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CLINICAL STUDY

Plasma levels of nitrite/nitrate and inflammation markers in elderly individuals

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Abstract: *Introduction:* Production of endothelial nitric oxide declines with advancing age. On the other hand, ageing itself is associated with a mild degree of chronic inflammation. Besides, asymptomatic infectious and non-infectious inflammation is frequent in old age. All this may lead to an increased formation of nitric oxide via inducible nitric oxide synthase. The plasma levels of nitric oxide metabolites in older age groups are not known. The aim of our study was to determine the plasma levels of metabolites of nitric oxide (nitrite and nitrate) and to correlate them with the levels of inflammation markers in clinically healthy individuals aged over 80.
Methods: The plasma levels of nitrite/nitrate as well as erythrocyte sedimentation rate, and the levels of C-reactive protein and tumor necrosis factor alpha were determined in a group of 30 clinically healthy individuals aged over 80 years. Results were compared with those obtained in a control group.
Results: Nitrate levels were increased and the levels of inflammation markers were significantly higher compared with those in a control group.
Conclusions: The levels of nitric oxide metabolites in elderly, clinically healthy individuals may be increased due to inflammation *(Tab. 2, Fig. 1, Ref. 32). Full Text (Free, PDF) www.bmj.sk.*
Key words: nitric oxide, nitrite, nitrate, inflammation, old age.

Nitric oxide (NO) is a multifunctional reactive mediator, which may act as a vasodilator, neurotransmitter, and the effector molecule of immune cells (1, 2). It is produced by nitric oxide synthase, which exists in three forms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS).

Endothelial and neural nitric oxide synthases are constitutively expressed and collectively referred to as constitutional NOS (cNOS). In contrast, NO production by neutrophils and macrophages, as mediated by iNOS, occurs under pathological conditions.

Given the short half-life, NO levels are determined indirectly by determining its metabolites, i.e., nitrites and nitrates. The plasma levels of nitrites, as compared with nitrates, are very low and, in addition, more sophisticated techniques are required for their determination (3, 4). Methods using the Griess reaction combined with spectrophotometric detection are not sensitive enough for these purposes. Hence, unless investigators are particularly interested in nitrite determination, total levels of nitrite and nitrate are determined as what is referred to collectively as nitrogen oxides (NOx). Recent studies have contributed to renewed interest in nitrites, considered a sensitive index of constitutional NOS; plasma nitrite levels could serve as a marker of endothelial function (5–7).

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NO production by endothelium decreases with increasing age. Molecular studies have suggested the decreased NO formation and its release from endothelial cells in aging is the result of decreased endothelial NOS activity (8). At the same time a role is played, by asymmetric dimethylarginine, an endogenous NOS inhibitor, whose increasing plasma levels correlate closely with age (9). On the other hand, aging has been associated with a mild degree of chronic inflammation. In addition, asymptomatic infection is also frequent in old age. Increased levels of pro-inflammatory cytokines have been reported in the highest age group (10), therefore an increase in NO production by inducible nitric oxide synthase (iNOS) would be expected during inflammation. The plasma levels of nitric oxide metabolites, as determined in clinical trials, is inconsistent. Some authors have reported levels declining with increasing age (11–13), while others have documented levels increasing with increasing age (14, 15).

The aim of our study was to determine the plasma levels of nitrites and nitrates and to correlate them with inflammation markers in clinical healthy individuals in the highest age group.

Subjects and methods

Selection of patients

We examined rest-home residents aged over 80, who were self-sufficient, were not taking any drugs on a regular basis, and considered themselves to be in good health. All had physical and laboratory examinations. Excluded from the study were all individuals with a newly diagnosed acute or chronic inflammatory

Tab. 1. Patient characteristics.

Characteristics	Elderly patients	Control group	p
Age (years)	86.4±5.8	36.9±9.1	
Women (n)	24	9	
BMI (kg/cm^2)	24.2±3.7	23.3±3.4	NS
Leukocytes (10^9/l)	9.5±2.8	7.0±2.3	0.02
Hemoglobin (g/l)	128±20	139±11	0.03
Creatinine (μmol/l)	80±24	79±12.2	NS
Glycemia (mmol/l)	6.9±2.6	4.9±0.61	0.01
Cholesterol (mmol/l)	4.6±1.0	4.7±0.6	NS

BMI – body mass index

disease, malignancy, renal insufficiency or liver cirrhosis. Only individuals with erythrocyte sedimentation rate and creatinine levels within the normal range were included into the study. A normal erythrocyte sedimentation rate was defined by standard values related to age, i.e., 20 mm/h and 30 mm/h for elderly males and females (16). Individuals with plasma C-reactive protein (CRP) levels over 30 g/l were also note eligible for inclusion.

Our patient group included 30 individuals (24 women and 6 men) aged 80 to 96 years, with a mean age of 86.7±5.6 years. The control group was made up by 15 clinically healthy individuals (9 women and 6 men) with a mean age of 36.8±9.1 years. Compared with controls, the elderly group had significantly higher mean leukocyte count and blood glucose levels and lower hemoglobin levels. Characteristics of both groups are given in Table 1.

The plasma levels of nitrites and nitrates, CRP and tumor necrosis factor alpha (TNF-alpha) were determined in both groups.

While patients had been instructed about their diet to adhere to for 3 days prior to blood sampling, no checks were made. The patients were not hospitalized as the study was conducted on a out-patient basis. Nitrate-containing drugs and smoking were not allowed throughout the study.

Blood sampling

Blood samples were withdrawn into heparinized test tubes in the morning after overnight fasting (for at least 8 hours). Samples were centrifuged at 1,000 g for 15 minutes. Plasma was frozen to -70 °C and samples were processed within one month since collection.

Methods

Determination of nitrate and nitrite

The levels of nitrites and nitrates were determined using a high-performance liquid chromatography (HPLC) system with two detectors. Nitrate ions were detected using a spectrophotometric UV/Vis detector, detecting these ions at absorbance of 212 nm, whereas nitrite ions were detected using an electrochemical detector featuring a glass/carbon working electrode at a potential of 0.8 V. The method is described in detail elsewhere (17).

Tumor necrosis factor-alpha (TNF-alpha) was determined using enzyme-linked immunosorbent assay (ELISA) with commercially available Human TNF alpha kits (BioSource International, Inc., Camarillo, Ca, USA.). The minimum detectable concentration was 1.7 pg/ml.

C-reactive protein levels were determined by immunoturbidometry (Roche Diagnostic, Mannheim, Germany). The lower detection limit was 0.3 mg/l.

Creatinine levels were determined using absorption spectrophotometry.

Body mass index (BMI) was calculated as weight divided by height squared.

Statistical analysis

Statistical significance of the differences in the parameters between both groups was determined using the two-sample test. p values lower than 0.05 were considered statistically significant. Correlation coefficients were calculated for individual parameters and linear regression analysis was performed.

Results

The group of elderly individuals was shown to have higher nitrate levels (29.3±12.0 μmol/l) compared with controls (21.38±5.81 μmol/l); however, the difference was not significant. An interesting finding in the elderly group were lower nitrite levels compared with younger controls (mean 1.1±1.0; median 0.8 μmol/l); with the difference again not being significant. Although erythrocyte sedimentation rates were still within normal in either group, CRP and TNF-alpha levels in the elderly were significantly raised ($p<0.05$ for CRP and $p<0.001$ for TNF-alpha). Results were listed in Table 2.

Further, a correlation was found between nitrate levels and TNF-alpha ($r=0.58154$; $p<0.001$) whereas there was no correlation between nitrite levels and inflammation markers. The correlation between nitrate and TNF-alpha is shown in Figure 1.

Discussion

Aging is an independent factor affecting endothelial function. Older age is associated with decreased endothelial nitric oxide production and, also enhanced sensitivity of endothelial cells to apoptotic stimuli (18). The lower mean nitrite levels

Tab. 2. Parameters studied in both groups.

Characteristics	Elderly patients	Control group	p
ESR (mm/h)	13±8	12±7	NS
CRP (mg/l)	8.3±6.5	4.4±3.2	0.02
TNF (ng/ml)	10.5±8.5	2.0±1.0	0.0001
NO_2 (μmol/l)	1.1±1.0	1.6±1.0	NS (0.08)
NO_3 (μmol/l)	29.4±12.1	23.1±8.9	NS

ESR – erythrocyte sedimentation rate, CRP – C-reactive protein, TNF – tumor necrosis factor, NO_2 – nitrite, NO_3 – nitrate

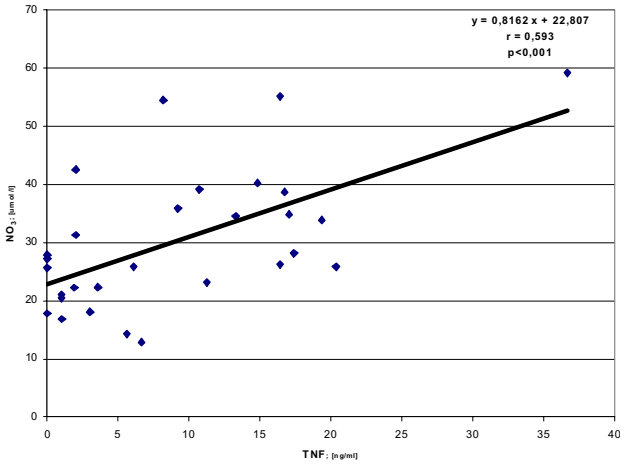

Fig. 1. Correlation between nitrate and TNF-alpha levels in the group of elderly patients.

shown in the group of elderly individuals is a most puzzling finding in this context, possibly indicating decreased endothelial NO production, as 70–90 % of nitrites circulating in the plasma are due to eNOS activity (19, 20). A precondition for the plasma levels of nitrites to serve as a marker of endothelial function is to avoid nitrite contamination during sample processing and use of validated method. In an effort to avoid contamination, the method we used reduces the manipulation with the sample to minimum. The baseline levels of both metabolites are in the range of values reported for other chromatographic methods (21). The plasma levels of nitrites are reported to be over a broad range depending on the method employed (4), although more recent studies with healthy individuals have reported a range of 100–600 nmol/l (5–7). This was the range of nitrite levels shown in young women when using the same method (22).

A different finding was made when detecting nitrate levels. The plasma levels can be affected by a variety of factors such a dietary nitrate intake, saliva production, bacterial synthesis in the bowel, denitrification action of liver enzymes, inhalation of atmospheric nitrogenic oxides, and impaired renal function (23). The plasma levels of nitrates of healthy volunteers rose by a factor of 2.5 after consuming celery while the nitrite levels remained virtually unaltered (14). Although the patients were instructed about their diet, the study was conducted on an outpatient basis and noncompliance cannot be ruled out. Our patients represent the highest age group. Their mean plasma levels of nitrates were higher compared with those of controls. An increase in inflammation markers was also seen, and a correlation between nitrates and TNF levels documented. These findings supported our hypothesis that the mean levels of nitrates in elderly individuals could be due to enhanced NO production by iNOS during inflammation. In fact, aging per se is associated with a mild degree of inflammation resulting in increases in inflammatory markers, in particular those of TFN alpha and CRP. This has been explained by a broad range of factors such as environmental ones including smoking, genetic factors, obesity, and hormonal changes (24). Asymptomatic infection (25) or non-infectious chronic inflammation may also be at play. Zhou et al (26) reported elevated plasma levels of NO metabolites in chronic cholecystitis. Compared with data reported by other authors (27–29), the mean levels of TNF alpha and CRP in our group of elderly patients were higher than would be consistent with the age-related mild degree of inflammation. Aging has been associated with a 2–4 fold increase in the plasma and/or serum levels of pro-inflammatory cytokines and acute phase proteins (30). In the past, elevated levels of both markers were associated with the inflammatory component of age-related disease such as atherosclerosis and dementia, abdominal aorta aneurysm, and so on (29, 31). Our group of patients was a selected one. Still, asymptomatic infectious inflammation cannot be ruled out and is more likely than other causes of inflammation. Factors potentially contributing to the increased nitrate levels may have included reduced glomerular filtration rate in elderly individuals and the relatively long half-life of nitrates (32), although creatinine levels were still within normal.

Conclusion

A group of apparently healthy elderly individuals aged over 80 has been shown to have increased plasma levels of nitrates in parallel with elevated levels of the inflammatory makers CRP and TNF alpha. In addition to dietary and renal factors, the increased levels may have been due to enhanced NO production by inducible NOS in asymptomatic inflammation.

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