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Investigation of *O*-Alkyl/*O*-Alkenyl Glycerolipid Ratios in Erythrocyte Lysates of Patients with Obstructive Sleep Apnea

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ABSTRACT

Background: Vulnerabilities of plasmalogens in erythrocyte membrane to hypoxic conditions and alteration induced by sleep restriction make them potential biomarkers for the evaluation of sleep apnea related sleep disorders.

Objective: We aimed to investigate and to compare ratios of alkyl/alkenyl glycerolipids of erythrocyte membranes (representing plasmanyl/plasmenyl plasmalogens) between controls and patients with sleep apnea and to evaluate possible differences, if any, which could be considered as a diagnostic tool.

Material and methods: The patients underwent polysomnography and categorized according to the severity of sleep apnea using Apnea-Hypopnea Index (AHI). Phospholipids in erythrocyte lysates were hydrolyzed by phospholipase C and the acylgroups were saponified. Then the *O*-alkyl/*O*-alkenyl glycerolipids were separated by thin-layer chromatography (TLC). Densitometric image analyses were performed on the lipid spots of TLC plates and the ratios were determined.

Results: No significant correlation was observed between the alkyl/alkenyl glycerolipid ratios of the eryhtrocyte lysates of sleep apnea patients and that of controls.

Conclusion: Results of this study warrants reinvestigation of alkyl/alkenyl glycerolipid ratios in erythrocytes of patients with sleep apnea, with each subjects sampled before and after the treatment of sleep apnea to better understand the potential of this ratio as a diagnostic tool.

Keywords: circadian rhythm; erythrocyte; obstructive sleep apnea; plasmalogen; hypoxia; thin-layer chromatography

Obstrüktif Uyku Apneli Hastaların Eritrosit Lizatlarında *O*-Alkyl/*O*-Alkenil Gliserolipid Oranlarının Araştırılması

ÖZET

Giriş: Eritrosit membran plazmalojenlerinin uyku apnesinin yol açtığı hipoksik şartlara ve uyku bozukluklarına bağlı değişimlere duyarlı olması, uyku apnesine bağlı uyku bozukluklarının değerlendirilmesinde onları potansiyel bir biyobelirteç yapmaktadır.

Amaç: Uyku apnesine bağlı uyku bozukluğu olan hastalarda ve kontrollerde eritrosit alkyl/alkenyl gliserolipid oranlarını (plasmanyl/plasmenyl plasmalojen) araştırarak karşılaştırmak ve varsa muhtemel farklılıkları tanı vasıtası olarak değerlendirmek.

Materyal ve metod: Hastalara polisomnografi uygulandı ve Apne-Hipopne Indeksi (AHI) kullanılarak uyku apnelerinin şiddetine göre kategorize edildiler. Eritrosit lizatındaki fosfolipidler fosfolipaz C ile hidrolize edilerek açil grupları saponifiye edildi. *O*-alkil/*O*-alkenil gliserolipidler ince tabaka kromatografisi (TLC) ile ayrıldı. TLC plakaları üzerindeki lipid bantları dansitometrik olarak analiz edilerek oranlar belirlendi.

Sonuçlar: Uyku apneli hastaların eritrosit lizatlarında *O*-alkil/*O*-alkenil gliserolipidler oranları ile kontroller arasında istatiksel olarak anlamlı bir korelasyon bulunamadı.

Sonuç: Bu çalışmanın sonuçları, uyku apneli hastaların eritrosit lizatlarında *O*-alkil/*O*-alkenil gliserolipidler oranlarının tedavi öncesi ve tedavi sonrası dönemde tespit edilerek karşılaştırılmasının, bu oranın bir tanı vasıtası olarak potansiyelinin daha iyi değerlendirilebilmesi için gerekli olduğuna işaret etmektedir.

Anahtar kelimeler: sirkadiyen ritim; eritrosit; obstrüktif uyku apne; plazmalojen; hipoksi; ince tabaka kromatografisi

Obstructive sleep apnea (OSA) is a sleep disorder pathogenesis of which is thought to be connected with intermittent hypoxia during period of apnea or hypopnea inducing oxidative stress and systemic inflammation with cardiovascular consequences (1). There have been some works indicating an association between sleep apnea and circadian rhythm disturbances. Hypoxia in OSA has been reported to cause an increase of HIF-1α which in turn disrupts the circadian rhythm in vitro (2). The plasmalogens are among the most altered plasma lipids as a function of sleep restriction (3-5).The authors related the metabolite changes to PPARα and more generally peroxisome involvement in sleep restriction (4).

Plasmalogens are a class of membrane glycerophospholipids. Both choline and ethanolamine glycerophospholipids in mammalian tissues consist of three subclasses; 1,2-diacyl-sn-glycero-3-phosphocholine (or ethanolamine), 1-alkyl-2-acyl-sn-glycero-3-phosphocholine (or ethanolamine), 1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphocholine (or ethanolamine). Those glycerophospholipids with alk-1-enyl groups at the sn-1 position of the glycerol are also referred to as plasmalogens (choline plasmalogens, ethanolamine plasmalogens) (6). In the course of synthesis in peroxisomes, plasmanyl plasmalogens which include an alkyl chain at sn-1 position of glycerol backbone are oxidized by a desaturase to yield the vinyl ether double bond in alkenyl chain of plasmenyl plasmalogens (7). Therefore, alkyl glycerolipids are considered as biosynthetic precursors of alkenyl glycerolipids (8) and as a consequence, alkyl/alkenyl glycerolipid ratio could be indicative of plasmalogen synthesis. We hypothesized an alteration in plasmalogen content of erythrocytes of patients with obstructive sleep apnea due to hypoxia and/or sleep disorder related circadian disturbances. Besides, plasmalogen levels in erythrocyte lysates are used as an diagnostic tool in inherited peroxisomal diseases (e.g. Rhizomelic chondrodysplasia punctata, Zellweger disorder) (9). To this end, we aimed to compare, for the first time, alkyl/alkenyl glycerolipid ratios in erythrocyte lysates of control and patients with sleep apnea associated sleep disorder groups and to evaluate the ratio as a potential biomarker for the diagnosis of obstructive sleep apnea conditions.

MATERIALS AND METHODS

Subjects and Experimental Design

The procedures were performed in accordance with the guideline set by İnönü University, Malatya Clinical Research Ethical Committee (Protocol number: 2016/198). The patient group consisted of 30 (22 male or 8 female) subjects. aging between 25-60. The control group included 30 age interval-matched subject (12 male and 18 female).

Inclusion criteria for the subjects: Subject is male or female between the age of 25 and 60 and provides written informed consent. Patient subject is having obstructive sleep apnea syndrome (OSAS). Exclusion criteria for subjects: Subject has an genetic disease, currently using CPAP machine, a history of malignancy or any autoimmune disease, undergone any major surgical procedure or trauma, any current medical condition which could interfere with the evaluation of the subject and control subject is currently having sleep disorders.

Blood samples were obtained from patients admitted to Sleep Disorder Clinic of Department of Chest Diseases, Turgut Özal Medical Center (Malatya/Turkey) and diagnosed as OSAS by polysomnography. General characteristics of the study subjects were given in Table 1.

Sleep Parameters

Patients underwent polysomnography in the hospital using a digital polysomnographic monitor (Alice 6 LDx Diagnostic Sleep System, Philips, Germany). The sleep records were scored and evaluated by an experienced, registered polysomnologist following the American Academy for Sleep Medicine 2012 criteria (10). An apneic episode was defined as a ≥90% reduction ofairflow lasting ≥10 seconds and hypopnea was characterized by a ≥30% reduction in breathing amplitude lasting ≥10 seconds and accompanied by an oxygen desaturation ≥ 3%. The sum of apnea and hypopnea events divided by the number of hours of sleepwere defined as apnea hypopnea index (AHI). The patients were categorized according to severity of sleep apnea by AHI into three groups as mild sleep apnea: AHI 5-15/h; moderate sleep apnea: AHI: 16-29/h and severe sleep apnea: AHI ≥ 30/h.

Sample Preparation

Preparation of Erythrocyte Lysates and Lipid Extraction

Blood samples were taken into tubes containing EDTA and centrifuged at 1500 g for 10 min at 15°C. After the removal of the plasma and buffy coat, erythrocytes were diluted

with an equal volume of 0.9% NaCl and centrifuged at 900 g for 10 min. Having discarded the supernatant, lower fraction was taken to Eppendorf tubes containing 1 mg of BHT (2,6-di-tert-butyl-4-hydroxytoluene) and equal volume of saline was added and vortexed. Then the resultant erythrocyte suspensions were kept at -80°C until analyzed. The method of Bligh and Dyer (11) was used for the lipid extraction of 1mL of the erythrocyte lysates. The final extracts were evaporated under nitrogen and resuspended in 0.2 mL of chloroform and kept at 4°C until analyzed.

Hydrolysis of Phospholipids by Phospholipase C

To the extract from 1 mL of erythrocyte was added 2 mL of diethyl ether and vortexed for two min. Phospholipids in the extracts were hydrolyzed using *C. perfringens* phospholipase C (14 U/mL) in 0.5 mL of 50 mM potassium phosphate buffer, pH 7.0. Following the vortexing, the tubes were placed on a roller mixer and incubated for 3.5 h at room temperature before centrifugation at 1500 g for 7 min at 15°C. The upper diethyl ether phase was taken to another tube and evaporated to dryness under nitrogen.

Saponification of Acyl Groups

To evaporated extract of phospholipid hydrolysis was added 2.5 mL of 0.5 M KOH in methanol. Following vortexing, the tubes were heated in a boiling water bath for 10 min. The tubes were then allowed to cool and 4.5 mL of 6% glacial acetic acid in methanol were added. The contents were transferred to vial using 7 mL of chloroform. Samples were left stand overnight after adding 6 mL of water. The lower chloroform layer was taken to a tube. Then the upper phase extracted again with another 7 mL of chloroform. The combined chloroform extracts were evaporated to dryness, redissolved in 0.2 mL of chloroform and stored at 4°C.

Separation of O-Alkyl and O-Alkenyl Glycerolipids by TLC

TLC plates (Merck KGaA TLC Silica gel 60, 105721, Darmstadt, Germany) were used to separate and identify alkyl and alkenyl glycerolipids. 20x20cm TLC plates were activated at 110°C for one hour. 1-*O*-hexadecyl-sn-glycerol was used as control standard. The chromatography was conducted as described elsewhere (12). Chloroform/methanol/acetic acid (98:2:1; v/v/v) was used as mobile phase. After separation of alkyl and alkenyl glycerolipids, the plates were sprayed with an aqueous solution of 10% copper (II) sulphate in 8% phosphoric acid and charred at 180°C for 20 mins.

Densitometric Image Analysis of *O*-Alkyl and *O*-Alkenyl Spots on TLC Plates

Images of the TLC plates were loaded in file format of TIFF and the spots were located manually on the images. After background correction, the spots were densitometrically analyzed and the data were converted to raw volumes by the SYNGENE GeneTools quantification software (GeneTools software, version 4.03.05, Syngene, Cambridge, UK). The densitometric raw volumes determined was used as a measure of spot signal intensity in arbitrary units (a.u.).

Statistical Analysis

Analysis of covariance (ANCOVA) was used to perform comparisons based on the alkyl/alkenyl ratio whilst controlling for covariates. Age and body mass index (BMI) were considered as covariates and gender was considered as a random factor. The group to be compared was taken as a fixed factor in ANCOVA. For the other independent group comparisons, age and BMI were always considered as covariate and gender considered as random factor where appropriate in ANCOVA. In tables only the significance of the interested group variable was presented as a result of ANCOVA. Basic characteristics of the groups based on continuous variables were compared by independent samples t test. For categorical variables continuity-corrected chi-square was used and distribution of these data was represented by count and percentage. In all analysis two-tailed significance value was considered to be 0.05. IBM SPSS Statistics for Windows version 22.0 (NY, USA) was used for analyses.

RESULTS

Comparison of Subject Characteristics for Control versus Patients with OSA Group

30 patients with obstructive sleep apnea (OSA) diagnosed by overnight polysomnography and 30 control subjects were included in the study. Comparison of gender, age, BMI, medication, cigarette smoking and AHI values (where applicable) characteristics of both control and patients with OSA groups were depicted in Table 1. Some of the patients in OSA group suffered from chronic diseases such as hypertension (n=7), diabetes (n=7), coronary heart disease (n=1), thyroid disease (n=4) and hypertension + diabetes mellitus (n=4) and as a result they were taking some medications. Average age of OSA group was found to be 10 years higher compared to that of control group. BMI of OSA group was also significantly higher (8 kg/m^2) compared to average control values. Of the patients, 11 and 18 were diagnosed as moderate and severe OSA. Only one of the patient's AHI indicated mild OSA.

Table 1. Basic Characteristics of Study Subjects						
		Control Group		Patient Group		
		n	%	n	%	p
Gender*	Female	18	60.00	8	26.66	0.019
	Male	12	40.00	22	73.33	
Smoking Status*	Current	10	33.33	9	30.00	=1.000
	Never	20	66.66	21	70.00	
Medication*	User	-	0.00	15	50.00	<0.001
	Nonuser	30	100.00	15	50.00	
Age (Mean±SD)**		30	36.20±7	30	49.70 ± 7.8	<0.001
Body Mass Index (Mean±SD)**		30	24.71± 3.44	30	32.38 ± 6.05	<0.001
Apnea-HypopneaIndex (Mean±SD)		-	-	30	42.16±20.58	-

*Continuity corrected chi-square
**Independent samples t test

Table 2. The Impact of OSA on Age and BMI Adjusted Erythrocyte Alkyl/Alkenyl Ratios of Subgroups			
	Sub-group	**Mean±SD**	**Test Statistics and p Values***
Total	Control (n=30)	6.92±3.91	$F_{group} = 2.380$
	Patient (n=30)	6.24±3.46	$p_{group} = 0.157$
Female	Control (n=21)	7.05±4.29	$F_{group} = 1.273$
	Patient (n=8)	6.36±4.81	$p_{group} = 0.270$
Male	Control (n=9)	6.60±3.04	$F_{group} = 0.209$
	Patient (n=22)	6.20±2.97	$p_{group} = 0.651$
Control	Female (n=21)	7.05±4.29	$F_{gender} = 0.002$
	Male (n=9)	6.60±3.04	$p_{gender} = 0.968$
Patient	Female (n=8)	6.36±4.81	$F_{gender} = 0.009$
	Male (n=22)	6.20±2.97	$p_{gender} = 0.925$
Control	Never smoked (n=20)	7.17±4.41	$F_{smoking\ status} = 0.013$
	Current smoker (n=10)	6.41±2.77	$p_{smoking\ status} = 0.934$
Patient	Never smoked (n=21)	6.75±3.80	$F_{smoking\ status} = 1.232$
	Current smoker (n=9)	5.06±2.26	$p_{smoking\ status} = 0.350$
Never Smoked	Control (n=20)	7.17±4.41	$F_{group} = 0.003$
	Patient (n=21)	6.75±3.80	$p_{group} = 0.959$
Current Smoker	Control (n=10)	6.41±2.77	$F_{group} = 0.974$
	Patient (n=9)	5.06±2.26	$p_{group} = 0.493$
Patient	AHI<30 (n=12)	6.55±3.97	$F_{AHI} = 3.585$
	AHI≥30 (n=18)	6.04±3.18	$p_{AHI} = 0.251$
Patient	No medication (n=15)	5.80±3.07	$F_{medication} = 2.204$
	Medication user (n=15)	6.68±3.87	$p_{medication} = 0.522$

*ANCOVA

O-Alkyl/O-Alkenyl Glycerolipid Ratios in Erythrocyte Lysates of Control and OSA Group

Separation of alkyl/alkenyl glycerol bands of an erythrocyte lysate sample on the TLC plate can be seen in Fig.1. Analysis of covariance (ANCOVA) was used to perform comparisions between alkyl/alkenyl ratios of control and OSA group whilst controlling for covariates, age and BMI. Gender was considered as a random factor. The impact of OSA on age and BMI adjusted erythrocyte alkyl/alkenyl ratios in erythrocyte lysates of subgroups were shown in Table 2.OSA group had lower alkyl/alkenyl glycerolipid ratio compared to that of the control group albeit not significantly. The alteration in the ratio could be the result of either decreased level of alkyl glycerolipid or increased level of alkenyl glycerolipids or both. Similarly, lower alkyl/alkenyl gliserolipid ratios in women of sleep disorder group compared to that in women of control group is discernable, albeit insignificant. The same tendency could be observed between men of control and sleep disorder group. Interestingly, sleep disorder patients with AHI ≥ 30 had lower alkyl/alkenyl ratio than those with AHI<30. Moreover, cigarette smoking also appears to influence similarly the ratio either between smokers and non-smokers of sleep disorder patients or between smokers of control and sleep disorder patients, i.e. cigarette smoking appears to lower the ratio possibly in addition to the lowering effect of sleep disorder observed. Although no statistically significant correlation was found between any of the groups in the preceeding data, nevertheless they all point in the same direction.

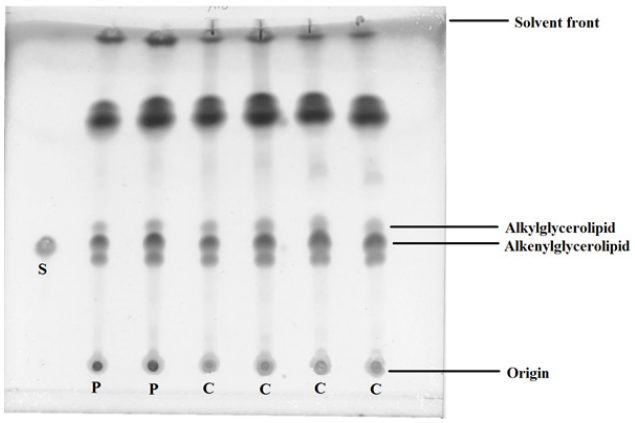

Figure 1. Chromatogram of Alkyl- and Alkenyl Glycerolipids of Eryhtrocyte Lysates
Phospholipids in erythrocyte lysates were hydrolyzed by phospholipase C and the acyl groups were saponified. The lipid extract was separated by TLC using chloroform/methanol/aceticacid (98:2:1; v/v/v) as mobile phase. After separation of alkyl and alkenyl glycerolipids, the plates were sprayed with an aqueous solution of 10% copper (II) sulphate and charred at 180 °C. Photometric image analyses were performed on the lipid spots of alkyl- and alkenylglycerolipids on TLC plates and the ratios were determined. S: Alkylglycerolipid standard (1-*O*-hexadecyl-sn-glycerol), C: Control, P: Patient.

DISCUSSION

The prevalence of OSA in the general adult population (at ≥ 15 events/h AHI) ranges from 6% to 17% which mounts to 49% in the advanced ages. The OSA prevalence is also greater in obese subjects (13). As the obesity epidemic continues to rise, the prevalence of OSA will likely to increase. Cardiovascular, neurocognitive and metabolic outcomes of OSA can adversly affect patients' health and quality of life. Epidemiologic studies suggest that OSA is under-diagnosed (14). Therefore appropriate testing is of importance. In the current work, alky /alkenyl glycerolipid ratios in erythrocyte lysates of patients with sleep apnea associated sleep disorders were compared to that of controls in an attemp to envisage any effect of OSA and to evaluate possible differences.

Mammalian erythrocytes contain both alkyl glyceryl ether and alk-1-enyl glyceryl ether (15). Plasmalogens constitute 15-20% of total phospholipids in cell membranes (including human erythrocytes), with ≥50% of glycerophosphoethanolamines in brain, heart, neutrophils and eosinophils (16). In our work, the alkyl/alkenyl ratio in control group was found to vary between 5.25-6.05 being higher compared to that have been reported in the literature. In human serum (≥40 years old), alkyl/alkenyl choline ratio (μM) was approx. 0.71-0.74, whereas alkyl/alkenyl ethanolamine ratio was 0.07-0.09 (deduced with calculation from the resuts) (17). In human erythrocytes however, alkyl/alkenyl ratio in choline glycerophospholipid was 0.60/0 whereas in ethanolamine glycerophospholipid was 0.60/9.20=0.07 (18). In Connor et al work, average alkylacyl/alkenylacyl ratio was found to be 1.19 in choline glycerophospholipids and 0.96 in ethanolamine glycerophospholipids (value deduced by calculation from the relevant results) (19). The difference between our results and the others' can be attributed to the fact that we measured alkyl/alkenyl glycerolipids as whole whereas others analyzed individual choline and ethanolamine alkyl- and alkenylglycerophospholipids separately.Besides differences in the analysis methods used could also have contributed to the disparities.The techniques used range from LC-MS/MS (17), lipid phosphorus analysis following TLC separation (18) to HPLC analyses (19).Whereas we used densitometric image analysis of lipid spots on TLC plates. Additionally, the extent to which alkyl- and alkenylglycerolipids react with visualization reagent (copper II sulphate) may not be identical.

One aspect of sleep apnea is sleep restriction which is associated with circadian rhythm disturbances (20), whereas the other aspect is the hypoxia stress to which cells expose. Both sleep restriction and hypoxia have been reported to influence tissue lipid/phospholipid compositions including plasmalogens. Lipid peroxidation of erythrocytes due to hypoxia can influence erythrocyte membrane plasmalogen content. Since vinyl ether bond of cellular plasmalogens are highly susceptible to oxidation (21), high ROS levels and oxidative stress were found to be related to the decreased erythrocyte plasmalogen levels in Alzheimer's disease (9). Hypoxic stress was reported to cause an increase in plasmalogen content of red blood cells. (22). Because of OSA, increase in erythropoiesisinduced by hypoxia (23) can also possibly alter erythrocyte membrane lipid compositions The most altered plasma lipids as a function of sleep restriction are various glycerophospholipids including plasmalogens. Researchers observed increased plasma levels of plasmalogens after 5 days sleep restriction in both humans and rats. Metabolite changes in humans points out peroxisome involvement in sleep restriction. Across the both species, various phospholipids were the most elevated as a function of sleep restriction (4). On the contrary, Chua et al reported that plasma choline plasmalogen levels decreased in sleep deprivation (5). However Davies et al also found that 13 glycerophospholipids exhibited significantly increased levels in humans during sleep deprivation (3).

As the preceeding data points out, various factors related to sleep apnea can implement a change in membrane lipid compositions. However in our work, comparisons of alkyl/alkenyl glycerolipid ratios in erythrocyte lysates between control and OSA group produced no significant differences. Some of the characteristics of our study subjects including age, gender, smoking and some diseases were found by other workers to alter plasmalogen content. Plasmalogen in human serum decreases with aging (24). On the other hand smoking is associated with plasmalogen deficiency in humans (25). Additionally, RBC plasmalogen content was observed to be increased in coronary heart disease (22) whereas decreased in diabetic patients (26). On the other hand, no sex-specific differences in human RBC plasmalogen levels or composition were observed (27). Nevertheless, in our work, the impact of OSA on age and BMI adjusted erythrocyte alkyl/alkenyl ratios in erythrocyte lysates produced no statistically significant differences between subgroups.

The findings of this study have to be seen in light of some limitations. BMI and age of control subjects do not match well those of patients with OSA. Since the prevalence in the general adult population is high in the advanced ages and obese men and women, we were unable to find control subjects matched with OSA patients in terms of BMI and age because of time constraint. This might have caused wide variations in alkyl/alkenyl ratios in erythrocytes of subjects. Besides, sensitivity of TLC method used in the current work is not comparable to the modern analytical techniques. Todays, lipidomic analyses by LC-MS/MS offers high sensitivity for detection of low abundance plasmalogen molecular species in human serum which enables measurement of nanomolar concantrations (28). Since dietary plasmalogens were reported to cause increases in relative plasmalogen composition of erythrocyte membranes (29) consumption of foods by subjects with varying plasmalogen level could be another factor causing high variation.

We did not find any significant difference between sleep apnea patients and controls in terms of alkyl/alkenyl ratios in erythrocytes, probably due to high variations between individuals. However, the current results warrant reinvestigation of erythrocyte alkyl-/ alkenyl-glycerolipid ratios in subjects with sleep apnea since we believe that individual variations in lipid parameters could have obscured the possible link between them. In order to overcome this, we suggest that the alkyl/alkenyl glycerol ratios of erythrocytes in samples of the same indiviual taken before and after the treatment of apnea (CPAP therapy) should be compared in order to better understand the potential of this ratio as diagnostic tool.

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DECLARATIONS

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Ethics

All the research procedures were conducted according to guidelines set by İnönü University Malatya Clinical Research Ethical Committee (protocol number: 2016/198).

Conflict of Interest

The authors declares no conflict of interest.

Authors' Contributions

Emine Koç collected the data, contributed the data analysis, performed the analyses, contributed to the manuscript writing. Hilal Ermiş collected the polysomnographic data. Gözde Harika Gözükara Bağ conducted statistical analyses. Muhammed Dündar performed the densitometric image analyses. Tayfun Güldür established the hypothesis, designed the experiments and contributed to the data analyses and the manuscript writing.

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