Effects of Nasal Continuous Positive Airway Pressure Treatment on Oxidative Stress and Adiponectin Levels in Obese Patients with Obstructive Sleep Apnea

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Key Words

Obstructive sleep apnea • Obesity • Oxidative stress • Adiponectin • Insulin resistance

Abstract

Background: Obesity and obstructive sleep apnea (OSA) are both associated with the prevalence of major cardiovascular illnesses and certain common factors they are considered responsible for, such as stress oxidative increase, sympathetic tonus and resistance to insulin. **Objective:** The aim of the present study was to compare the effect of continuous positive airway pressure (CPAP) on oxidative stress and adiponectin levels in obese patients with and without OSA. **Methods:** Twenty-nine obese patients were categorized into 3 groups: group 1: 10 individuals without OSA (apnea-hypopnea index, AHI ≤ 5) who did not have OSA diagnosed at polysomnography; group 2: 10 patients with moderate to severe OSA (AHI ≥ 20) who did not use CPAP; group 3: 9 patients with moderate to severe OSA (AHI ≥ 20) who used CPAP. **Results:** Group 3 showed significant differences before and after the use of CPAP, in the variables of diminished production of superoxide, and increased nitrite and nitrate synthesis

and adiponectin levels. Positive correlations were seen between the AHI and the superoxide production, between the nitrite and nitrate levels and the adiponectin levels, between superoxide production and the HOMA-IR, and between AHI and the HOMA-IR. Negative correlations were found between AHI and the nitrite and nitrate levels, between the superoxide production and that of nitric oxide, between the superoxide production and the adiponectin levels, between AHI and the adiponectin levels, and between the nitrite and nitrate levels and the HOMA-IR. **Conclusions:** This study demonstrates that the use of CPAP can reverse the increased superoxide production, the diminished serum nitrite, nitrate and plasma adiponectin levels, and the metabolic changes existing in obese patients with OSA.

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Introduction

Obesity is strongly associated with obstructive sleep apnea (OSA). Current criteria identify mild OSA as an apnea-hypopnea index (AHI) of 5–15, moderate OSA as an AHI of 15–30 and severe OSA as an AHI higher than

30 events per hour [1]. Several studies have shown that both the weight excess and the repeated episodes of hypoxia/reoxygenation cause increased production of free radicals [2, 3].

Another condition of morbimortality involving OSA and obesity is insulin resistance. Insulin sensitivity is also influenced by the levels of adiponectin, which, contrary to other adipocytokines, is inversely related to the fat mass, and shows negative correlation with insulin resistance [4]. Moreover, adiponectin displays anti-inflammatory [5] and anti-atherosclerotic properties [6].

Continuous positive airway pressure (CPAP) therapy is widely employed in the treatment of OSA, and its efficacy has been demonstrated in several studies, being considered the treatment of choice for OSA [7–9]. The effects of CPAP in different pathological conditions associated with OSA, such as neuroendocrine function changes [10] and endothelial dysfunction [11, 12], on the levels of resistin, ghrelin and leptin [13–15], and on sympathetic hyperactivity [16] are now being investigated.

The aim of the present study was to compare the effect of CPAP on oxidative stress and adiponectin levels in obese patients with and without OSA.

Subjects and Methods

Subjects

All subjects were selected from the Sleep Laboratory at the Clínica Neurológica Luiz Ataide. They were consecutive patients presenting to the sleep center. The inclusion criteria were obese males (BMI ≥ 30) with suspected OSA with AHI >20 for OSA subjects and AHI <5 for control subjects ($n = 10$). Exclusion criteria included type 1 or type 2 diabetes, acute or chronic inflammatory processes, central sleep apnea or Cheyne-Stokes respiration, no medical history of chronic obstructive lung disease, asthma, thyroid dysfunction, and use of glucocorticoids, β -agonists or β -blockers.

The study was approved by the local Ethics Committee and written informed consent was obtained from each participant. All subjects underwent overnight polysomnography testing (Alice 3®; Healthdyne Technologies, Respironics) at the Clínica Neurológica Luis Ataide Sleep Laboratory. The parameters assessed were: electrocardiogram, electroencephalogram, blood pressure, chest and abdominal movements (plethysmography), peripheral oxygen saturation (pulse oximetry), nasal/oral airflow (thermistor) and body position and snoring (microphone). Apneas were defined as complete cessation of airflow for at least 10 s and hypopneas as reduction of respiratory signals for at least 10 s associated with a minimum of 4% of oxygen desaturation. After polysomnography, subjects who had diagnosis of OSA (AHI >20) were asked to receive CPAP treatment ($n = 29$), at least 4 h per night, during 2 months. Therefore, some of them did not adapt to the device ($n = 10$).

Use of CPAP

After the diagnostic polysomnography, the OSA patients underwent another polysomnography for CPAP (Somnotron 41®; Weinmann) pressure titration (quantifying). Each patient received the least amount of pressure needed for eliminating apneas, hypopneas and snoring in all body positions and in all sleep phases. Initially, the pressure used was 0.4 kPa (4 mbar) and, every 5 min (at least) after the occurrence of obstructive events, the pressure was raised by 0.1 kPa (1 mbar). If such events were aborted in the following 30 min, the pressure was reduced by 0.1 kPa (1 mbar), every 10 min, until obstructive events occurred again, when the entire procedure was repeated. Thereafter, in patients who were treated with CPAP (same machine type used during the titration study), the compliance data (days/time used) were evaluated by the readings of the built-in time counter of the CPAP machine. All subjects underwent clinical assessment and blood testing, as described below.

Blood Sample Collection

Separation of Peripheral Blood Monocytes

Venous blood was drawn in EDTA, between 7:00 and 8:00 a.m., and then diluted in sterile RPMI 1640 culture medium (15 ml of blood plus 15 ml of RPMI 1640), at a room temperature of 22–25°C. To the 30-ml suspension, 15 ml of Histopaque (1077; Sigma) were added and the entire mixture was centrifuged (2–8°C) at 1,400 rpm for 30 min.

The plasma was immediately aspirated, and the cell layer was harvested (PBMC) and transferred to another test tube, into which 15 ml of RPMI 1640 were added; this mixture was centrifuged at the same settings for 10 min. The supernatant was aspirated and discarded. Two more washes with RPMI 1640 were done, each centrifuged for 5 min. The supernatant was discarded and the precipitate resuspended in 2 ml of complete RPMI 1640 culture medium, containing 3% fetal bovine serum and antibiotics (penicillin 100 U/ml and streptomycin 100 μ g/ml). Cell counting was done in a Neubauer chamber, from an aliquot of the cell suspension and 1:10 trypan blue. This dye is used for counting cells and assessing their viability. Cell concentration was adjusted to 2×10^6 cells in 2 ml of medium. The cells were plated in six 35-mm well Falcon plates, in which 2 ml of the suspension (2×10^6 cells in 2 ml of complete RPMI 1640 culture medium) were dispensed in each well. Then, the wells were washed with RPMI 1640 culture medium, for removing nonattached cells. The material was kept at a humid atmosphere, 37°C and 5% CO₂ incubator for another hour.

Superoxide Anion Production

This anion can be detected by using its capacity of chemically reducing an electron acceptor compound though the action of ferricytochrome C [30 mg/ml in Hanks' balanced salt solution (Gibco), 2.4×10^{-3} M, equine mitochondria ferricytochrome C, type 3 (Sigma)]. Superoxide dismutase (SOD, bovine erythrocyte superoxide dismutase enzyme; Sigma) containing 3,000 μ g/mg of protein in final 3 mg/ml distilled water solution was used.

To assess the production of superoxide (SO), 2 discontinuous analysis systems were prepared, with the first assessment done right after the beginning of cell stimulation with phorbol myristate acetate (PMA; Sigma) and a second assessment, 2 h later.

For preparing these systems, cultured monocytes were used, with the addition of SOD to the first system (negative control), distilled water to the second (positive control). They were kept in a 37°C and 5% CO₂ humid atmosphere incubator for 10 min for SOD activation. Cytochrome C and PMA were added to the medium (PMA was prepared in a concentrated solution of 3,000 µg/ml in sodium dimethyl-sulfoxide (Sigma), diluted to a 2-µg/ml solution in 2,145 ml Hanks' balanced salt solution and put in the wells of the culture plate.

Cytochrome C and PMA were also added in the second system, containing distilled water. After that, 600-µl samples were drawn concomitantly from each system and put in Eppendorf tubes. The first aliquot collected relative to the time zero of each system is the blank. The subsequent sample was collected in a 2-hour interval in the same conditions. The samples were centrifuged at 10,000 rpm for 5 min at room temperature, in a microcentrifuge (25,000 g, rotor Ra-1M; Kubota).

The supernatant was used to determine the degree of ferricytochrome C reduction (relative to the production of the SO radical); it was transferred to a 1-ml quartz cuvette and read in a spectrophotometer with wavelength adjusted for the red band (550 nm).

The final calculation was done with the formula: $[O_2] = K \times OD \times V$, where K is the constant calculated for the final volume in each well, OD is the optical density and V is the sample volume.

In the present study, K, calculated for the final volume in each well, was 205.49.

Nitric Oxide Production in Cultured Monocytes Treated with *Escherichia coli* Lipopolysaccharide

In each group, the concentration was adjusted to 1×10^6 cells in 1 ml of culture medium in each well. After this, the cells were treated with lipopolysaccharide 10 µg/ml for 24 h. Nitric oxide (NO) release was assessed by the method of Griess. This method indirectly quantifies NO production by determining nitrites and nitrates accumulated in the supernatant, after the treatment. The samples and the standard (NaNO₂ 1 mM, 251-4; Sigma) were placed in a vinyl plate at fixed concentrations (1.56–50 µM), diluted in RPMI 1640 (50 µl per well). After, 50 µl Griess reagent (1 g sulphanilamide, Sigma 9251; N-(naphthyl) ethylenediamine dihydrochloride, Sigma 5889 0.1 g; phosphoric acid PA 2.5 ml and distilled water q.s.p. 100 ml) were added. The plate was incubated for 10 min in the dark. Readings at 540 nm were done in ELISA reader (Dynatech MR 5000). The test sensitivity threshold was 1.56 µM.

Insulin Resistance

To assess insulin resistance, the HOMA-IR was used, determined from the formula [17, 18]: $HOMA-IR = \text{fasting blood insulin } (\mu\text{U/ml}) \times \text{fasting blood glucose (mmol/l)} / 22.5$. The HOMA-IR is strongly correlated with insulin resistance determined by euglycemic clamp [19, 20].

Adiponectin Assay

The plasma adiponectin levels were determined in the beginning of the study and 2 months later, using the Human Adiponectin ELISA KIT (Linco Research). The intra- and interassay coefficients of variation were 5.8 and 7.3%, respectively.

Statistical Analysis

Statistica/W 5.0 was the software used for statistical analysis. ANOVA test was used to assess the differences between and within groups. The Pearson coefficient was used to assess correlations. The results are presented as median and standard error. The level of significance adopted was $p < 0.05$.

Results

The characteristics of the sample are depicted in table 1. There were significant differences in the minimal oxygen saturation and in the AHI between the OSA obese group and the non-OSA obese group. It can also be seen that the BMI values were not significantly different among the groups. Likewise, the BMI at the start and 2 months later were not different within the groups.

Table 2 shows that SO production and HOMA-IR were higher in OSA patients when compared to the control group, while the levels of serum nitrite and nitrate and plasma adiponectin were lower in OSA patients, when compared to the control group.

There were significant differences in SO production (fig. 1), serum nitrites and nitrates (fig. 2), plasma adiponectin levels (fig. 3) and insulin resistance (fig. 4) in OSA patients treated with CPAP for 2 months. It could also be seen that the SO production, the HOMA-IR, the serum nitrite and nitrate levels as well as plasma adiponectin levels in OSA subjects treated with CPAP, after 2 months of CPAP use, were similar to those of the control group.

By analyzing the relationship between AHI and HOMA-IR (fig. 4) in OSA obese individuals, it can be seen that the higher the AHI values the higher the HOMA-IR levels, constituting a positive correlation.

In table 3, the relationship between serum nitrite and nitrate production and SO production ($r = -0.927$; $p < 0.05$), AHI ($r = -0.867$; $p < 0.05$), adiponectin levels ($r = 0.688$; $p < 0.05$) and HOMA-IR ($r = -0.813$; $p < 0.05$) in OSA obese individuals is demonstrated (basal levels), and we found strong correlations between these variables.

The relationship between SO production ($r = -0.927$; $p < 0.05$) and AHI ($r = -0.867$; $p < 0.05$), adiponectin levels ($r = 0.688$; $p < 0.05$) and HOMA-IR ($r = -0.813$; $p < 0.05$) in OSA obese individuals is demonstrated (basal levels) in table 4.

Table 1. Sample characteristics

	Control group (group 1)	OSA group not treated with CPAP (group 2)	OSA group treated with CPAP (group 3)
n	10	10	9
Age, years	56.8 ± 4.7	57 ± 10.5	55.8 ± 10.6
Sex, male/female	10/0	10/0	9/0
BMI	33.1 ± 2.5	34.1 ± 1.3	37.3 ± 4.9
SO production, nmol O ₂ /10 ⁶ PMN	9.6 ± 1.2	15.9 ± 3.7*	14.6 ± 3.5*
Serum nitrite and nitrate production, μM	50.5 ± 2.9	30.3 ± 7.9*	25.8 ± 5.2*
Adiponectin, μg/ml	5.5 ± 0.1	4.6 ± 0.5*	4.5 ± 0.4*
Hypertension	4 (13.8%)	5 (17.2%)	6 (20.7%)
Smokers	1 (10%)	2 (10.5%)	1 (10%)
Coronary artery disease	2 (6.9%)	1 (3.9%)	1 (3.9%)
Minimum O ₂ saturation, %	84.5 ± 3.2	65.3 ± 4.7*	71.3 ± 7.5*
AHI	3.6 ± 0.1	29.5 ± 3.7*	29.9 ± 8.6*
CPAP adherence, hours/night	–	–	5.3 ± 1.7
CPAP adherence, days	–	–	54.2 ± 3.9

PMN = Polymorphonuclear leukocytes. * p < 0.05, significant difference relative to group 1.

Table 2. Correlation between serum nitrites and nitrates production and SO production, AHI, adiponectin levels, O₂ saturation and HOMA-IR in OSA obese individuals

	Serum nitrite and nitrate production, μM	
	r	p
Superoxide production, nmol O ₂ /10 ⁶ PMN	–0.27	0.000000
AHI	–0.867	0.000002
Adiponectin, μg/ml	0.688	0.001127
O ₂ saturation, %	0.671	0.002321
HOMA-IR	–0.813	0.000002

PMN = Polymorphonuclear leukocytes.

Table 3. Correlation between superoxide production and AHI, adiponectin levels, O₂ saturation and HOMA-IR in OSA obese individuals

	SO production, nmol O ₂ /10 ⁶ PMN	
	r	p
AHI	0.726	0.000431
Adiponectin, μg/ml	–0.773	0.000102
O ₂ saturation, %	–0.642	0.002933
HOMA-IR	0.833	0.000009

Discussion

It is believed that the major causative factors of metabolic and cardiovascular dysfunctions induced by OSA are intermittent hypoxemia and the increased sympathetic tonus. Obesity is also associated with a higher prevalence of cardiovascular diseases, and obese individuals have a greater predisposition for developing OSA. A novel and important finding from our study is that the antioxidant/oxidant equilibrium in OSA is disturbed, as the OSA obese patients had lower NO and higher SO levels than non-OSA obese patients.

There may be a synergism between obesity and sleep apnea in the mediation of oxidative stress. Furukawa et al. [21] reported that the oxidative stress in obese individuals is the factor responsible for the establishment of the metabolic syndrome, by the following mechanisms: (1) increased production of free radicals causes disequilibrium in the production of adipocytokines, and (2) the selective increase in oxygen reactive species in areas with fat deposits leads to the increase in systemic oxidative stress.

In the present study, the NO and SO production in OSA obese individuals were correlated with AHI, which highlights the role of the hypoxia/reoxygenation events on the oxidative stress. It could also be observed that the greater the severity of OSA, characterized by high AHI values, the lower the NO production and higher the SO

Fig. 1. SO production in obese individuals without OSA (control group), untreated OSA obese patients and OSA obese patients treated with CPAP for 2 months. NS = Not significant. * $p < 0.05$.

Fig. 2. Nitrite and nitrate levels in obese individuals without OSA (control group), untreated OSA obese patients and OSA obese patients treated with CPAP for 2 months. NS = Not significant. * $p < 0.05$.

Fig. 3. Adiponectin levels in obese individuals without OSA (control group), untreated OSA obese patients and OSA obese patients treated with CPAP for 2 months. NS = Not significant. * $p < 0.05$.

Fig. 4. HOMA-IR in obese individuals without OSA (control group), untreated OSA obese patients and OSA obese patients treated with CPAP for 2 months. NS = Not significant. * $p < 0.05$.

levels. Ip et al. [11] showed an inverse correlation between AHI and NO production in OSA individuals, even after BMI adjustments were done, indicating that OSA causes changes in the synthesis of serum nitrites and nitrates, irrespective of obesity. Concerning SO levels in OSA, Schulz et al. [12] assessed healthy OSA individuals and those with other diseases, and verified that patients with OSA showed higher SO levels than those of the other 2

groups. In the same study, it was also shown that the use of CPAP both in the short term and at follow-up promoted decreases in SO levels.

As to the relationship between the production of NO and SO, and adiponectin, we suggest that the increased levels of adiponectin is one of the changes responsible for reducing oxidative stress. Magalang et al. [22] reported that the treatment of human neutrophils with human ad-

iponectin inhibits the release of SO by these cells, possibly due NADPH oxidase regulation.

There are some studies that assessed adiponectin levels in OSA patients. In the study by Makino et al. [23], the adiponectin levels were lower in OSA patients than in healthy controls, but the OSA severity did not interfere in the adiponectin levels. On the other hand, Wolk et al. [24] found higher adiponectin levels in OSA patients than in healthy individuals, and attributed this finding to a possible activity of adiponectin in attempting to protect the cardiovascular system of these patients.

The present study measured the adiponectin levels in obese patients with OSA and treated with CPAP. In 2004, Zhang et al. [25] found that obese individuals with apnea had lower adiponectin levels, regardless of the degree of obesity. Masserini et al. [26] also found lower adiponectin levels in obese individuals with OSA than in healthy controls, but did not report significant changes in the adiponectin levels of OSA obese patients after the use of CPAP during one night.

There are several studies correlating sleeping disorders with insulin resistance; however, the results about the effects of CPAP on this variable are controversial. Ip et al. [27] studied insulin resistance in patients with mild, moderate and severe OSA, compared to healthy individuals. They demonstrated that insulin resistance was a risk factor for hypertension and could enhance atherogenesis in these patients.

However, West et al. [28] analyzed the effect of CPAP on blood glucose, insulin resistance; glycated hemoglobin and adiponectin in diabetics with OSA, and did not find significant differences in the variables analyzed after the use of CPAP for 3 months (3.6 ± 2.8 h per night).

The number of hours of CPAP use may be a determining factor in the results obtained. In the present study, the device was used for 5.3 ± 1.7 h per night. Studies on the minimum CPAP time per night are scarce [29]. According to Campos-Rodriguez et al. (2005) [30], the use of CPAP for, at least, 1 h per night is efficacious in reducing the mortality rate, but for improving the clinical variables, the longer the CPAP is used per night, the greater the benefits.

As to the relationship between insulin resistance and OSA, a significant correlation was found between AHI and HOMA-IR (fig. 4). Punjabi et al. [31] showed that the severity of OSA is related with the HOMA-IR. Studies like the ones by Larsen et al. [32] in high altitudes and Braun et al. (2001) [33] in hyperbaric chambers corroborate the role of hypoxia in the establishment of insulin resistance. They reported that hypoxia lowers insulin

sensitivity by 50%. Moreover, there is an increased production of inflammatory mediators such as IL-6, TNF- α , hypoxia-induced factor-1 and NF- κ B, which impair the metabolic actions of insulin, contributing to the development of insulin resistance.

Another factor that influences the occurrence of insulin resistance in OSA is sympathetic hyperactivity. Regarding the intermittent hypoxia episodes, CNS activation occurs to promote wakening. Also, sleep fragmentation acts on the hypothalamic-pituitary axis promoting increased release of serum catecholamines and activation of the rennin-angiotensin-aldosterone system, causing sodium retention and increase in blood volume [34, 35].

The increase in sympathetic activity also affects the adipocytokines [36, 37]. According to Fasshauer et al. (2001) [38], β -adrenergic stimulation inhibits the mRNA expression of adiponectin in mice adipocyte cultures.

The dependent relation between obesity and insulin resistance in OSA is still controversial. In the present study, the presence of sleep disorders worsens insulin resistance irrespective of obesity. Similar results were found by Ip et al. [27] and Punjabi et al. [39], who identified the insulin resistance present in OSA as a factor independent from obesity. Gruber et al. (2006) [40] reported that OSA is independently related with the metabolic syndrome, but not with insulin resistance, suggesting that in apneic patients factors other than insulin resistance would be the major ones responsible for the pathophysiology of the metabolic syndrome.

We acknowledge that there are a number of limitations to our study. The main limitation of this study was the lack of randomization. Second, we studied a relatively small number of patients, but we could detect the differences between study participants. In addition, we could analyze a nonobese sleep apnea group.

According to the results of the present study, it may be concluded that:

- (1) OSA increases the inflammatory state in the presence of obesity;
- (2) obese individuals with OSA have insulin resistance, increased production of SO and lower serum adiponectin as well as nitrite and nitrate levels;
- (3) the changes of insulin sensitivity, adiponectin levels, and serum nitrites and nitrates as well as SO production in OSA occur independently from the degree of obesity;
- (4) the use of CPAP can reverse the reduction of plasma adiponectin as well as serum nitrite and nitrate levels, the increased SO production and improve insulin resistance occurring in OSA obese patients.

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