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Original article

Assessment of oxidative stress markers and hearing thresholds in patients with obstructive sleep apnea-hypopnoea treated with cysteine and superoxide dismutase therapy

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Summary. *Background and aim of the work:* In OSAHS, the hypoxia and reoxygenation cicles, maintain a state of oxidative stress, which seems to cause a change in the oxidative balance. Our aim is to compare the markers of oxidative stress with audiological findings and OSAHS severity, in OSAHS patients untreated and also treated ones, with cysteine and superoxide dismutase. *Methods:* 65 patients (42 Men, 23 Women) with 30-65 years age range have been enrolled, with a mean age of 52.6 ± 13.3 years with moderate OSAHS. We have analyzed plasma and lymphocyte markers of oxidative stress (glutathione, thioredoxin and heat shock protein) and they were underwent tonal audiometry. Patients were divided in two groups: Group A (32 patients) included patients treated for 8 weeks with cysteine and superoxide dismutase; Group B (33 patients) included patients untreated. *Results:* The research showed a significant increase in reduced glutathione levels ($p<0.05$) in OSAHS patients treated; conversely, it showed a decrease of oxidized glutathione level ($p<0.05$) in treated patients than OSAHS untreated ones. The thioredoxin values, in untreated OSAHS patients, appear to be reduced than in OSAHS patients treated ($p<0.05$), and that the heat shock protein values were more elevated in untreated OSAHS patients ($p<0.05$). Finally, it was found that a correlation exists between the severity of OSAHS and auditory dysfunction. *Conclusions:* The study of the oxidative stress markers has produced results which lead to support the idea that, in a personalized therapy context, the use of antioxidant therapy can cooperate effectively the first choice treatment. (www.actabiomedica.it)

Key words: Obstructive Sleep Apnea Hypopnoea Syndrome, chronic intermittent hypoxemia, hearing loss, thioredoxina, heat shock protein, gluthatione, cysteine and superoxide dismutase therapy, antioxidant-nutritional therapy

Introduction

Obstructive Sleep Apnea/Hypopnea Syndrome (OSAHS) is a constantly increasing disease, both for a refinement in diagnostic means (more sophisticated polysomnography, use of drug induced sleep endoscopy, use of questionnaires to screening) and for an increased awareness of the consequences that can afflict OSAHS patients. In fact, the untreated OSAHS patients have an increased risk of neurodegenerative diseases (1, 2), metabolic (3,4) and cardiovascular diseases (5), such as diabetes, hypertension (6), stroke (7) and myocardial infarction (7). One of the most severe signs of OSAHS is chronic intermittent hypoxia, caused by recurrent episodes of partial or complete obstruction of the upper airways during sleep,

with subsequent oxygen desaturation, arousals during sleep (8) and excessive daytime sleepiness. As already seen for other neurodegenerative disorders (9, 10), the hypoxia and reoxygenation cicles maintain a state of oxidative stress, which seems to cause a change in the oxidative balance inducting production of excess reactive oxygen species (2, 11, 12). Furthermore, the hypoxemia determines peripheral nerve damage. In the early stages of ischemic event, mechanisms to reduce peripheral neuropathy are activated but these become inadequate over time, due also to the accumulation of oxidative markers and of chronic hypoxia. An adequate oxygen supply is a necessary condition in order that the transduction mechanism of the inner ear and the transmission of nerve impulses take place properly (13). In these conditions of hypoxia, like OSAHS disease (14), eukaryotic cells have developed mechanism of cellular stress response, in order to control the biochemical balance induced by stress. This mechanism of cellular stress response include the heat shock protein synthesis (15, 16) and the stimulation of the thioredoxin system (17, 18). Together, these two systems, through intracellular and extracellular processes form a complex system, mainly including the redox regulation of gene expression and signal transduction, anti-apoptotic functions, protection against oxidative stress, the regulation of the redox state of the extracellular environment, growth factor and cytokine like effect (19). In addition, is also important to evaluate the glutathione status. In fact, the reduced glutathione (GSH) is considered to be one of the most important scavengers of reactive oxigen species, and its ratio with oxidised glutathione (GSSG) may be used as a marker of oxidative stress. In this study, in OSAHS patients, we have analyzed plasma and lymphocyte markers of oxidative stress such as glutathione, thioredoxin (TRX) and heat shock protein (HSP 72) (9). Furthermore, to evaluate the possible improvement of the redox state of the OSAHS patients, we have administered a nutraceutical treatment to some of them, with cysteine and superoxide dismutase, a product based on milk serum proteins with high cysteine content, aminoacid, a precursor in the synthesis of glutathione and superoxide dismutase; This nutraceutical treatment posseses an activity of "pro-activation", which follows the induction of defense bio-cellular mechanisms. We hypothesized that the GSH and TRX values, in OSAHS patients treated with cysteine and superoxide dismutase, are more elevated than OSAHS patients untreated, and in OSAHS patients untreated the HSP 72 values are more elevated than OSAHS patients treated. Furthermore, the aim is also to assess if a correlation exists between the level of hypoxia induced by OSAHS and auditory dysfunction severity.

Methods

Sixty-five patients were enrolled (42 Men, 23 Women) age range 30-65 years, with a mean age of 52.6±13.3 years (49.4 in men; 55.9 in women) with moderate OSAHS with Apnea Hypopnea Index (AHI) between 16 and 30 events/h. Were excluded from the study subjects that:

- could not tolerate cysteine or superoxide dismutase;
- had a history of treatment for OSAHS or for hearing impairment;
- had an active acute or chronic infection;
- had been diagnosed with a cardiovascular, metabolic or chronic respiratory disease;
- used anti-inflammatory terapy, or lipid-lowering drugs, psychiatric terapy or other medications that lower oxidative stress;
- that used drugs or alcohol abusers.

All patients were subjected to tonal audiometry to assess hearing thresholds. Audiometric threshold was obtained as the pure tone average for the frequencies 0.5-1-2-4 kHz and subdivided in: normal hearing (<20 dB); mild hearing loss (21-40 dB); moderate hearing loss (41-70 dB); severe hearing loss (71-90 dB); profound hearing loss (>90 dB). OSAHS patients were required to complete questionnaires to confirm daytime sleepiness through the Epworth Sleepiness Scale (ESS) (20). OSAHS patients were randomly divided in two groups: Group A (32 patients) included patients treated for 8 weeks with oral intake of cysteine and superoxide dismutase (oral tablets twice daily); Group B (33 patients) included patients without terapy. Blood samples were collected from all the participants. All of the experimental evaluations were repeated 8 weeks after the end of the terapy for all patients.

Sleep Studies

The Polygraphic System Embletta MPR System, according to the American Academy of Sleep Medicine standards (AASM 2012), allows monitoring of Polygraphic cardiorespiratory. Apnea was defined as a significant decrease (>90%) in oronasal flow for at least 10 seconds. Hypopnea was defined as an airflow decrease of 30% for at least 10 seconds with ≥4% desaturation from the baseline, or a decrease of ≥50% from the baseline for at least 10 seconds with ≥3% desaturation from the baseline and/or arousal. An oxygen desaturation event was defined as a ≥3% decrease in oxygen. According to AASM, OSAHS severity was based on the Apnea and Hypopnea events/hour (AHI, Apnea/Hypopnea Index) and was graded as mild (5 AHI 15 /h), moderate (15<AHI 30/h), or severe (AHI>30/h) (21).

Measurement biomarkers of oxidative stress

Blood Sampling

Blood (6 ml) was collected after an overnight fast by venopuncture from an antecubital vein into tubes EDTA. Immediately after sampling, two blood aliquots were separated: first 2 ml were centrifuged at 10.000 g for 1 min at 4°C to separate plasma from red blood cells; the remaining aliquot (4 ml) was utilized for lymphocytes purific. All samples were stored at -80°C until analysis.

Lymphocytes Purification

Lymphocytes from peripheral blood were purified by using the *Ficoll Paque System* following the procedure as suggested by the manufacturer (*GE Healthcare, Piscataway, NJ*).

Western blot analysis

Plasma samples were ready to use, while the lymphocyte pellet was homogenized and centrifuged at 10,000 × *g* for 10 min and the supernatant was used for analysis after dosage of proteins. In brief, an equal amount of proteins (40 μg) for each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred overnight to nitrocellulose membranes, also the nonspecific binding of antibodies was blocked with 3% nonfat dried milk in phosphate-buffered saline. Immunodetection of Hsp72 was performed by using a monoclonal mouse anti-Hsp70 antibody (SPA-810, Stressgen). When probed for TRXr protein, we used a polyclonal rabbit anti-TRXr-1 antibody (07-613, Upstate). All blots were then visualized by using a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG. A goat polyclonal antibody specific for actin was used as a loading control (sc-1615 product of Santa Cruz; 1:1,000 dilution in PBS; pH, 7.5). Immunoreactive bands were scanned by a laser densitometer (*LKB Ultroscan XL, Pharmacia, Uppsala, Sweden*). Molecular weights of the proteins were determined by using a standard curve obtained with proteins of known molecular weight.

Reduced Glutathione and Oxidised glutathione assay

GSH and GSSG were measured by the NADPH-dependent GSSG reductase method, as previously reported (22). Lymphocytes were homogenized on ice for 10 s in 100 m*M* potassium phosphate, pH 7.5, which contained 12 mM disodium EDTA. For total glutathione, aliquots (0.1 ml) of homogenates were immediately added to 0.1 ml of a cold solution containing 10 mM DTNB and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The samples were then mixed by tilting and centrifuged at 12,000 g for 2 min at 4°C. An aliquot (0.05 ml) of the supernatant was added to a cuvette containing 0.5 U of GSSG reductase in 100 mM potassium phosphate and 5 mM EDTA, pH 7.5. After 1 min of equilibration, the reaction was initiated with 220 nmol of NADPH diluted for a final reaction volume of 1 ml. The formation of a GSH-DTNB conjugate was then measured at 412 nm. The reference cuvette contained equal concentrations of DTNB, NADPH and enzyme, but not the sample. For assay of GSSG, aliquots (0.5 ml) of homogenate were immediately added to 0.5 ml of a solution containing 10 mM N-ethylmaleimide (NEM) and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The sample was mixed by tilting and centrifuged at 12,000 g for 2 min

at 4°C. An aliquot (0.5 ml) of supernatant was passed at drop/s trough a SEP-PAK C_{18} Column that had been washed with methanol followed by water. The column was then washed with 1 ml of buffer 1. Aliquots (0.865 ml) of the combined eluates were added to a cuvette with 250 nmol of DTNB and 0.5U of GSSG reductase. The assay then proceeded as in the measurement of total GSH. GSH and GSSG *standards* in the ranges between 0 and 10 nmol and 0.010 and 10 nmol, respectively, added to control samples, were used to obtain the relative standard curves, and the results were expressed in nmol of GSH or GSSG, respectively, per mg protein.

Statistic analysis

Continuous variables are expressed as mean ± standard deviation (SD), and categorical variables are expressed as the numbers of individuals and percentages. Significance was set at *P* values of less than 0.05.

Table 2. Hearing loss in group A and group B

Hearing loss	Group A	Group B
Mild	5 (15.6%)	7 (21.2%)
Moderate	3 (9.3%)	3 (9%)
Severe	/	/

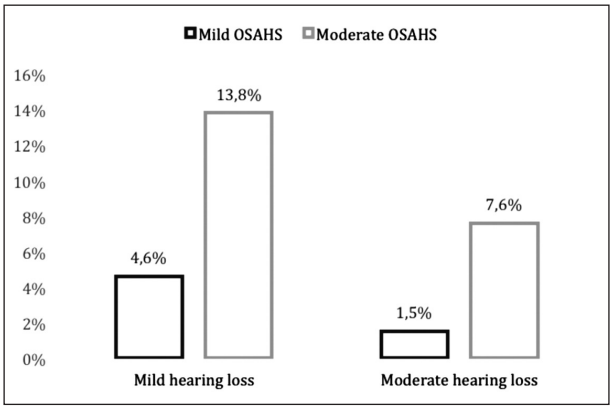

Figure 1. Correlation between OSAHS and hearing loss severity. P<0.05

Results

In group A, after treatment, the scores of ESS questionnaires showed an increase of the patients without daytime sleepiness from 21.8% to 34.3%, with an improvement of the average oxygen saturation, mean of AHI/h, mean of OD/h and mean of lowest oxyhemoglobin saturation (Table 1). However, in group B, has been noticed a slight worsening of the patients without daytime sleepiness from 21.2% to 18.1%, and a worsening of the average oxygen saturation, mean of AHI/h, mean of OD/h and mean of lowest oxyhemoglobin saturation (Table 1). Also, we observed 10 patients in group A and 8 patients in group B with sensorineural hearing loss (Table 2). Among these 18 patients, there was a positive relationship (p<0.05) between OSAHS severity and hearing loss severity (Figure 1). These data were also confirmed by even worse values, in terms of increase of oxidative stress, compared to all patients with OSAHS (Table 3). Moreover, we observed that in lymphocytes of OSAHS patients untreated, there is an increased level of cytoprotective proteins Hsp72 (p<0.05) and a significant decreased levels of thioredoxin reductase (TRX) in peripheral lymphocytes of OSAHS untreated patients compared to OSAHS treated patients (p<0.05) (Figures 2, 3). In addition, we demonstrated that peripheral lymphocytes from OSAHS patients treated showed significantly increased GSH levels (p<0.05) and, conversely, a decreased level of oxidized glutathione (GSSG)

Table 1. Characteristics of Group A and Group B before and after 8 weeks. P<0.05

	Mean BMI	Mean AHI/h	Mean OD/h	Mean SPO_2	Mean-Lowest oxyhemoglobin saturation
Group A baseline	29.2 ± 5.6	23.2 ± 8.1	29,6 ± 18.3	92.6% ± 4.2	81% ± 9
Group A after 8 weeks	28.8 ± 6.4	21.6 ± 9.3	24.8 ± 11.7	93.4% ± 2.8	83% ± 7,8
Group B baseline	29.1 ± 7.8	22,1 ± 9.4	28.6 ± 15.9	93.8% ± 5.3	82% ± 11.2
Group B after 8 weeks	27.1 ± 6.4	22,9 ± 10.2	29.4 ± 16.1	93% ± 5.7	81.3% ± 11

Table 3. Comparision between TRX: thioredoxin means and HSP: heat shock protein means in all groups (A + B) and TRX means and HSP means in groups (A+B) with hearing loss. P<0.05.

	Group A - Before treatment		Group B - Before treatment	
	TRX- T0	HSP- T0	TRX- T0	HSP- T0
Mean	116,18	24,53	118,80	25,618
Mean in hearing loss patients	108,87	33,75	111,71	28,79

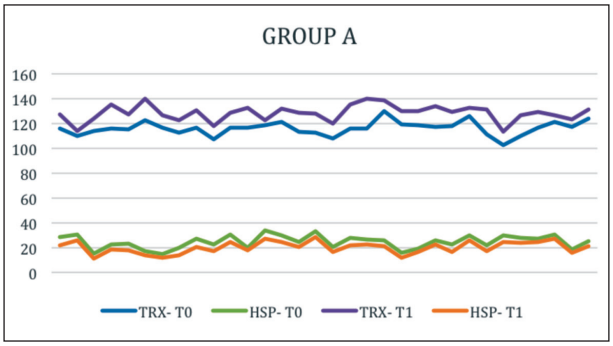

Figure 2. Stress oxidative markers before (T0) and after 8 weeks of treatment (T1) in group A. TRX: thioredoxin; HSP: heat shock protein. P<0.05

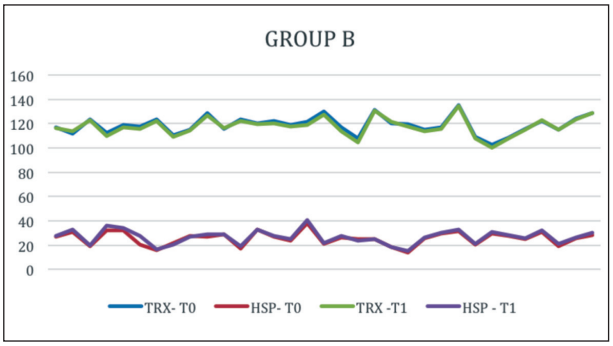

Figure 3. Stress oxidative markers before (T0) and after 8 weeks of treatment (T1) in group B. TRX: thioredoxin; HSP: heat shock protein. P<0.05.

(p<0.05) (Table 4). This results in a significant reduction the GSH/GSSG ratio in lymphocytes of OSAHS untreated patients compared with OSAHS treated patients (p<0.01) (Table 4).

Conclusions

According to some studies (23, 24) which reported increased oxidative stress levels associated to modified tiolic status and reduced plasmatic antioxidant defences in OSAHS patients, our results showed an important involvement of thioredoxin system in the regulation of oxidative balance. Through the results obtained we may consider the OSAHS desease as an important negative factor: at first it may take to an excessive daytime sleepiness and gives possible association with hearing loss induced by hypoxia, while, by molecular appearance, it also alters the balance of oxidative stress markers. If the hypothesis that antioxidant therapy may reduce the seriousness of OSAS -in terms of reduction of daytime sleepiness, increasing in average SPO2 and reduction of AHI- will overtime be confirmed by other studies, it could have profound implications for prevention and/or clinical management. In OSAHS disease, as is clear from the review of Lira and coll. (25), oxidative stress plays a key role, both because the worsening intermittent hypoxia in turn worsens the oxidative balance, and also because today the study of oxidative markers could get the as-

Table 4. Values of reduced glutathione, oxidized and ratio in all groups (A+B) after 8 weeks. GSH: Reduced glutathione; GSSG: oxidized glutathione

	Plasma			Lymphocyte		
	Group A	Group B	P value	Group A	Group B	P value
Total GSH	15.7 ± 4.1	10.2 ± 4.3	p<0.05	12.87 ± 0.5	10.98 ± 0.3	7.7 ± 0.7
GSH	13.88 ± 3.2	9.09 ± 5.8	p<0.05	10.93 ± 0.4	9.82 ± 0.6	5.88 ± 0.5
GSSG	0.346 ± 0.02	0.412 ± 0.001	p<0.05	0.091 ± 0.01	0.081 ± 0.01	0.103 ± 0.01
Ratio GSH/GSSG	89 ± 12	65.1 ± 15	p<0.01	132 ± 11.2	121 ± 10	64.7 ± 7.9

sumption that antioxidant therapy definitely makes an important contribution especially in the management of a patient OSAHS for it to be efficient as possible and it should also be multidisciplinary. Therefore, the details of the patient's choice of the therapeutic plan should lead to a choice increasingly targeted and personalized therapy, and that can not be ignored even by the assessment of an antioxidant treatment choice, which would be of assistance and cooperation to the first treatment chosen.

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