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# Oxidative stress in obstructive sleep apnoea

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**Aims** Any sustained elevation of oxidative stress in patients with obstructive sleep apnoea (OSA) might help explain their increased risk for cardiovascular diseases. We tested the hypothesis that measures of oxidative stress are increased in otherwise healthy subjects with OSA when compared to closely matched OSA-free control subjects.

**Methods and results** Plasma indices of oxidative stress and lipid peroxidation [thiobarbituric acid-reactive substances (TBARS), oxidized LDL (oxLDL), isoprostanes] were measured in 41 moderate-severe OSA males without other diseases and in 35 matched controls first before sleep, then after 4 h of untreated OSA, and again in the morning after 4 h of effective treatment with continuous positive airway pressure (CPAP). Plasma levels of oxLDL, TBARS, and isoprostanes in OSA patients ($n = 34$, 26, 17, respectively) were comparable to the controls ($n = 28$, 27, 15 for the three markers, respectively). Neither untreated OSA nor CPAP treatment nor normal sleep affected levels of any of the three measures of oxidative stress. There was no association between the severity of sleep apnoea and any measure of oxidative stress.

**Conclusion** Otherwise healthy OSA patients, without any other co-morbidities, do not manifest evidence for higher oxidative stress and lipid peroxidation. Thus, oxidative stress and lipid peroxidation do not appear to be key mediators of increased cardiovascular disease in OSA patients.

## Introduction

Increased oxidative stress has been associated with development of cardiovascular and cerebrovascular diseases.[1–3] Oxygen-free radicals, produced within the vasculature, contribute to the pathogenesis of hypertension, coronary artery disease, stroke, chronic heart failure, diabetes, chronic inflammatory diseases, and neurodegenerative disorders such as Alzheimer's disease.[4–8]

Obstructive sleep apnoea (OSA) is associated with increased cardiovascular morbidity,[9–11] independent of obesity and percentage of body fat.[12,13] Patients with OSA experience repeated episodes of cessation of breathing, which leads to hypoxia and reoxygenation. These events may represent a form of oxidative stress leading to increased generation of reactive oxygen species (ROS) that could injure the vascular endothelium[14] and thus may contribute to the association between OSA and cardiovascular disease.[15,16] Furthermore, therapies directed at opposing oxidative stress[17,18] may be a potential strategy for intervention in the large population of OSA patients who do not tolerate standard therapy with continuous positive airway pressure (CPAP).

Whether there is indeed increased oxidative stress in OSA is controversial.[19–29] Some studies suggest that lipid peroxidation is present in sleep apnoea. Barcello *et al.*[19] and Lavie *et al.*[20] observed higher levels of thiobarbituric acid-reactive substances (TBARS) in patients with OSA than in controls. *In vitro* studies by Dyugovskaya *et al.*[21] showed increased adhesion molecule expression and production of ROS in leukocytes of OSA patients. Schulz *et al.*[22] demonstrated enhanced neutrophil superoxide release in OSA patients when compared with controls, which was reversed by CPAP therapy.[22] Further, Saarelainen *et al.*[23] reported increased oxidized LDL (oxLDL)-autoantibodies in OSA patients and Carpagnano *et al.*[24,25] observed elevated 8-isoprostane levels in OSA patients when compared with obese or healthy subjects.

However, there are also contrary observations on oxidative stress and OSA. *In vivo* studies by Wali *et al.*[26] did not show differences in susceptibility of LDL to oxidative stress between OSA patients and controls and Ozturk *et al.*,[27] by evaluation of glutathione, lipid peroxidation concentration, and osmotic fragility of red blood cells, also recently failed to support the notion of increased oxidative stress in OSA patients. Further, Muns *et al.*[28] found no differences in the number of blood neutrophils in OSA patients, nor in their oxidative burst activity/capacity when compared with healthy controls.

Thus, there appear to be inconsistencies in prior studies of oxidative stress in OSA patients. These conflicting results may be explained by several factors including the presence of co-morbidities and/or medications in OSA patients, both

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of which can have significant effects on measurements of oxidative stress; the absence of controls matched closely for BMI and obesity, as obesity may elicit oxidative stress independent of OSA; the presence of undiagnosed OSA in control subjects; and finally, the timing of oxidative stress measurements. It is important to differentiate between any acute effect of hypoxaemia immediately resulting from apnoeic sleep and any chronic state of heightened oxidative stress that may be sustained in OSA patients even during the daytime.

We therefore tested the hypothesis that plasma indices of lipid peroxidation, oxLDL, TBARS, and isoprostanes, are increased in otherwise healthy subjects with OSA, when compared with closely matched control subjects, proved to be free of OSA. Any sustained elevation of ROS in OSA patients might help explain their increased prevalence of cardiovascular diseases.

## Methods

Subjects undergoing sleep polysomnography were recruited from the Mayo Clinic Sleep Disorders Center. The night before polysomnography was performed, the study personnel screened subjects who did not have any co-morbidities, were taking no medications, and were non-smokers. Informed written consent was obtained. The following morning, after completing overnight polysomnography and obtaining the subject's complete sleep report, we identified the subjects either as healthy controls [Apnoea–Hypopnoea Index (AHI) $\leq$5 events/h) or as OSA patients (AHI $\geq$20 events/h]. AHI was calculated as the total number of apnoeas and hypopnoeas per hour of sleep. We excluded subjects with mild OSA (AHI $>$ 5 and $\leq$20 events/h) or subjects with sleep disturbed breathing other than OSA and subjects with unexpectedly high cholesterol levels or co-morbidities that were not identified during the initial screening process. Selection of only obese and overweight controls helped assure comparability between groups.

By this selection process, 12 subjects were excluded because the diagnostic sleep study revealed the presence of mild sleep apnoea or other sleep disturbances or because the subjects had unexpected co-morbidities. As a result, we studied 41 males with newly diagnosed OSA, who were free of other diseases, had never been treated for OSA, and were taking no medications, and 35 healthy males of similar age and body mass index (BMI), in whom occult OSA was excluded by overnight polysomnography. All participants were non-smokers and fasted for at least 4 h before the first blood draw. The study was approved by the Human Subjects Review Committee.

Sleep studies followed a split-night protocol according to the standard of care in our institution. The first half of the study was for the diagnosis of OSA. A therapeutic CPAP trial followed in the second half of the night. In moderate-severe OSA patients, plasma levels of oxLDL ($n = 34$), TBARS ($n = 26$), and isoprostanes ($n = 17$) were measured at 9 p.m. (before sleep), at 2 a.m. (after 4 h of untreated OSA, before CPAP therapy started), and at 6 a.m. (after waking in the morning, after 4 h of CPAP treatment). Measurements were obtained at similar times in control subjects (oxLDL, $n = 28$; TBARS, $n = 27$; isoprostanes, $n = 15$).

Plasma oxLDL was assessed by enzyme-linked immunosorbent assay (ELISA) (Mercodia oxLDL ELISA kit).[30] The intra-assay precision values (co-efficient of variation) for oxLDL at 38 and 81 U/L were 6.7 and 9.3%, respectively. The inter-assay precision values at 41 and 80 U/L were 12.5 and 10.1%, respectively. Serial dilution of plasma samples for oxLDL demonstrated that the method was linear down to at least 5 U/L. TBARS levels were determined by a standard colorimetric method.[31] Free isoprostanes in plasma were measured using extraction and enzyme immunoassay procedures described in the isoprostanes measurement kit (Cayman Chemical). The intra-assay co-efficient of variation for free isoprostanes was 2.25 $\pm$ 0.6%, whereas the inter-assay co-efficient of variation was $<$10%. The lower limit of detection for isoprostane assay was 5 pg/mL.

Results are reported as mean $\pm$ SEM, except for values of the three markers of oxidative stress. OxLDL, TBARS, and free isoprostane levels are reported as mean and 95% confidence interval. Continuous variables were compared between groups using one-way analysis of variance (ANOVA). A split-plot analysis of variance for repeated measures was used to test the hypotheses about the group means and their interactions with time. All statistical tests used were two-sided. Bonferroni's method was used to account for the inflation of the experiment-wise type I error due to multiple comparisons. The following section reports *P*-values for split-plot ANOVA for repeated measures associated with group–time interaction. Statistical significance was defined as $P < 0.05$.

## Results

The OSA patients and control groups were very similar with regard to demographics, haemodynamics, and metabolic characteristics (including lipid levels) (*Table 1*). OSA subjects suffered a severe hypoxic burden, with nocturnal oxygen saturation falling to a nadir of 78 $\pm$ 1%. AHI after CPAP treatment in the OSA patients decreased from 47 $\pm$ 3 to 5 $\pm$ 3 events/h. Within each substudy (i.e. oxLDL, TBARS, isoprostanes), OSA patients and control subjects had similar age and BMI, and the severity of OSA was also similar in each substudy. Plasma oxLDL levels were similar in patients with moderate-severe OSA and in the controls at all three time points [mean and 95% confidence interval: 9 p.m.: 46 (40, 52) vs. 50 (44, 56) U/L; 2 a.m.: 44 (38, 50) vs. 45 (39, 51) U/L; 6 a.m.: 47 (41, 53) vs. 47 (41, 53) U/L, respectively; $P = 0.26$] (*Figure 1*). The levels of TBARS in sleep apnoeics were also similar to that in controls [mean and 95% confidence interval: 9 p.m.: 6.0 (5.0, 7.0) vs. 6.3 (5.3, 7.3) nmol/mL; 2 a.m.: 5.7 (4.9, 6.5) vs. 5.8 (5.0, 6.6) nmol/mL; 6 a.m.: 6.3 (5.5, 7.1) vs. 6.3 (5.5, 7.1) nmol/mL in OSA and control subjects, respectively; $P = 0.65$] (*Figure 2*). Baseline plasma-free isoprostane levels in 17 OSA patients were again comparable to the control group [36 (28, 44) vs. 34 (26, 42) pg/mL, respectively; $P = 0.74$]. These isoprostane levels in sleep apnoeic patients remained comparable to the control group throughout the night [2 a.m.: 39 (33, 45) vs. 38 (32, 44) pg/mL; 6 a.m.: 42 (34, 50) vs. 37 (29, 45) pg/mL, respectively; $P = 0.76$) (*Figure 3*).

## Discussion

In the present study, to quantitatively assess the degree of lipid peroxidation and oxidative injury *in vivo*, we analysed three markers of oxidative stress, namely, oxLDL, TBARS, and isoprostanes. LDL is generally believed to be important in the development of atherosclerosis, and its atherogenicity may be due to oxidative modifications.[32] OxLDL, if produced in high concentrations, has toxic effects on endothelial cells. Its quantification is a common method to assess lipid peroxidation.[30,33] Oxidative injury can be assessed further by quantification of TBARS.[34,35] TBARS are one of the earliest markers of lipid oxidation in human studies and are used as predictors for atherosclerosis. Isoprostanes, produced by free radical-induced peroxidation of arachidonic acid, have also been proposed as a sensitive measure of oxidative stress.[36] Acute hypoxic events along

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**Table 1** Baseline characteristics of OSA patients and controls

| | Moderate-severe OSA patients ($n = 41$) | Controls ($n = 35$) | *P*-value |
|---|---|---|---|
| Demographics | | | |
| Age (years) | 47 ± 2 | 47 ± 2 | 0.96 |
| BMI (kg/m$^2$) | 33 ± 1 | 31 ± 1 | 0.06 |
| Systolic BP (mmHg) | 132 ± 2 | 133 ± 2 | 0.66 |
| Diastolic BP (mmHg) | 79 ± 2 | 79 ± 2 | 0.94 |
| Heart rate (b.p.m.) | 74 ± 2 | 70 ± 2 | 0.13 |
| Biochemical measurements | | | |
| HDL (mg/dL) | 40 ± 2 | 42 ± 2 | 0.56 |
| LDL (mg/dL) | 111 ± 8 | 114 ± 8 | 0.77 |
| Triglycerides (mg/dL) | 225 ± 40 | 258 ± 40 | 0.62 |
| Creatinine (mg/dL) | 1.2 ± 0.1 | 1.0 ± 0.05 | 0.08 |
| Glucose (mg/dL) | 99 ± 3 | 102 ± 3 | 0.41 |
| Diagnostic sleep study | | | |
| AHI (events/h) | 47 ± 3 | 4 ± 3 | <0.0001 |
| Arousal index (events/h) | 51 ± 4 | 22 ± 4 | <0.0001 |

Values are mean ± SEM.

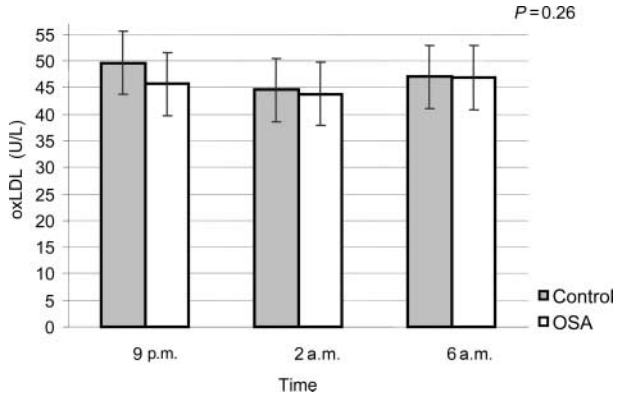


**Figure 1** Plasma oxLDL in 34 OSA males before sleep, after 4 h of untreated OSA, and after 4 h of CPAP treatment compared with 28 control subjects in whom blood samples were taken at similar time points. CPAP was applied between 2 and 6 a.m. Data are mean and 95% confidence interval. *P*-value is for ANOVA for repeated measures associated with group–time interaction.

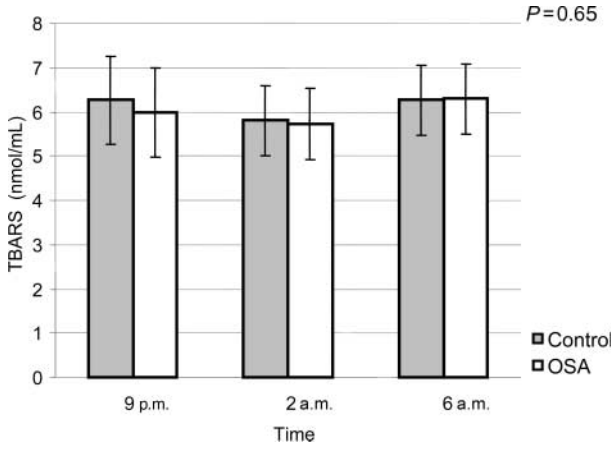


**Figure 2** Plasma TBARS in 26 moderate-severe OSA patients before sleep, after 4 h of untreated OSA, and after 4 h of CPAP treatment compared with 27 healthy males. Data are mean and 95% confidence interval. *P*-value is for ANOVA for repeated measures associated with group–time interaction.

with other stresses have been previously shown to increase the burden of these tissue and plasma oxidative stress markers within short periods of time.[37–42]

In our present study, we have shown that patients with moderate-severe OSA (characterized by elevated AHI and high arousal index) do not have evidence for greater oxidative stress and lipid peroxidation than healthy normal subjects. The mean levels of oxLDL, TBARS, and isoprostanes in otherwise healthy patients with OSA were comparable to the control group. Furthermore, neither untreated OSA nor CPAP treatment nor normal sleep acutely affected plasma levels of any of the three measures of oxidative stress. There was no association between the severity of sleep apnoea and any measure of oxidative stress.

We anticipated that the indices of oxidative stress would be increased in OSA patients for several reasons. First, sleep apnoeic patients are subjected repetitively each night to disturbed hypoxic sleep.[43] These episodes of hypoxia/reoxygenation could facilitate free radical production, which would propagate lipid peroxidation and vascular damage. Secondly, increased inflammatory leukocytes in OSA patients have been shown to trigger free radical production.[21] Thirdly, catecholamine-induced changes, secondary to increased sympathetic nerve activity in OSA,[44] can promote lipid peroxidation. Finally, long-term sleep deprivation, also a cardinal feature of OSA, has been shown to activate lipid oxidation, inhibit antioxidant defence systems, and inactivate mitochondrial enzymes.[45]

The role of free radical scavengers in sleep apnoeic patients is unclear. Our present study was not structured to measure antioxidants. However, two preliminary reports suggest that antioxidant defence mechanisms are unaffected or even decreased in OSA.[26,46] Therefore, the absence of any evidence for oxidative stress in our patient population is unlikely to be explained by a compensatory increase in the activity of antioxidative enzymes in OSA patients. Christou *et al.*[46] showed that in 14 patients with severe OSA (AHI > 20), antioxidant capacity was reduced.

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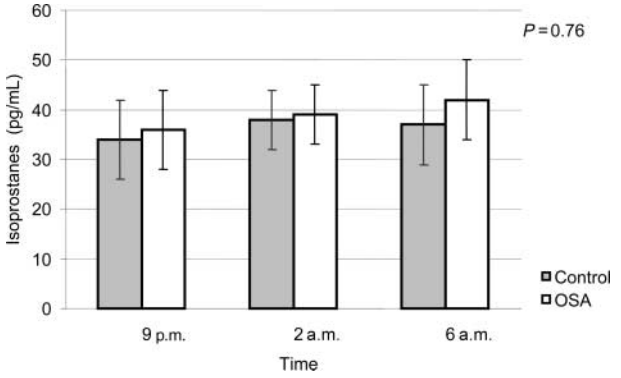


**Figure 3** Plasma free isoprostanes in 17 moderate-severe OSA patients before sleep, after 4 h of untreated OSA, and after 4 h of CPAP treatment compared with 15 healthy control subjects. Data are mean and 95% confidence interval. *P*-value is for ANOVA for repeated measures associated with group–time interaction.

However, both the OSA and control groups included smokers and ex-smokers. Wali *et al.*[26] showed no significant differences in glutathione peroxidase and catalase activities in red blood cells in hypoxic and non-hypoxic patients. CPAP therapy also did not elicit any change in antioxidant enzymes. Therefore, the lack of increased oxidative stress and lipid peroxidation in our sleep apnoeic patients suggests that in the absence of significant co-morbidities, sleep apnoea does not, in and of itself, initiate the generation of oxidative stress or lipid peroxidation. However, it is possible that in the setting of co-morbidities such as hypertension, vascular disease, and the metabolic syndrome, the oxidative consequences of sleep apnoea may become apparent.

Strengths of our study are, first, that in contrast to many prior studies, we included only normotensive, newly diagnosed, and untreated OSA patients, who had no co-existing diseases apart from OSA. Secondly, the patients and controls were not taking any medications or vitamins and were not smokers. Thirdly, the control subjects had similar age and BMI, thus ruling out any potential confounding influence of age, and especially obesity, on our data. Finally, complete overnight polysomnography excluded any possibility that occult sleep apnoea in our obese control subjects could be obscuring differences in oxidative stress between our OSA patients and control subjects. A potential limitation of this study is the split-night protocol, which may have underestimated the effects of CPAP therapy. We did not observe any acute or chronic effects of OSA on any of the indices of oxidative stress. Other studies have reported increased levels of oxidative stress at 9 p.m. before sleep in OSA patients with cardiovascular comorbidities;[19] however, oxidative stress was not evident in our otherwise healthy OSA patients at any time of measurement. A further limitation is that plasma isoprostanes were not measured in all subjects. However, in our study, isoprostane measurements were performed only to further confirm the absence of any evidence of oxidative stress in OSA, as had been suggested by the negative results of TBARS and oxLDL measurements.

## Conclusion

In otherwise healthy subjects, OSA is not associated with abnormal lipid peroxidation or other measures suggesting increased oxidative stress. Our data do not support the notion of increased oxidative stress mediating the development of cardiovascular and cerebrovascular diseases reported in OSA patients.

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