Title: **EFFECTS OF CPAP UPON OXIDATIVE STRESS AND NITRATE EFICIENCY IN SLEEP APNOEA. A RANDOMIZED TRIAL**

Brief title: Alonso-Fernández-OSAS and oxidative stress and NO

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ABSTRACT

Background: Previous studies present contradictory data concerning obstructive sleep apnoea syndrome (OSAS), lipid oxidation and nitric oxide (NO) bioavailability.

Objectives: This study was aimed: (1) to compare the concentration of 8-isoprostane and total nitrate and nitrite (NOx) in plasma of middle aged males with OSAS and no other known comorbidity and healthy controls of the same age, gender and body mass index; and (2) to test the hypothesis that nasal continuous positive airway pressure (CPAP) therapy attenuates oxidative stress and nitrate deficiency.

Methods: In this prospective randomized, placebo-controlled, double-blind, cross-over study, 31 consecutive newly diagnosed middle-aged OSAS men and 15 healthy control subjects were selected. OSAS patients were randomized to sham CPAP and effective CPAP application for 12 weeks. Blood pressure, urinary catecholamine levels and 8-isoprostane concentrations and NOx in plasma were obtained before and after both treatment modalities.

Results: OSAS patients had significantly higher 8-isoprostane concentration (median (interquartile range (IQR)) 42.5 (29.2-78.2) vs. 20.0 (12.5-52.5) pg/ml, p=0.041, Mann-Whitney test) and lower NOx (264 (165-650) vs. 590 (251-1465) μmol/l, p=0.022) than healthy subjects. Body mass index, blood pressure or urinary catecholamines were unchanged by CPAP therapy, but 8-isoprostane concentration decreased (38.5 (24.2-58.7) pg/ml at baseline and 22.5 (16.2-35.3) pg/ml on CPAP, p=0.0001), and NOx increased (280 (177-707) vs. 1373 (981-1517) μmol/l, p=0.0001) after it.

Conclusions: OSAS is associated with an increase in oxidative stress and a decrease in NOx that is normalized by CPAP therapy.

INTRODUCTION

Cardiovascular morbidity and mortality is increased in patients with Obstructive Sleep Apnoea Syndrome (OSAS).[1-6] Oxidative and nitrosative stress are believed to contribute to this association[7-9] because markers of lipid peroxidation appear increased in the plasma, exhaled breath condensate and urine of these patients.[10-14] However, not all studies have been able to replicate these observations.[15-17] Potential confounding factors explaining this discrepancy may include age differences between patients and controls, the smoking history of the patients and/or the presence of co-morbidities such as obesity, chronic obstructive pulmonary disease (COPD) or cardiovascular disease, all of which are common in OSAS and can be associated to oxidative stress by themselves.[2,4,5] Likewise, the effect of continuous positive airway pressure (CPAP) therapy upon these biological abnormalities is also controversial. Whereas some un-controlled studies showed that CPAP therapy decreases oxidative stress in these patients,[10,12,13,18-21] other groups failed to demonstrate any significant change after it.[16,17]

To address these limitations, we designed a prospective, randomized, double-blind, placebo-controlled and cross-over study that sought to: *(1)* compare the concentration of 8-isoprostane and total nitrate and nitrite (NOx) in plasma of middle aged males with OSAS in whom lung and cardiovascular diseases were carefully excluded and in healthy controls of the same age, gender and body mass index; and *(2)* test the hypothesis that CPAP therapy improves oxidative stress and nitrate deficiency in these patients.

METHODS

Subjects and Ethics

Thirty-one consecutive newly diagnosed males with OSAS and 15 healthy male volunteers were included in the study. All patients with OSAS fulfilled all the following inclusion criteria: (1) apnoea-hypopnea index (AHI) $\geq$10 h^{-1}; (2) excessive daytime sleepiness defined by an Epworth scale score $\geq$11 points; and (3) no treatment for OSAS. Inclusion criteria for healthy control subjects were AHI <5 h^{-1} and Epworth sleepiness scale <10. Exclusion criteria for both study groups were: (1) unwillingness or inability to participate in the study; (2) obstructive or restrictive lung disease as identified by pulmonary function testing; (3) use of cardioactive drugs; (4) cardiac rhythm disturbances, including sinus bradycardia and sinus tachycardia; (5) known arterial hypertension, or 24-h blood ambulatory pressure monitoring (ABPM) showing blood pressure values equal to or higher than 135 / 85 mm Hg; (6) left ventricular ejection fraction <50%, ischemic or valve heart disease, hypertrophic, restrictive or infiltrative cardiomyopathy, pericardial disease or stroke, by history, physical examination, ECG, chest radiography, conventional exercise stress testing, and echocardiography; (7) diabetes mellitus, by history or 2 random blood glucose levels $\geq$126 mg/dl; (8) morbid obesity (body mass index >40 Kg/m^2); and/or (9) daytime hypoxemia (PaO2 <70 mm Hg) or hypercapnia (PaCO2 >45 mm Hg). Other exclusion criteria during the study period were: (1) need to change medication; (2) hospital admission for 10 or more days; and (3) average nightly CPAP usage less than 3.5 hours. Control subjects were recruited from a list of healthy subjects from our sanitary area that had had a routine health test in the previous three months. We randomly selected a control subject similar in gender, age ($\pm$ 2 years), weight ($\pm$ 2 Kg) and height ($\pm$ 5 cm) with regard to the two preceding patients included in the study. The study was approved by the Institutional Ethics Committee at the hospital and all subjects gave their written informed consent.

Design

We performed a single-center, prospective, randomized, double-blind, placebo-controlled and cross-over clinical study, in which patients received CPAP and sham therapy[22] for two 12-week periods. In healthy controls measurements were obtained once. At recruitment, ABPM, echocardiography, catecholamines concentration in urine and a sleep study were determined in all participants. Also, after fasting overnight, a venous blood sample (anti-coagulated with dipotassium EDTA, for 8-isoprostane and total nitrate and nitrite concentration (NOx) determinations) was collected in all of them between 08:00 and 10:00 hours. Within 30 minutes of blood collection, plasma was obtained by centrifugation at 3000 rpm for 15 min. All plasma samples were stored at −60°C until analysis.

Patients with OSAS underwent a full-night CPAP titration study at home using an automated pressure setting device (Auto Set; ResMed, Sydney, Australia).[23] Patients were given detailed instructions on the appropriate use of the CPAP equipment and were randomized to receive either effective or sham CPAP therapy during 12 weeks. The sham CPAP device was a modified conventional CPAP device, in which the exhalation port diameter had been enlarged to nearly cancel nasal pressure and an orifice resistor was connected between the tubing and the CPAP unit that loads the blower with the same airflow resistance as in effective CPAP.[22] No information about the type of therapy they were receiving was given. Compliance with therapy was obtained from a built-in run-time counter. After 12 weeks, CPAP device was switched to the alternate mode of therapy and ABPM, and plasma and urine sampling were repeated (Figure 1).

Measurements

A validated portable recording device (Sibel Home-300; Sibel S.A., Barcelona, Spain)[24] that records oronasal airflow using a thermistor and nasal cannula prongs, chest wall impedance, oxygen saturation, snoring and body position was used to perform a sleep study in patients and healthy controls. Respiratory events were classified as either obstructive or central on the basis of presence or absence of respiratory effort. Respiratory events were scored as apnoeas when there was a cessation of oronasal airflow lasting ≥10 seconds. Hypopnea was defined as a decrease of 50% in oronasal airflow lasting >10 seconds, associated with a fall in arterial oxygen saturation (SaO_2) >4% of the preceding baseline level. Mean nighttime SaO_2, minimum SaO_2 (lowest values recorded during sleep), desaturation index and percentage of time with SaO_2 <90% on nocturnal oximetry were computed as indexes of nocturnal oxygen saturation.

24-h Ambulatory pressure monitoring (ABPM) was obtained using an oscillometric method (Spacelabs device, model 90207, Redmond, WA, USA).[25] Blood pressure was measured every 30 min during the day (08:00am to 11:00pm) and every 60 min during the night (11:00pm to 8:00am) on a workday. An appropriate cuff was placed on the non-dominant arm. Patients were instructed to carry out their ordinary daily activities, to go to bed no later than 11:00 pm and not to move their arm during recordings.

Echocardiography was performed in the supine and left-lateral positions after a minimum rest period of thirty minutes, using high-quality echocardiograph with 2.0 to 4.0 MHz probes (Hewlett Packard Sonos 5500, Andover, MA). Echocardiographic images were obtained in the paraesternal long and short axes, apical two chamber and four chamber, and subcostal views, using two-dimensional, M-Mode and Doppler echocardiographic techniques. The parameters were measured from at least three cardiac cycles. All echocardiograms were performed by the same experienced echocardiographer, who was unaware of both the subject's group and the patient's treatment assignment at each visit. Systolic function was

assessed by left ventricular shortening fraction (LVSF) and left ventricular ejection fraction (LVEF).[26] LVSF ≥28% and LVEF ≥50% were considered normal.

Forced spirometry was performed as previously described using a MasterScope system (Jaeger, Würtzburg, Germany).[25]

In each visit, subjects were requested to collect separate urine samples from 8 AM until going to bed (day) and all urine during the night and the first after getting up in the morning (night). The urinary excretion of norepinephrine and epinephrine were determined as previously described.[27]

A specific enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) was used to measure 8-isoprostane concentrations in plasma. The intra-assay and interassay variability were 5% and 7%, respectively, and the detection limit of the assay was 5 pg/mL.
Total nitrate and nitrite (NOx) were measured in plasma by capillary electrophoresis using a Beckman capillary electrophoresis system (P/ACE MDQ). Absorbance was read at 200 nm, and nitrate concentration was determined using a NaNO3 standard. The lower detection limit was 10 µmol/L, and the intra-assay and inter-assay variability were 5% and 8%, respectively.

Statistical Analysis
Values are expressed as mean ± SD or median (interquartile range, IQR) depending on their distribution. All statistical tests were two-sided. Comparisons between groups were performed using the t-Student test or the U-Mann-Whitney test. Categorical variables were compared using the chi-square test. Bivariate relationships between variables were determined by Pearson's or Spearman's correlation. To investigate the effect of the CPAP therapy over time in patients with OSAS we used repeated-measures ANOVA with treatment (effective CPAP vs sham CPAP) as a within-subject factor and order of therapy as a between-subject factor. Whenever ANOVA results indicated the existence of significant differences between treatment conditions, post-hoc multiple comparisons were performed with the Bonferroni test. A p value lower than 0.05 was considered statistically significant. All analyses were performed using the Statistical Package for the Social Sciences for Windows Release 11.0 software (SPSS Inc., Chicago, IL).

RESULTS
Characteristics of the Subjects
Figure 1 presents subjects flow chart. Seven subjects refused to take part in the study. Three patients deemed ineligible for inclusion after initial assessments (one had diabetes mellitus and two because they had unknown mitral stenosis), therefore 31 OSAHS patients were randomized.
Table 1 shows the main demographic and functional characteristics of all participants at baseline. Demographic data, smoking habits, spirometric results and ABPM results were not different between patients with OSAS and healthy controls. As expected, sleep parameters were abnormal (and consistent with the diagnosis of OSAS) in patients, whereas they were normal in healthy subjects. Patients with OSAS showed higher nocturnal levels of norepinephrine and epinephrine than control subjects but LVSF and LVEF were similar in both groups.
Plasma concentrations of 8-isoprostane and total nitrate and nitrite (NOx)
At recruitment, before treatment with CPAP, patients with OSAS showed higher plasma levels of 8-isoprostane (42.5 (29.2-78.2) vs. 20.0 (12.5-52.5) pg/ml, p=0.041) and lower NOx concentration (264 (165-650) vs. 590 (251-1465)µmol/l, p=0.022) than healthy subjects. We

did not find any correlations between 8-isoprostane or NOx plasma levels and sleep parameters.

Effects of CPAP

Six patients failed to complete the trial, leaving 25 patients for the final analysis. Two of them were excluded because, on average, they use CPAP at night for less than 3.5 hours, whereas the remaining four patients were lost for follow-up (Figure 1). The baseline anthropometric characteristics, smoking habit, lung function data, sleep study indices, sympathetic tone, ABPM or left ventricular systolic function of these five patients were not significantly different from those who completed the trial. Mean CPAP pressure value was 10 ± 2 cm H_2O and average nightly use of CPAP and sham CPAP was 6.2 ± 1.1 and 6.3 ± 1.6 h, respectively.

Body mass index (BMI), ABPM and urinary catecholamines were not modified by CPAP (Table 2). However, effective CPAP (but not sham CPAP) significantly decreased 8-isoprostane concentration (from 38.5 (24.2-58.7) to 22.5 (16.2-35.3) pg/ml, p=0.0001) and increased NOx concentration in plasma (from 280 (177-707) to 1373 (981-1517) µmol/l, p=0.0001) (Figure 2). The decrease of 8-isoprostane concentration was similar in the OSAS subgroup treated with optimal CPAP and sham CPAP than in de OSA patients treated with sham CPAP and optimal CPAP (18.3 [1.1-25.0) vs. 21.6 (4.0-25.6) pg/mL, p=0.276). Indeed, the increase in NOx concentration was not affect by the treatment order (827 (563-974) vs. 961 (630-1309) µmol/l, p=0.921).Values after CPAP therapy were not significantly different from those determined in healthy subjects.

DISCUSSION

Our study provides two main findings of interest. First, the plasma concentration of 8-isoprostane is higher and that of NOx is lower in patients with OSAS carefully selected to exclude pulmonary and cardiovascular disease than in healthy subjects matched for gender, age and body mass index. Second, these abnormalities are normalized after 12 weeks of CPAP therapy. Despite that some previous studies have provided similar results, to the best of our knowledge, ours is the first prospective, randomized, placebo controlled, cross-over study that evaluates oxidative stress and NO bioavailability in patients with OSAS and the effects of CPAP therapy on these parameters. Our results, therefore, provide a definite answer to some, still open, questions in this field.

Oxidative stress

Many previous studies have identified an association between OSAS and cardiovascular disease.[1,3] Oxidative stress is often proposed as a likely pathogenic mechanism.[28,29] However, earlier studies of lipid peroxidation in OSAS provide conflicting results. Some of them could not find differences in the susceptibility to lipid peroxidation in these patients.[15-17] This is best exemplified by the study of Svatikova et al.[16] who reported that the concentration of thiobarbituric acid-reactive substances, oxidized LDL autoantibodies and free isoprostanes levels in 41 severe OSAS patients without cardiovascular comorbidity were similar to those of 35 healthy controls matched for BMI and age. However, they did not measure lung or left ventricular function, and controls were recruited from those who attended their sleep unit and proved to have an AHI lower than 5, albeit they had a relatively high sleep fragmentation index (22 ± 4 h^{-1}). Moreover they employed a split-night protocol which is in our opinion unsuitable to investigate biomarker changes in these patients.[16] On the other hand, several other studies reported abnormal lipid peroxidation in OSAS.[10-14] For instance, Carpagnano et al.[11] showed increased levels of 8-isprostane in plasma and exhaled breath condensate in OSAS than controls, and Minoguchi et al. demonstrated that the urinary

levels of 8-isprostane in OSAS were related to the AHI.[14] Isoprostanes are a complex family of compounds produced from arachidonic acid via a free radical catalyzed mechanism.[30] They are often used as clinical markers of lipid peroxidation in human diseases because their stability and specificity.[29] We found that patients with OSAS in whom pulmonary and cardiac co-morbidities were carefully excluded had higher 8-isprostane plasma levels than healthy controls and that CPAP therapy (but not treatment with sham CPAP) normalized these values. Results of studies investigating the effects of CPAP therapy upon oxidative stress in OSAS also yield conflicting results. On the one hand, several uncontrolled studies showed that short,[11] medium[12,14,21] and long term[10,13,20] CPAP reduces oxidative stress in OSAS patients. In addition, Barceló et al.[18] found that treatment with CPAP during one year increases the plasma antioxidant status. In contrast, other groups failed to demonstrate that CPAP had any effect whatsoever upon oxidative stress and antioxidant capacity.[16,17] Differences between studies may be due to the effects of several potential confounders and the fact that none of them was controlled by sham CPAP. In our study, we sought to avoid these limitations by selecting participants (both patients with OSAS and controls) very carefully, such that all of them were free of any other comorbid disease and none was receiving medication, neither before nor during the study period. Further, we recruited into the study healthy subjects in whom subclinical pulmonary or cardiac disease was carefully excluded and, most importantly, we designed a placebo-controlled, cross-over study, which is the most powerful design in order to test the efficacy of any therapeutic intervention. Under these circumstances, our results clearly show that effective CPAP therapy improves oxidative stress in patients with OSAS (Table 2, Figures 2-3). Taken into account all the above mentioned arguments we postulate that our results clearly demonstrate that OSAS produces oxidative stress and that this is treatable with CPAP.

Nitrate and nitrite (NOx) deficiency

Impaired nitric oxide (NO) release from endothelial cells is also regarded as an initiator and promoter of cardiovascular disease in patients with OSAS. Nitrate and nitrite (NOx) are stable derivatives of NO, and their levels reflect overall NO production.[31] Previous studies have demonstrated that NO production is lower in OSAS patients than in controls.[7-9,32] In keeping with these results, we also found that NOx levels were lower in OSAS than in an age and BMI matched control group. The mechanisms explaining this observation are unclear. Hypoxemia is a likely candidate since it is known to suppress the expression of endothelial NO synthase,[33] and previous studies found that NOx was inversely related to nocturnal desaturation,[8,9] and oxygen administration increases serum NOx in OSAS.[9] On the other hand, previous studies have shown that effective CPAP therapy increases nitric oxide levels in OSAS.[7,32] Further, Lavie et al.[32] reported a significant decrease in circulating NO levels after CPAP withdrawal for just one night. Our study confirms these previous results using a highly robust design (randomized, placebo-controlled cross-over trial). Thus, our results demonstrate that treatment with CPAP during three months of CPAP led to significant improvement in NO metabolites compared with placebo.

Strengths and potential limitations

As discussed above, we think that the experimental design (randomized, placebo-controlled cross-over trial) and the carefully selection of patients and controls (in whom co-morbidities were carefully excluded) are the main strengths of our study. Yet, as any study, it has some potential limitations that deserve comment. First, sample size was relatively small. This was due to the difficulty of including only middle-aged patients with newly diagnosed OSAS, having no other diseases and taking no cardiovascular medication. And, second, because our study included only males, our results may not be directly applicable to females.

Conclusions
Our results demonstrate that OSAS causes oxidative stress and reduces NOx bioavailability and also that CPAP therapy normalizes these biological abnormalities.

Disclosures
We have no conflicts to disclose.

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FIGURE LEGENDS

Figure 1. Subjects' flow chart.

Figure 2. Individual plasma 8-isoprostane (A) and nitrate and nitrite (B) concentrations at baseline, after sham CPAP and optimal CPAP in OSAS patients.

TABLE 1: Baseline clinical and functional characteristics of the subjects studied.

	Control subjects (n=15)	OSAS patients (n=31)
Age, yr	48 ± 10	52 ± 13
Body mass index, Kg/m^2	28.7 ± 4.7	30.5 ± 4.0
Smokers , %	27	35
Pack/yr	20 ± 7	20 ± 12
Apnoea-hypopnea index, h^{-1}	3.7 ± 3.3	43.8 ± 27.0∗
Mean SaO_2 , %	94 ± 2	91 ± 6‡
Minimum SaO_2 , %	85 ± 5	72 ± 15‡
CT90%SaO_2	0.0 (0-0.05)	6.3 (2.0-29.8)∗
Desaturation index, h^{-1}	4.0 (3.0-7.5)	41.2 (18.9-63.3)∗
FVC, % pred	99 ± 16	105 ± 17
FEV_1 , % pred	108 ± 16	111 ± 15
FEV_1/FVC, %	88.2 ± 3.6	85.8 ± 7.2
Daytime ambulatory BP, mm Hg		
Systolic	122 ± 9	126 ± 10
Diastolic	78 ± 5	79 ± 6
Nighttime ambulatory BP, mm		
Systolic	110 ± 10	117 ± 11
Diastolic	67 ± 6	70 ± 7
Norepinephrine, µg/g		
Diurnal	22.5 (19.6-30.1)	33.2 (26.6-46.5)
Nocturnal	11.3 (7.2-16.7)	22.2 (13.9-29.4)†
Epinephrine, µg/g		
Diurnal	6.9 (3.4-10.3)	7.2 (4.0-11.8)
Nocturnal	3.4 (2.2-4.9)	6.3 (3.8-9.9)‡
LVSF, %	40.1 ± 4.8	37.6 ± 3.1
LVEF, %	70.1 ± 5.9	67.1 ± 3.7

Values represent mean ± SD or median (interquartile range) depending on the distribution.
SaO_2, oxygen saturation; CT90%, % total time study with SaO_2 < 90%; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second; BP, blood pressure; LVSF, left ventricular shortening fraction; LVEF, left ventricular ejection fraction.
∗ $p<0.001$, † $p<0.01$, ‡ $p<0.05$ (t-Student or U-Mann-Whitney tests).

TABLE 2: Effects of CPAP on BP and urinary catecholamines, oxidative stress and nitrate and nitrite concentration in patients with obstructive sleep apnoea-hypopnea syndrome (OSAS).

	Baseline	Sham CPAP	Therapeutic CPAP
Weight, kg	88.8 ± 14.5	88.6 ± 15.2	88.4 ± 15.8
Daytime ambulatory BP, mm Hg			
Systolic	126 ± 10	126 ± 12	127 ± 9
Diastolic	79 ± 6	77 ± 6	78 ± 5
Nighttime ambulatory BP, mm Hg			
Systolic	118 ± 12	117 ± 12	117 ± 17
Diastolic	70 ± 8	70 ± 6	69 ± 9
Norepinephrine, µg/g			
Diurnal	32.9 (26.6-47.5)	32.2 (25.3-41.8)	32.8 (21.1-37.5)
Nocturnal	20.6 (15.4-32.7)	16.4 (13.9-27.5)	19.8 (14.8-29.4)
Epinephrine, µg/g			
Diurnal	7.0 (3.9-11.5)	7.2 (4.7-11.2)	7.3 (4.9-10.5)
Nocturnal	6.2 (3.9-11.3)	4.6 (3.7-7.5)	5.4 (3.7-10.9)
8-isoprostane, pg/ml	38.5 (24.2-58.7)	42.1 (34.7-50.1)	22.5 (16.2-35.3)†‡
NOx, µmol/l	280 (177-707)	494 (292-940)	1373 (981-1517)†‡

Values represent mean ± SD or median (interquartile range) depending on the distribution.
NOx indicates total nitrate and nitrite concentration in plasma; BP, blood pressure.
† $P<0.001$ vs. baseline; ‡ $P<0.001$ vs. sham CPAP.