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The affection of plasma exosomes on airway obstruction and endothelial function in OSA patients

Lin Li[1,2#], Qing Wang[1,2#], Wenshu Xin[1,2], Kaixue Wen[1,2], Yuhao Zhang[1,2*]

[1]*Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, Shanxi, China*

[2]*Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, China*

 # They are both the first author

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ABSTRACT

This study aimed to investigate the effects on airway obstruction and endothelial function by extracting and analyzing plasma exosomes with OSA patients. For this purpose, the clinical data and imaging data of 60 patients with OSA were retrospectively analyzed, who were admitted to the Central Hospital from Apr. 2018 to Jul. 2021. By using an electron microscope for the observation of exosomes, the degree of airway obstruction was compared by pulmonary function instrument, and HE staining was performed to analyze them. The results showed that the diameter of exosome particles was concentrated at 80.5 ~ 158.6 nm, the diameter of OSA exosomes was concentrated at about 121.9 nm, and the diameter of exosomes in the control group was concentrated at about 145.0 nm. Compared with the patients in the control group, the level of miRNA-33b-3p in the control group was significantly different ($P < 0.05$). The content of exosomal miRNA-33b-3p in OSA patients decreased significantly, and the corresponding airway obstruction increased. The results of HE staining showed that there were obvious atherosclerotic plaques in the arterial endothelium of the OSA group, and the atherosclerotic plaques were significantly reduced after miRNA-33b-3p injection ($P < 0.05$). In general, OSA patients can regulate airway obstruction and endothelial cell function by controlling the expression of Plasma exosomes and miRNA-33b-3p, resulting in increased airway obstruction and endothelial cell atherosclerosis.

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Introduction

Obstructive Sleep Apnea (OSA) is a common and complex respiratory disease characterized by repeated complete or incomplete upper airway collapse during sleep and has a high incidence. According to relevant statistics, the incidence rate is about 1%~3% (1) and about 1 billion people around the world are affected (2).

Patients suffer frequent hypoxia during night sleep due to the collapse of the upper respiratory tract due to muscle relaxation in the oropharynx, resulting in inadequate oxygen supply. Snoring is caused by airflow through the narrow upper respiratory tract and is a surrogate marker for OSA. In order to restore oxygen supply, patients constantly wake up from sleep, resulting in sleep interruptions. Patients suffer from intermittent hypoxia and sleep fragmentation during sleep, resulting in daytime sleepiness and cardiovascular damage. But its internal mechanism is not clear. Its severity was measured by the Apnea-Hypopnea Index (AHI), which reflects the number of apnea/hypopnea per hour of sleep (3). The current first-line treatment method for continuous positive airway pressure (CPAP), curative effect is better, but poor patient compliance; it is lack of effective drugs (4).

Extracellular vesicles are phospholipid bimolecular vesicles secreted by cells. Exosomes are a kind of extracellular vesicles with a diameter of about 30-100nm (5), which can be secreted by endothelial cells, macrophages and other cells. The formation process of intracellular vesicles is more complex than that of ordinary extracellular vesicles. First, intracellular vesicles are formed in multi-vesicles endosomes

*Corresponding author. E-mail: Yuhaoz@gmail.com

inside cells and released after the latter is fused with the plasma membrane, namely exosomes (6, 7). In the process of formation, it carries cell-derived active molecules, such as proteins, miRNA, mRNA and DNA, etc. The type and quantity of specific contents are related to the type of cell but are also affected by the physiological or pathological state of the cell. Some studies have found that exosomes can destroy the functional integrity of endothelium (8, 9). At present, exosomes are usually extracted and then observed and studied by Fluorescence microscopy (10).

In this study, plasma exosomes were extracted from OSA patients by membrane affinity method to explore the influence of plasma exosomes on upstream airway obstruction and endothelial function, providing a reference for subsequent clinical treatment. Here's the story.

Materials and methods

General Information

A total of 60 OSA patients admitted to our hospital from April 2018 to July 2021 were selected, including 23 females and 37 males, aged from 15 to 78 years, with an average age of (46±2.1) years. The number of hospitalizations abandoned and referrals were excluded. All patients were confirmed by multichannel sleep recorder (PSG) monitoring and subsequently grouped according to apnea-hypopnea index (AHI) ranges. At the same time, 60 normal people in the outpatient department were selected as a non-OSA control group. There were no significant differences in age, height and weight between the OSA group and control group.

On the morning of the second day, blood was taken from the patient's cubital vein on an empty stomach. 10 mL of venous blood was collected from all the subjects, followed by a 1 000×g centrifugation for 10 min for plasma separation. Then it was centrifuged at 4°C for 15 min at 13 000×g, filtered with a 0.22μm filter, packed by 1 mL/ tube, frozen at -80 °C. The study was approved by the Medical Ethics Committee of the hospital, and all subjects signed informed consent.

Inclusion criteria: Meet OSA diagnostic criteria; Obtain informed consent signed by patients/family members; All patients had symptoms such as snoring, open-mouth breathing and daytime sleepiness.

Exclusion criteria: Accompanied by cerebrovascular diseases, blood system diseases, autoimmune diseases; Patients with acute and chronic systemic inflammatory diseases, chromosomal disorders, neuromuscular diseases, congenital heart disease, liver and kidney defects and other diseases; Patients with other types of sleep disorders; Recent presence of ent surgery, positive airway pressure ventilation treatment, taking drugs to promote sleep and respiratory infections; recurrent chronic tonsillitis; Withdrawal or incomplete clinical data.

Main Instruments

The main used instruments were Zetasizer Nano ZS Zeta Potentiometer, ExoRNeasy Serum/Plasma Midi Kit (QIAGEN), Alice NightOne Polysomnography Monitor (Royal Philips Electronics, Netherlands), and MasterScreen BODY Lung Function Instrument (JAEGER, Germany).

Polysomnography monitoring

The polysleep monitor was used to explain the precautions for detection. The patient's head, face and abdomen were routinely cleaned to avoid irritant food, and the patient was placed in a supine or lateral position. Each lead electrode was connected and fixed firmly according to the operating requirements. Apnea hypopnea index (AHI), oxygen reduction index (ODI), minimum oxygen saturation (SAO2-L), mean oxygen saturation (SAO2-M) and other indicators were automatically analyzed by computer, and sleep events were analyzed by professional sleep monitoring personnel. OSA was diagnosed when AHI > 5 times /h.

Extraction of plasma exosomes by membrane affinity method

The plasma frozen at -80°C was taken out, melted on ice, and 10mL was taken. According to the instructions, the Buffer XBP was mixed with the filtered 10mL plasma sample at 1:1, then transferred to the separation column, centrifuged at 500×g for 1 min, and the waste liquid was discarded. 10 mL Buffer XWP was added to the separation column and centrifuged at 5 000×g for 5 min. The separation column was transferred to a new collection tube, and 100μL PBS was added to the center of the membrane. After incubation at room temperature for 5 min, the

samples were centrifuged at 5 000×g for 5 min. Exosomes were obtained.

Extraction of exosomes

The 20μL exocrine weight suspension drop was placed on a special copper wire for electron microscopy and suspended with 10μL PBS for 3 min. Then, the cells were redyed with 2% phosphotungstate solution and stood at room temperature for 5 min. PBS was replaced to clean the grid three times. The residual excess liquid was dried with absorbent filter paper and stood for 2-3 min under a 60 W incandescent lamp.

Identification and analysis of exosome proteins

The bicinchoninic acid (BCA) method was used for quantitative detection of exosome protein suspension. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page) was followed by wetting for 1.5 hours and 5% BOVINE serum albumin (BSA) was blocked at room temperature for 2 hours. Tris-hydroxymethyl aminomethane-Tween (TBST) buffer solution was washed and primary antibody (1:1000) was incubated overnight at 4°C. TBST buffer was washed and corresponding secondary antibodies were added and incubated at room temperature for 1 h, followed by electrochemiluminescence development.

Dynamic light scattering analysis exosome particle diameter determination

The principle analysis of samples by using the dynamic light scattering particle size distribution of the particles due to Brownian motion in the system of the scattering light intensity change, the detector will be scattered light signal is converted into a current signal, again through the digital correlator algorithm to get the diffusion coefficient of particles in solution, and finally calculate the particle diameter size and its distribution. The prepared exosome suspension was diluted with PBS, mixed evenly by eddy shock, and placed in the sample pool.

Detection of exosome Mir-33B-3p

Rt-PCR was used to detect mir-33B-3p in exosomes from OSA patients and healthy subjects. 200 μ L plasma exosomes were taken and total RNA was extracted with TRIzol reagent. PrimeScript First-Strand cDNA Synthesis Kit (Takara, Japan) reverses into cDNA. PCR was performed using 2.0μ L cDNA template and cDNAs PrimeScript RT Master Mix kit (Takara, Japan): 95°C, 15 min, 94°C, 15 s, 55°C, 30 s, 70°C, 1 min, 40 cycles. The relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method. Determine whether the difference is statistically significant.

MiR - 33-3 p b:

RT CTCAACTGGTGTCGTGGAGTCGG CAATTCAGTTGAGGGGCTGCA

Forward: CAGTGCCTCGGCAGTGC

Reverse: CTCAACTGGTGTCGTGGAGTC

Comparison of the relationship between plasma exosome Mir-33B-3p level and the severity of airway obstruction

Under the guidance of professional technicians, a pulmonary function instrument was used to measure the lung function of the subjects according to the operating guidelines of ATS/European Respiratory Society (ERS), and VC, FVC, FEV1, FEV1% PRED, FEV1 /FVC and other indicators were obtained. According to the detection results, the patients were divided into severe, moderate and mild airway obstruction, and the mir-33B-3p level and lung function test data of the patients were compared, and the difference was analyzed to determine whether there was statistical significance.

Comparison of plasma exosome Mir-33B-3p levels and endothelial cells in patients

After grouping, endothelial cells of patients were collected, and after receiving cells from OSA patients with severe airway obstruction, a part of mir-33B-3p was injected, and the nucleus and cytoplasm were dyed blue purple and red by hematoxylin and eosin dyes, respectively. HE staining can be used to observe vascular endothelial and smooth muscle proliferation and plaque formation. Artery atheromatous plaque under the microscope for dyeing shallow foamy structure, endothelial cells on the patients for regular materials, fixation, dehydration, paraffin embedding, sectioning (5 microns), contrast testing the endothelial cells of microRNAs expression level, analysis of patients and healthy subjects outside the plasma miRNA expression of vascular endothelial cells secrete body exists.

Statistical methods

SPSS 22.0 software was used, and the measurement data were expressed as mean ± standard deviation (X ± S). T-test was used for inter-group comparison with test level α= 0.05. Analysis of variance and SNK-Q test were used for inter-group comparison. Statistical data were compared by χ2 test. Pearson correlation was used for correlation analysis, and P < 0.05 was statistically significant.

Results and discussion

Identification results of exosomes

Under electron microscopy, exosomes were round and oval, with a complete double-layer lipid envelope structure (Figure 1). Exosomes express special proteins such as HSP70, CD9 and CD63 due to their special invagination process. The three special proteins were detected by the WB technique (Figure 2). Determination of exosome diameter by dynamic light scattering method showed that the diameter of exosome particles was concentrated in the range of 80.5 ~ 155.6 nm, among which, the diameter of exosome in OSA patients was concentrated at about 121.9nm, and that in healthy people was concentrated at about 145.0nm, as shown in Figure 3

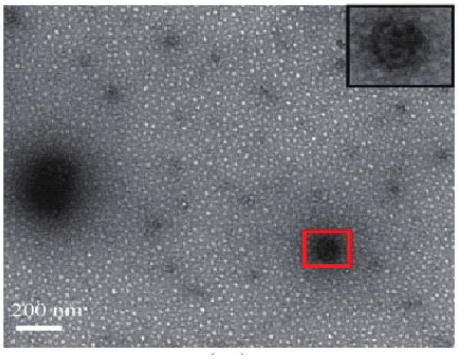

Figure 1. Electron microscopic observation of exosomes

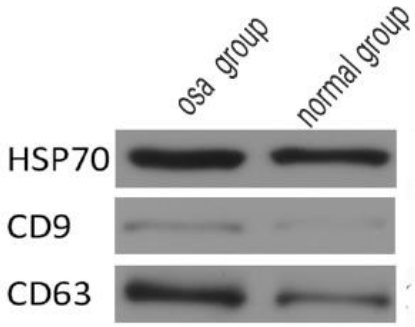

Figure 2. HSP70\CD9\CD63 expression levels in each group

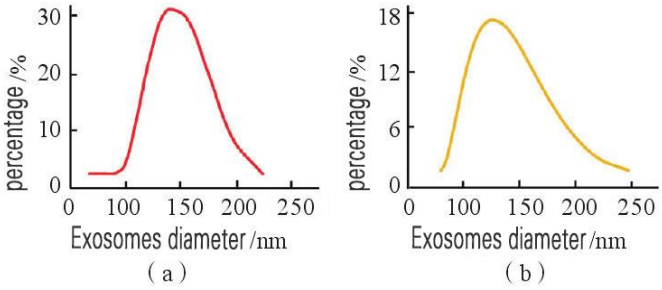

Figure 3. Determination of exosome diameter by dynamic light scattering method
Note: a: NC group，b: OSA group

Identification results of exosome miRNA-33B

Qrt-PCR was used to verify the test results. After random grouping, the SOA group and the control group were tested and it was found that the content of exosome miRNA-33B-3p in OSA patients was significantly reduced compared with that in healthy subjects (Figure 4).

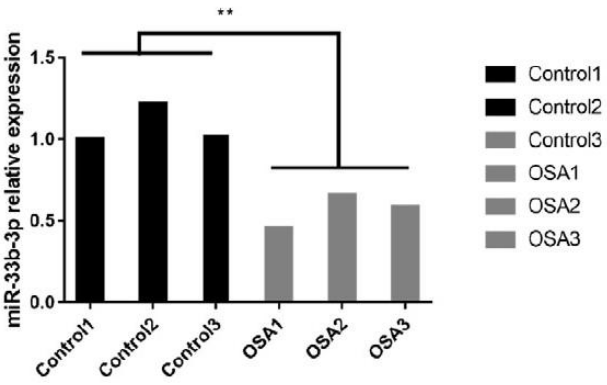

Figure 4. The miRNA-33b expression levels in each group

Relationship between plasma exosome miRNA-33B-3p level and severity of airway obstruction in patients

Pulmonary function instrument detection showed that among 60 OSA patients, there were 24 patients with mild to moderate pulmonary function (FEV1% PRED ≥50%), accounting for 40.0%. Thirty-six patients (60.00%) were with severe disease (FEV1% PRED < 50%). Comparison of patients in the above two subgroups showed that miRNA-33B-3P levels in patients with mild-moderate obstructive ventilate dysfunction were significantly different from those in patients with severe or above obstruction (P < 0. 05) (Table 1).

Table 1. The relationship between the expression levels of miRNA-33b and FEV1 % pred in OSA patients

Indicators	Mild to moderate obstructive ventilatory dysfunction	Severe obstructive ventilatory dysfunction
FEV1 % pred (%)	64.95 ± 11.52	39.09 ± 7.69 **
miRNA-33b (%)	72.34±13.42	54.18±18.67 **
FVC /VC (%)	97.14 ± 3.94	91.31 ± 7.75 **

Note: *shows Significant difference compare with mild and moderate group

Differences between plasma exosome Mir-33B-3p levels and endothelial cells in patients

HE staining was used to observe the formation of arterial plaque. HE staining results showed that there was no obvious atherosclerosis in the control group and the patients with mild and moderate OSA obstructive ventilation dysfunction group, while atherosclerosis plaque appeared in the arterial endothelium in the severe OSA group. The results showed that the miRNA-33B-3P injection group had significantly less atherosclerosis than the OSA group ($P < 0.05$).

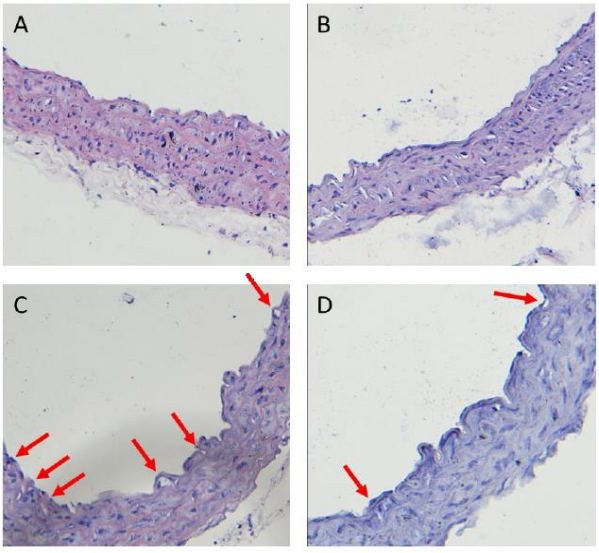

Figure 5. Observation of formation of arterial plaque by HE staining. Note: A: NC group. B: Mild to moderate OSA group. C: Severe OSA group. D: miR-33b-3p group.

Over the past few decades, the incidence of moderate to severe OSA have increased to 23.4% in women and 49.7% in men, due to the increase in obesity as a result of technological development (11). In recent years, the incidence of OSA is still increasing year by year with the increasing degree of social aging. It can cause abnormal secretion of a variety of hormones in the body and then affect the change of metabolic level (12), and the influence on children is increasingly significant (13, 14). Modern medical cognition of OSA pathogenesis is derived from the balance theory of upper airway opening and closing during sleep, and the four pathophysiological elements of pathogenesis are gradually attracting attention. The pathogenesis model based on anatomical and non-anatomical factors, namely PALM model, included critical closed pressure (Pcrit, P) representing upper airway structure, low arousal threshold (A), and high loop gain (LOOP) representing respiratory control instability Gain,L) and upper airway muscle responsiveness (M). Studies have shown that the severity of OSA increases with the severity of obesity (15). At present, the main cause of OSA is hypertrophy of the tonsil and adenoid. In addition, once the disease occurs, it can cause a series of systemic pathophysiological changes in the body, leading to cardiovascular, kidney, central nervous system and other diseases, and even death in serious cases (16). Therefore, early diagnosis and treatment are crucial.

It was initially thought that exosomes were just a way for cells to excrete unwanted products from metabolism, but now further research shows that exosomes are involved in the communication of signals between cells and play an important role in the occurrence and development of diseases. Exosomes can act directly on themselves in paracrine form, or they can be transferred to distant areas with body fluids. It acts on cells in the following three ways: 1. Directly interacts with target cells through the membrane surface (17); 2. Function through functional proteins in inclusions; 3. Target cell functions are regulated through mRNA, lncRNA (18) and microRNA (19) in inclusions. Exosomes have been detected in blood, sputum (20), urine (21) and other body fluids. Studies have found that exosomes have the potential to become biomarkers for some diseases, and their functions are still being further explored.

Endothelial dysfunction is common in cardiovascular diseases such as heart failure and is also an important link in the process of OSA leading to cardiovascular diseases. OSA patients may have endothelium-dependent diastolic dysfunction due to

decreased NO secretion. Endothelial dysfunction can also be caused by increased apoptosis of endothelial cells in the process of disease, resulting in cardiovascular diseases (22). Studies have shown that continuous CPAP therapy can alleviate this apoptosis and reduce cardiovascular complications.

Khalyfa et al. (23) found in exosome studies that plasma exosomes can lead to a significant destruction of endothelial permeability, reduce the mRNA synthesis and secretion of nitric oxide synthase, and significantly up-regulate the expression of intercellular adhesion factor-1. The injury of endothelial cells is closely related to the development of cardiovascular dysfunction. Another related study (24) showed significant differential expression of HSA-Mir-630 in the exosomes of patients with endothelial dysfunction, and endothelial function returned to normal after the resumption of hSA-Mir-630 expression. It was further demonstrated that exosomal miRNA plays an important role in endothelial cell dysfunction induced by OSA (25).

In this study, plasma exosomes were extracted from OSA patients by venous blood collection using the membrane affinity method, and their protein and miRNA-33B-3p expression levels were detected. The results showed that the exosomes were round and oval and had a complete double-layer lipid envelope structure, which expressed special proteins such as HSP70, CD9 and CD63. The diameter of exosome particles is concentrated in 80.5 ~ 158.6 nm, among which, the diameter of exosome in OSA patients is concentrated in 121.9nm, and the diameter of exosome in healthy people is concentrated at 145.0 nm. Comparison of Mir-33B-3p level with pulmonary function test data by pulmonary function instrument showed that miRNA-33B-3p level in patients with mild-moderate obstructive ventilator dysfunction was significantly different from that in patients with severe or above obstruction ($P < 0.05$). The content of exosome miRNA-33B-3P in OSA patients was significantly reduced compared with healthy subjects, and the corresponding degree of airway obstruction was higher. HE staining results showed that no obvious atherosclerosis was found in the control group and the mild to moderate OSA group, while atherosclerosis plaque was found in the arterial endothelium of the severe OSA group. The results showed that the mirNA-33B-3P injection group had significantly less atherosclerosis than the OSA injection group ($P < 0.05$). These results indicate that plasma exosomes in OSA patients can regulate the degree of airway obstruction and endothelial cell function by controlling miRNA expression. Low miRNA-33B-3p levels can lead to increased airway obstruction and endothelial atherosclerosis. In connection with existing studies, it has been confirmed that obesity, diabetes, hypertension, male gender and age are all risk factors for OSA (26), and even lead to acid and alkali poisoning and cardiovascular disease in severe cases, which have a serious impact on patients' life and work. We can monitor patients' plasma exosomes and regulate miRNA expression to assist patients' treatment and prediction, so as to help medical staff pay enough attention to patients' conditions and give reasonable and effective treatment (27).

There are some deficiencies in this study. First, the results of previous high-throughput detection were directly quoted in the study, and high-throughput detection was not carried out on this sample. Therefore, some exosomal miRNAs with different expressions may be missed, and there may still be other exosomal miRNAs that play an important role in the effect studied. Second, the sample size selected for this qPCR to verify the expression of exosome miRNA is small, and there may be some errors. Therefore, the sample size should be increased before relevant detection and verification. Third, only a small number of patients were selected in this experiment. If the selected areas and the number of patients are more extensive and diverse, the results will be more convincing. Future well-designed clinical trials are needed to examine the role of plasma exocrine in the clinical development of OSA patients.

In conclusion, plasma exosomes in OSA patients can regulate the degree of airway obstruction and endothelial cell function by controlling the expression of miRNA (including but not limited to miRNA-33B-3p), resulting in an increased degree of airway obstruction and formation of endothelial cell atherosclerosis. The expression levels of plasma exosomes and miRNA-33B-3p can provide a basis for the early diagnosis and treatment of OSA patients.

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None.

Interest conflict

The authors declare no conflict of interest.

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