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Nitric oxide metabolites and erythrocyte deformability in a group of subjects with obstructive sleep apnea syndrome

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Abstract. Our aim was to evaluate nitric oxide metabolites (nitrite and nitrate), expressed as NOx, and erythrocyte deformability, expressed as elongation index, in a group of subjects with obstructive sleep apnea syndrome (OSAS). We enrolled 48 subjects (36 men and 12 women; mean age 50.3 ± 14.68 yrs) with OSAS diagnosed after a 1-night cardiorespiratory sleep study. OSAS severity was assessed evaluating the apnea/hypopnea index (AHI) and subjects were subdivided in two subgroups: Low (L = AHI <30) and High (H = AHI >30). NOx was examined converting nitrate into nitrite with a nitrate reductase and then assessing nitrite with spectrophotometry after the addition of Griess reagent. The elongation index was obtained using the diffractometer Rheodyn SSD of Myrenne at shear stresses of 30 and 60 Pa and it was expressed as elongation index (EI). We found no difference in NOx among the entire group of OSAS subjects and normal controls, while we observed a NOx decrease in the H subgroup in comparison with L subgroup, but not in comparison with normal controls. We noted a significant decrease in EI at each shear stress in the entire group and also in the two subgroups in comparison with controls. The decrease in NO bioavailability and in erythrocyte deformability might contribute to explain the increased cardiovascular risk in OSAS subjects.

1. Introduction

The obstructive sleep apnea syndrome (OSAS) is characterized by repeated obstructions of upper airways, partial or complete, during sleep, and consequent episodes of apnea or hypopnea, with intermittent arterial oxygen desaturation [4, 39]. The OSAS is diagnosed via polysomnography and its severity is expressed as apnea/hypopnea index (AHI). Continuous positive airway pressure (cPAP) therapy, with or without associated oxygen therapy, is the gold standard for its treatment [14]. The most important complications are cardiovascular diseases, resulting in severe morbidity and mortality. OSAS is associated in fact with an higher risk of arterial hypertension, coronary artery disease, and cerebrovascular accidents [21, 23], which often occur during morning hours [25, 32]. OSAS has been proposed as an independent risk factor for the development of essential hypertension and the isolated increase in diastolic pressure is often the earliest pressure modification in these subjects [24, 37]. Some author have described higher incidence of myocardial infarction in OSAS subjects during night-time (from 10 pm to 6 am) [46] suggesting that OSAS could precipitate myocardial ischemia during sleep in patients with coronary disease. Untreated

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OSAS may also worsen the prognosis of subjects with coronary disease increasing cardiovascular death [24]. The strong relationship between OSAS and stroke is demonstrated by its prevalence in 44–72% of patients with stroke and by the evidence of a 4-fold increase in risk of cerebral accidents in OSAS with an AHI >20 [24].

The pathogenesis of cardiovascular disease in subjects with OSAS probably depends on several factors [37]. In OSAS, an impaired autonomic nervous system activity has been demonstrated: during apneas and hypopneas an enhanced parasympathetic tone is evident, while sympathetic nervous system tone increases after the apneic events [20]. In addition, several papers have demonstrated an impaired hemorheological profile [12, 41, 42], an increased blood coagulation [43] and also an altered inflammatory [13, 19, 34] and oxidative status [2, 3, 7, 10, 26, 35, 40] in OSAS subjects. The frequent hypoxia-reoxygenation episodes are presumed to play a key role in the pathogenesis of endothelial dysfunction. The intermittent hypoxia may induce the production of ROS that contribute to the generation of adhesion molecules, leukocyte activation, and an enhanced systemic inflammation. In addition, untreated sleep apnea is associated with increased levels of endothelin, which may contribute to vasoconstriction, and with an increased endothelial cell apoptosis [37]. A significant negative correlation between brachial artery flow-mediated dilation and OSAS severity has been also demonstrated [37].

Plasma nitric oxide (NO) metabolites, such as nitrites and nitrates, usually expressed as NOx, are significantly reduced in subjects with OSAS [1, 9, 27, 29, 31]: the hypoxia-reoxygenation phenomena influence NO production by NO synthase (NOS) because intermittent hypoxia induces a down-regulation of eNOs expression [44, 47], and consequently NO synthesis is inversely related to the severity of the disease [9, 27]. The cPAP therapy seems to improve the endothelial function as it increases NOx levels in the long-term [1, 22, 28, 29, 31, 33] and even after an overnight application [16].

Few papers have taken into account the effect of OSAS on blood rheology and the methodological differences among these studies make difficult to compare their results. Chin et al. [8] showed increased levels of fibrinogen and hematocrit in the morning in a small group of OSAS subjects, suggesting an increment of blood viscosity. By other authors plasma fibrinogen was correlated with AHI value and with nocturnal minimal oxygen saturation (SO_2) [38]. Tazbirek et al. found elevated blood viscosity and erythrocyte aggregation in obese men with OSAS in comparison with those without OSAS [42]. However, other authors [12] observed an increase only in plasma viscosity, inversely correlated with mean nocturnal SO_2, but no modification of erythrocyte deformability. In overweight OSAS subjects, Sinnapah et al. [36] found increased erythrocyte aggregation, positively correlated with AHI and BMI. This paper underlined that BMI is more predictive of erythrocyte aggregation than AHI, suggesting that overweight influences blood rheology more than OSAS severity. Treatment with cPAP reduces plasma fibrinogen [8], and blood and plasma viscosity [42] improving the blood rheological properties.

Considering all these data, the purpose of our study was to evaluate nitric oxide metabolites (NOx) concentration and erythrocyte deformability in subjects with OSAS.

2. Subjects

We consecutively recruited 48 subjects (36 men and 12 women; mean age 50.3 ± 14.68 yrs) with obstructive sleep apnea syndrome from those with suspected OSAS referred to our center. Clinical history and physical examination were performed in all subjects and Epworth Sleepiness Scale (ESS) was also given. OSAS was diagnosed after a 1-night cardiorespiratory sleep study and its severity was assessed evaluating the apnea/hypopnea index (AHI). OSAS subjects were subdivided according to the AHI value

Table 1
Mean ± S.D. of age, anthropometric characteristics and OSAS parameters in the whole group of OSAS patients and in the two subgroups with respectively AHI 5–30 and AHI ≥30

	All OSAS patients	AHI 5–30	AHI ≥30
Age (years)	50.3 ± 14.6	45.3 ± 14.4	52.8 ± 14.2
BMI (kg/m^2)	35.4 ± 7.3	35.7 ± 8.5	35.1 ± 6.5
Waist circumference (cm)	118.8 ± 16.1	114.2 ± 14.5	122.5 ± 16.6
Neck circumference (cm)	44.4 ± 4.5	41.5 ± 3.2	46.6 ± 4.1***
AHI	38.5 ± 25.7	15.1 ± 8.1	56.6 ± 18.9***
mSO_2 (%)	91.1 ± 3.7	93.4 ± 2.7	89.5 ± 3.4***
EPS	11.1 ± 5.1	9.2 ± 3.7	12.4 ± 5.6*
ODI	39.3 ± 29.0	14.3 ± 9.4	55.4 ± 25.7***

*$p < 0.05$, ***$p < 0.001$ vs. OSAS 5–30 (Student's t test for unpaired data). BMI = Body Mass Index; AHI = Apnea-hypopnea index; mSO_2 = mean oxygen saturation; EPS = Epworth sleepiness scale; ODI = oxygen desaturation index.

in two subgroups: Low (L = 21 subjects with AHI <30) and High (H = 27 subjects with AHI >30), therefore the Low subgroup included subjects with mild to moderate OSAS, while the H subgroup included the subjects with severe OSAS. Means and S.D. of age, BMI, waist circumference, neck circumference, AHI, oxygen desaturation index (ODI), mean nocturnal SO_2 and mean heart rate (HR) are reported in Table 1 (Table 1); 23 of the OSAS subjects had arterial hypertension, 10 subjects had diabetes mellitus and 6 had cardiovascular disease (history of myocardial infarction or stroke). Regarding the evaluation of NOx, the control group consisted of 31 subjects (14 women and 27 men, mean age 41.3 ± 7.4 years), while regarding the evaluation of erythrocyte deformability, the control group included 29 subjects (13 women and 16 men, age range 35–52 years); both groups of subjects were free of medical diseases as assessed by clinical history, physical examination, electrocardiography, and routine hematological and urine analysis.

All the subjects gave their informed consent before entering the study and the study was approved by the Ethical Committee.

3. Methods

Venous blood samples were collected in the morning by venous puncture from the antecubital vein of fasting subjects and immediately transferred to anticoagulated glass tubes for evaluation of NOx and erythrocyte deformability.

3.1. NO metabolites (NOx)

Considering that *in vivo* NO has a very short life (less than 0.1 sec) and it is converted into nitrite (NO_2^-), which has a half-life of few minutes, and into the more stable nitrate (NO_3^-), NOx represents almost only the nitrate concentration. In the laboratory method adopted by us at first nitrate was converted into nitrite by a nitrate reductase, and then nitrite was assessed by spectrophotometry after addition of Griess reagent.

3.2. Elongation index (E.I.)

To evaluate erythrocyte deformability, we mixed 30 μl of anticoagulated blood with 2 ml of dextran solution at a viscosity of 24 mPa. The measurement was obtained by using the diffractometer Rheodyn SSD of Myrenne, which measures the diffraction pattern of a laser beam passing through erythrocytes suspended in a viscous medium and deformed by a force with defined shear stress. The shear stresses employed by us were 6, 12, 30 and 60 Pa. The erythrocyte deformation was expressed as elongation index (EI) = (l − w/l + w) × 100, were l = length and w = width of the erythrocytes.

4. Statistical analysis

Data were expressed as means ± S.D.; the difference between normal subjects and OSAS patients was evaluated according to the Student's *t* test for unpaired data. The statistical difference between normal subjects and OSAS subjects subdivided according to the apnea/hypopnea index (AHI) was estimated using the 1-way analysis of variance (ANOVA) integrated with the Bonferroni test. The correlations were performed employing the linear regression test. The null hypothesis was rejected for p values <0.05.

5. Results

In the entire group of OSAS subjects no difference in NOx was found in comparison with normal controls, while a significant decrease in EI at each shear stress was observed (Table 2). Subdividing OSAS subjects according to the AHI value in the two subgroups, we noted a NOx decrease in the H subgroup (AHI >30) in comparison with L subgroup, but not in comparison with normal controls. However, the EI, at each shear stress, was significantly reduced in the two subgroups in comparison with normals (Table 3). In the entire group of OSAS subjects we observed a negative correlation between NOx and AHI ($r = -0.61$, $p < 0.001$), a positive correlation between NOx and mean nocturnal SO_2 ($r = 0.418$, $p < 0.01$) and a negative correlation between AHI and mean nocturnal SO_2 ($r = -0.56$, $p < 0.001$). We also noted a positive correlation between AHI and neck circumference ($r = 0.60$, $p < 0.001$), and a negative correlation between neck circumference and mean nocturnal SO_2 ($r = -0.47$, $p < 0.01$). No significant correlation was found between EI, at each shear stress, and NOx nor between EI and AHI, mean nocturnal SO_2, or neck circumference.

Table 2
Mean ± S.D. of nitric oxide metabolites (NOx) and elongation index (EI) at two shear stresses, in control subjects and the whole group of OSAS patients

	Control subjects	All OSAS patients
NOx (μmol/l)	28.07 ± 18.83	27.49 ± 10.25
EI 30	43.7 ± 6.1	39.2 ± 4.3***
EI 60	46.9 ± 5.4	43.2 ± 3.6**

**$p < 0.01$, ***$p < 0.001$ vs. Control subjects.

Table 3
Mean ± S.D. of nitric oxide metabolites (NOx) and elongation index (EI) at two shear stresses, in control subjects and the two subgroups of OSAS patients, with respectively AHI 5–30 and AHI ≥30

	Control subjects	FAHI 5–30	AHI ≥30	F
NOx (μmol/l)	28.07 ± 18.83	33.47 ± 10.05	22.84 ± 7.79*	3.246[a]
EI 30	43.7 ± 6.1	38.4 ± 5.2[¶]	39.6 ± 3.7[#]	6.502[b]
EI 60	46.9 ± 5.4	42.5 ± 4.4[#]	43.5 ± 3.0[#]	6.044[b]

[a]$p < 0.05$, [b]$p < 0.01$ (ANOVA). *$p < 0.05$ vs. mild OSAS (Bonferroni's post-test). [#]$p < 0.05$, [¶]$p < 0.01$, [§]$p < 0.001$ vs. control subjects (Bonferroni's post-test).

6. Discussion

The trend of NOx observed by us in OSAS subjects is confirmed by some authors [11], but conflicts with the results of others [1, 9, 22, 29, 31, 33]. The subdivision according to the AHI value showed a marked decrease in NOx only in the subgroup with AHI >30. Agreeing with other authors [15, 18], we found a negative correlation between NOx and AHI in the group of OSAS subjects, in which a correlation between NOx and mean nocturnal SO_2 and between AHI and mean nocturnal SO_2 was evident. Also the neck circumference seems to influence AHI and mean nocturnal SO_2. These data suggest that the behavior of NOx in OSAS depends especially on its severity. As oxygen is a substrate of NOS, the frequent desaturation in OSAS subjects could reduce NOS activity; in addition, hypoxia is responsible for alterations in gene regulation, so it could suppress the transcription of endothelial NOS (eNOS) gene [9]. Some authors have examined the effect of intermittent hypoxia on cultured human umbilical vein endothelial cells and they have observed significantly lower levels of NO, NOS activity and NOS mRNA expression [47]. Jelic et al. [17], in freshly venous endothelial cells of newly diagnosed OSAS subjects, found a reduced expression of eNOS and reduced levels of phosphorylated eNOS (the activated form) associated with an increased expression of inducible NOS (iNOS). Treatment with cPAP for 4 weeks significantly increased eNOS and phosphorylated eNOS, and decreased iNOS expression, improving flow-mediated dilation [17]. Other authors, in animal models, have demonstrated that chronic intermittent hypoxia down-regulates the endothelial NOS expression inducing NF-kB activity and the consequent overproduction of inflammatory mediators, such as TNF-α, able to inhibit eNOS expression [44]. It has been also suggested that the increased production of ROS in OSAS might cause eNOS uncoupling with consequent decreased activity of this enzyme [45]. The reduced availability of NO may be involved in the pathogenesis of arterial hypertension and cardiovascular diseases, especially in severe OSAS.

In the entire group and in the subgroups of OSAS subjects we found a significant decrease in EI at each shear stress. This datum is different from which of others [12, 42], who did not find any difference about erythrocyte deformability between OSAS subjects and normal controls. It must be mentioned that Dikmenoglu et al. [12] have examined this rheological determinant using a filtration thecnique, while Tazbirek et al. [42] employing a laser optical rotational cell analyzer. In this group of OSAS subjects, no correlation between NOx and EI has been observed although, theoretically, a link between these two parameters could subsist. In fact, *in vitro* NO donors increase the erythrocyte deformability [5], whereas the NOS inhibitors reduce this rheological determinant [5, 6]. In our study no correlation between EI and some parameters of OSAS severity (AHI, mean nocturnal SO_2) was found while other authors noted a correlation between erythrocyte deformability and nocturnal minimal SO_2 [42]. If we

consider that, up to now, in OSAS subjects neither an alteration of osmotic fragility of erythrocytes [30] nor abnormalities of red cell metabolism [12, 30] or erythrocyte membrane peroxidation [12, 30] have been demonstrated, we must suppose that the behavior of erythrocyte deformability is due to exogenous factors, such as hydrogen concentration, NO or intermittent oxygen desaturation, although we did not observe any statistical correlation between some of these factors and the EI. Considering the several cardiovascular complications accompanying OSAS, this haemorheological alteration, that influences the microcirculation, seems to assume a particular role. In the next future, it will be useful to evaluate if also erythrocyte deformability might be improved by cPAP treatment.

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