 221－222.

21) Olivenza, R., Moro, M.A., Lizasoain, I., Lorenzo, P., Fernandez, A.P., Rodrigo, J., Bosca, L. and Leza, J.C. (2000) Chronic stress induces the expression of inducible nitric oxide synthase in rat brain cortex. J. Neurochem. 74 : 785－791.

22) Efron, D.T. and Barbul, A. (1998) Modulation of inflammation and immunity by arginine supplements. Curr. Opin. Clin. Nutr. Metab. Care. 1 : 531－538.

23) Wanchu, A., Khullar, M., Sud, A. and Bambery, P. (1999) Nitric oxide production is increased in patients with inflammatory myositis. Nitric Oxide. 3 : 454－458.

24) Ashutosh, K. Nitric oxide and asthma: a review. Curr. Opin. Pulm. Med. 6 : 21－25.

25) Wong, H.R., Carcillo, J.A., Burckart, G. and Kaplan, S. S. (1996) Nitric oxide production in critically ill patients. Arch. Dis. Child. 74 : 482－489.

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