Plasma exosomes in OSA patients promote endothelial senescence: effect of long-term adherent continuous positive airway pressure

Abdelnaby Khalyfa[1], Jose M. Marin[2], Zhuanhong Qiao[1], David Sanz Rubio[2], Leila Kheirandish-Gozal[1] and David Gozal[1,*]

[1]Department of Child Health and the Child Health Research Institute, University of Missouri School of Medicine, Columbia, MO and [2]Translational Research Unit, Hospital Universitario Miguel Servet & IISAragon, CIBERES, Zaragoza, Spain

*Corresponding author. David Gozal, Department of Child Health, University of Missouri School of Medicine, 400 N. Keene Street, Suite 010, Columbia, MO 65201. Email: gozald@health.missouri.edu.

Abstract

Obstructive sleep apnea (OSA) is associated with increased risk for end-organ morbidities, which can collectively be viewed as accelerated aging. Vascular senescence is an important contributor to end-organ dysfunction. Exosomes are released ubiquitously into the circulation, and transfer their cargo to target cells facilitating physiological and pathological processes. Plasma exosomes from 15 patients with polysomnographically diagnosed OSA at baseline (OSA-T1) after 12 months of adherent continuous positive airway pressure (CPAP) treatment (OSA-T2), 13 untreated OSA patients at 12-month intervals (OSA-NT1, OSA-NT2), and 12 controls (CO1 and CO2) were applied on naïve human microvascular endothelialcells-dermal (HMVEC-d). Expression of several senescence gene markers including p16 (*CDKN2A*), *SIRT1*, and *SIRT6* and immunostaining for β-galactosidase activity (x-gal) were performed. Endothelial cells were also exposed to intermittent hypoxia (IH) or normoxia (RA) or treated with hydrogen peroxide (H_2O_2), stained with x-gal and subjected to qRT-PCR. Exosomes from OSA-T1, OSA-NT1, and OSA-NT2 induced significant increases in x-gal staining compared to OSA-T2, CO1, and CO2 (p-value < 0.01). p16 expression was significantly increased ($p < 0.01$), while *SIRT1* and *SIRT6* expression levels were decreased ($p < 0.02$ and $p < 0.009$). Endothelial cells exposed to IH or to H_2O_2 showed significant increases in x-gal staining ($p < 0.001$) and in senescence gene expression. Circulating exosomes in untreated OSA induce marked and significant increases in senescence of naïve endothelial cells, which are only partially reversible upon long-term adherent CPAP treatment. Furthermore, endothelial cells exposed to IH or H_2O_2 also elicit similar responses. Thus, OSA either directly or indirectly via exosomes may initiate and exacerbate cellular aging, possibly via oxidative stress-related pathways.

Statement of Significance

Obstructive sleep apnea (OSA), short sleep duration, and obesity are associated with increased risk of several highly prevalent disorders, which can be viewed as resulting from accelerated aging. The functional integrity of endothelial cells is fundamental for cardiovascular system homeostasis, and vascular senescence is an important contributor to cardiovascular dysfunction. Exosomes are released ubiquitously into the circulation, and transfer their cargo to target cells facilitating both physiological and pathological processes. We show that circulating exosomes in untreated OSA induce significant increases in senescence in naïve endothelial cells, which are only partially reversible after adherent continuous positive airway pressure (CPAP) treatment. Thus, OSA either directly or indirectly via exosomes may initiate and exacerbate cellular aging, possibly via oxidative stress-related pathways.

Key words: OSA; CPAP; endothelium; cardiovascular; extracellular vesicles; exosomes; senescence; aging; intermittent hypoxia; oxidative stress

Introduction

Obstructive sleep apnea (OSA) is characterized by increased upper airway resistance and recurrent upper airway obstruction during sleep. The recurrent narrowing or collapse of the upper airway leads to episodic hypoxemia, hypercapnia, and arousals, all of which have been associated with increased risk of multisystem morbidity and overall mortality. Among the several important morbid consequences of OSA, increased risk for cardiovascular disease (CVD) such as hypertension, atherosclerosis, ischemic heart disease, peripheral vascular disease, myocardial infarction, and stroke have all been reported, leading to increased long-term overall mortality [1–6]. Several intermediary mechanisms, such as sympathetic activation, endothelial dysfunction, vascular oxidative stress, inflammation, increased coagulation, and metabolic dysregulation, link OSA to vascular disease [7]. Endothelial dysfunction is an important consequence of OSA [8, 9], and is frequently measured as impaired endothelium-dependent vasodilatation [10]. Endothelial dysfunction has been linked to a variety of diseases, including atherosclerosis, diabetes mellitus, coronary artery diseases, hypertension, and hypercholesterolemia [11–13].

Cell senescence has been widely reported to occur during organismal aging and aging-related diseases, and increased numbers of senescent cells are recognized as a hallmark of aging, and as an important contributor to aging-related diseases [14–16]. For example, cellular senescence has been shown to play a role in cardiac fibrosis after myocardial infarction, as well as being increasingly linked to the development of vascular pathologies, in particular atherosclerosis [17–20]. Cell senescence can be triggered by a variety of senescence stressors, such as replicative stress, oxidative stress, and DNA instability, which affect gene expression and activate multiple signaling pathways that give rise to various senescence phenotypes [21]. Oxidative stress is not only strongly associated with aging and aging-related diseases, but also plays a crucial role in the development of endothelial dysfunction [22], since it is now clear that the endothelium represents not only a physical barrier between circulating blood and the underlying tissues, but also constitutes an important cellular system with autocrine, paracrine, and endocrine functions that are highly relevant to vascular homeostasis [23, 24].

Several studies have investigated the potential of extracellular vesicles (EVs) to serve as biomarkers for various diseases including cancer, neurodegenerative, metabolic, and immunologic disorders [25–29]. Cell-to-cell communication is pivotal for all multicellular organisms, and cells exchange information through the secretion of soluble factors or by direct interaction. Most eukaryotic cells release membrane-derived vesicles that can have an impact on both neighboring and distant cells [30]. EVs are classified based on their cellular origin and/or biological function or based on their biogenesis. Exosomes are a subtype of EVs secreted into the extracellular space through fusion of multivesicular bodies with the plasma membrane [31]. We have previously shown that plasma exosomes from children with endothelial dysfunction induce substantial alterations in naïve endothelial cells, and that targeted modifications of their cargo can abrogate some of these effects [32]. Interestingly, it was recognized that circulating exosomes are able to produce reactive species during pathological conditions [33, 34]. Thus, it is reasonable to posit that OSA is a condition that facilitates senescence [35, 36] and that OSA-induced changes in circulating exosomes could be involved in promoting aging-related oxidative stress [37, 38]. Our hypothesis is that circulating exosomes from patients with OSA will induce senescence in endothelial cells. We aimed to examine the effect of plasma exosomes derived from healthy controls and untreated OSA patients, as well as OSA patients before and after long-term adherent continuous positive airway pressure (CPAP) treatment on senescence markers in naïve human endothelial cells in vitro.

Methods

Human subjects

Human studies were conducted at the Sleep Clinic of the Hospital Universitario Miguel Servet, a large teaching hospital in Zaragoza, Spain, as part of the EPIOSA study (NCT02131610) as previously described [39]. Selection criteria in the EPIOSA study are reported in detail in Supplementary Table 1, and the selection process for the present study is illustrated in Figure 1. Age range of participants was 18–60 years, and all underwent an overnight in-lab polysomnographic evaluation. For patients with symptomatic OSA, CPAP therapy was offered, and when accepted by the patient, titration was performed using either conventional polysomnography or an automatic CPAP following a validated protocol [39]. The initial 15 patients with polysomnographically diagnosed OSA at baseline (OSA-T1) and after 12 months of adherent CPAP treatment (6.16 ± 0.88 hours/night throughout the duration) (OSA-T2) were selected into this study. The adequate adherence to CPAP was >4 hours/night. As untreated OSA, subjects who were diagnosed with OSA (OSA-NT1) but who opted for not receiving treatment for 12 months (OSA-NT2) were also included (n = 13) [39]. In addition, nonsnoring controls (CO1) with similar age and body mass index (BMI) to OSA patients whose overnight polysomnography (PSG) was normal (apnea-hypopnea index (AHI) < 5 events/hour sleep) were enrolled, and reevaluated after 12 months (CO2; n = 12). Mean age of CO subjects was 36.2 ± 5.4 years and for OSA 41.7 ± 8.3 years, while BMI for CO was 27.3 ± 2.6 kg/m^2 and for OSA was 30.1 ± 0.6 kg/m^2. The participants closest in age and BMI to each of the patients in the OSA group treated with CPAP were identified and selected. The criteria used to select subjects from the control group and from the untreated OSA group consisted in matching them for age (±2 years) and BMI (± 2 kg/m^2) with each of the initial 15 patients in the OSA-T1 group. Data from all sleep studies were scored using AASM guidelines [40] by trained personnel that were blinded to the aims or nature of the study. Optimal titration of CPAP is obtained by using auto-CPAP (Autoset-T; ResMed, Sydney, Australia), according to the guidelines of the Spanish Sleep and Breathing Group [41]. Adherence to CPAP therapy was measured using the machines' internal timers.

At baseline and at every annual visit, smoking status was evaluated with co-oximetry and questionnaire. The number of smokers was as follows: for CO there were three smokers and three heavy smokers and the average of smoking tobacco was 20 pack/year. For OSA-NT, there were three smokers and three heavy smokers and the average of smoking was 8 pack/year. For OSA-T, there were four smokers and four heavy smokers and the average of smoking was 10 pack/year.

Blood samples were drawn at baseline and after 12 months (for OSA subjects only) using a 21-G butterfly needle into

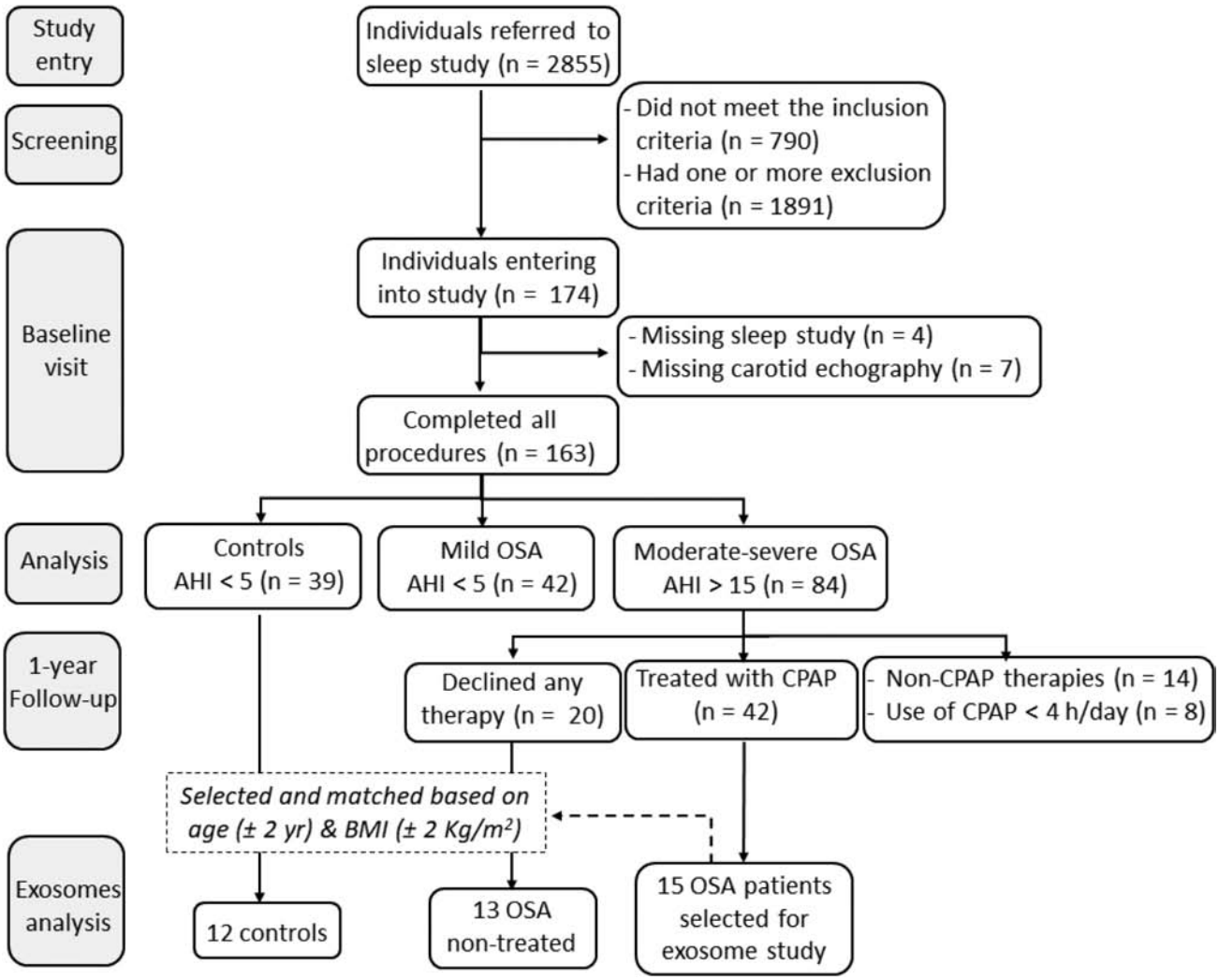

Figure 1. Flow chart illustrating subject enrollment. From January 1, 2014 to March 30, 2017, 2,855 individuals were referred to the sleep clinic, and 174 were recruited to the EPIOSA study. As untreated OSA, subjects who were diagnosed with moderate–severe OSA (OSA-NT1) but who opted for not receiving treatment for 12 months (OSA-NT2) were also included (n = 13). In addition, we also included a nonsnoring control group whose overnight PSG was normal (AHI < 5 events/hour sleep) at baseline (CO1) and who were reevaluated after 12 months (CO2; n = 12).

ethylenediaminetetraacetic acid (EDTA) (PreAnalytix GmbH, Switzerland). Serum glucose, triglycerides, total cholesterol, and high-density lipoprotein cholesterol were measured by spectrophotometry (Chemical Analyzer ILAB 650, Instrumentation Laboratory). Plasma was separated by centrifugation and stored at −80°C.

Circulating plasma exosome isolation and quantification

Human plasma exosomes were isolated using the Total Exosome Isolation Kit according to the manufacturer's standard protocol (Life Technologies, Grand Island, NY) [42]. We implemented all necessary steps to characterize the purity of the exosomes as previously reported [43]. The number of human exosomes in each of the samples was determined using an enzymatic fluorescent assay (FluoroCet #FCET96A quantitation kit; System Biosciences, Mountain View, CA) as described by the manufacturer's guidelines.

Flow cytometry

Human plasma exosomes were incubated with Exo-Flow kits (System Biosciences) to identify selective subpopulations of exosome surface markers utilizing FACS analysis (FACSCalibur, BD Biosciences, San Jose, CA). For more details, please see Supplementary Methods.

Endothelial cell culture

Human microvascular endothelial cells-dermal (HMVEC-d) were purchased from Lonza (Allendale, NJ; catalog # CC-2543). Cells (0.3×10^6 cells/well) were grown in endothelial growth medium (EGM-2-MV; Clonetics) supplemented with 5% fetal bovine serum (FBS; Clonetics), and further incubated at 37°C and 5% CO_2 in cell culture incubator. The cells were trypsinized and centrifuged at 220 × g for 7 min, diluted, and replated at appropriate densities. All cells were used before passage 4.

Exosome cellular uptake

Human exosomes were labeled using PKH26 (Sigma-Aldrich, St. Louis, MO), and images were captured using a confocal microscope. Exosomes were labeled for 10 min at 37°C. To remove unbound dyes, samples were filtered through a microspin column

G-25 (Sigma-Aldrich). For more details, please see Supplementary Methods.

Intermitted hypoxia in vitro

Human microvascular endothelial cells-dermal (HMVEC-d) were grown and maintained in growth medium (EGM-2-MV; Clonetics) containing depleted FBS and further incubated at 37°C and 5% CO_2 in cell culture incubator. Cells were grown in T-25 flasks and incubated either in hypoxia (0.1%–21% O_2, balance N_2) or normoxia (RA; 21%–21% O_2) every 30 min for 1–6 days.

Hydrogen peroxide treatment in vitro

Human microvascular endothelial cells (HMVEC-d) were grown in 12-well plates and hydrogen peroxide (H_2O_2) (prepared in PBS) was added to the cells at final concentration of 0, 10, 30, 60, 80, 100, and 120 μM at 37°C in 5% humidified CO_2. For more details, please see Supplementary Methods.

Senescence-associated β-galactosidase activity assay in vitro

HMVEC-d cells were grown in 12-well plates for 24 hours and cells were treated with either exosomes from healthy subjects, OSA diagnosed OSA at baseline (OSA-T1) and after 12 months of adherent CPAP treatment (>4 hours/day) (OSA-T2) were obtained, or untreated OSA (OSA-NT1) for 24 hours in depleted FBS. In parallel, two additional experiments were also pursued. Endothelial cells were exposed to intermittent hypoxia (IH) or treated with different doses of H_2O_2. Senescence-associated b-galactosidase (SA-b-gal) activity was measured using a senescence B-galactosidase staining kit, SA-β-Gal, activity (BioVision Inc., Milpitas, CA). For more details, please see Supplementary Methods.

qRT-PCR

Total RNA was isolated from HMVEC-d cells and prepared using RNeasy Mini Kit (Qiagen, Valencia, CA) as described by the manufacturer's protocol. qRT-PCR analysis was performed for selected mRNAs using ABI PRISM 7500 System (Applied Biosystems, Foster City, CA). For more details, please see Supplementary Methods.

Data analysis

Data are expressed as means ± standard error (*SE*) unless otherwise indicated. Statistical analyses were performed using SPSS statistical software (version 21.0; Chicago, IL). Comparisons between exosome-treated and untreated cells were performed using with either one- or two-way analysis of variance (ANOVA) test for multiple comparison tests. A value of $p < 0.05$ was considered a statistically significant difference unless otherwise indicated.

Results

Subject characteristics

A total of 40 subjects were recruited in this study, and the subjects were classified into three groups: (A) healthy controls (CO; $n = 12$), (B) subjects with OSA but untreated (OSA-NT; $n = 13$), and (C) OSA subjects (OSA-T; $n = 15$) treated with CPAP for a period of 12 months, as shown in Figures 1 and 2, and Table 1. There were no significant differences in age or BMI between the three groups. However, AHI was markedly higher among OSA-T subjects (70.1 ± 16.7 events/hour) compared to OSA-NT (32.9 ± 17.3 events/hour; $p < 0.01$), and CPAP treatment resulted in normalization of their AHI (OSA-T2: 2.7 ± 2.1/events/hour; $p = 0.001$; Table 1). In addition, significant differences emerged in diastolic blood pressure (dBP) between OSA-T1 (82.3 ± 5.3 mmHg) and OSA-T2 (73.6 ± 10.2 mmHg; $p = 0.01$). In contrast, OSA-NT subjects, who were of similar age, gender, and BMI, showed no improvements in either AHI or blood pressure after 12 months (Table 1). As indicated in Table 1, the SpO_2 during wake state was statistically significantly lower in OSA-T1 (90.50 ± 2.87%) compared to OSA-T2 (94.70 ± 1.60%; $p = 0.01$); however, no significant differences emerged in CO or OSA-NT.

Exosomes characterization and cellular internalization

First, we purified exosomes and characterized them using electron microscopy (EM) to determine the size of the plasma isolated vesicles, which were in the 30–120 nm range as shown in Supplementary Figure 1A [27, 42, 44]. Next, using different established human exosome markers including tetraspanins (CD63; for exosome formation and secretion), targeting/adhesion (CD31; for exosome maturation and target cell binding), antigen presentation (HLA-G; for immune modulation), and membrane transport (Rab5b; for exosome biogenesis, secretion, and cell fusion) (see Supplementary Figure 1B), we confirmed the purity and specificity of the isolation procedures. Furthermore, exosomes were quantified, and used in equal numbers (Supplementary Figure 1C), whereby the average number of exosomes across all groups and all experiments was 4.48×10^{e8}/mL ± 0.42×10^{e8}/mL.

In order to ascertain that human plasma-derived exosomes are incorporated into human naïve endothelial cells, the culture media of the cells was supplemented with PKH26-labeled exosomes (Figure 3), and was readily detected in the lipid cell membrane, whereas no signal was observed in cells grown in medium supplemented with PKH26 but without exosomes (Figure 3).

Effects of exosomes on senescence markers

We examined the effects of exosomes derived from all six subject groups on naïve human endothelial cells and evaluated the expression of the senescence markers in p16, and Sirt1 and Sirt6 and also the changes in X-Gal (Figures 4 and 5). Compared to controls (CO1, CO2), the expression of p16 was significantly increased in untreated OSA untreated (OSA-T1, OSA-NT1, OSA-NT2; $p < 0.01$) and was reduced following adherent CPAP treatment (OSA-T2; Figure 4A), albeit to levels that remained significantly higher than CO ($p < 0.01$). Such findings were also corroborated with the quantification of cellular senescence-associated expression of SA-β-Gal activity (Figure 5A). Indeed, the ratio of positive SA-β-Gal/DAPI for healthy subjects was 5.8 ± 0.25 compared to OSA-NT or OSA-T1 (7.02 ± 0.35), with significant improvements in OSA-T2 (6.1 ± 0.36; $p = 0.02$ vs. OSA-T1, OSA-NT1, OSA-NT2; $p < 0.05$ vs. CO2).

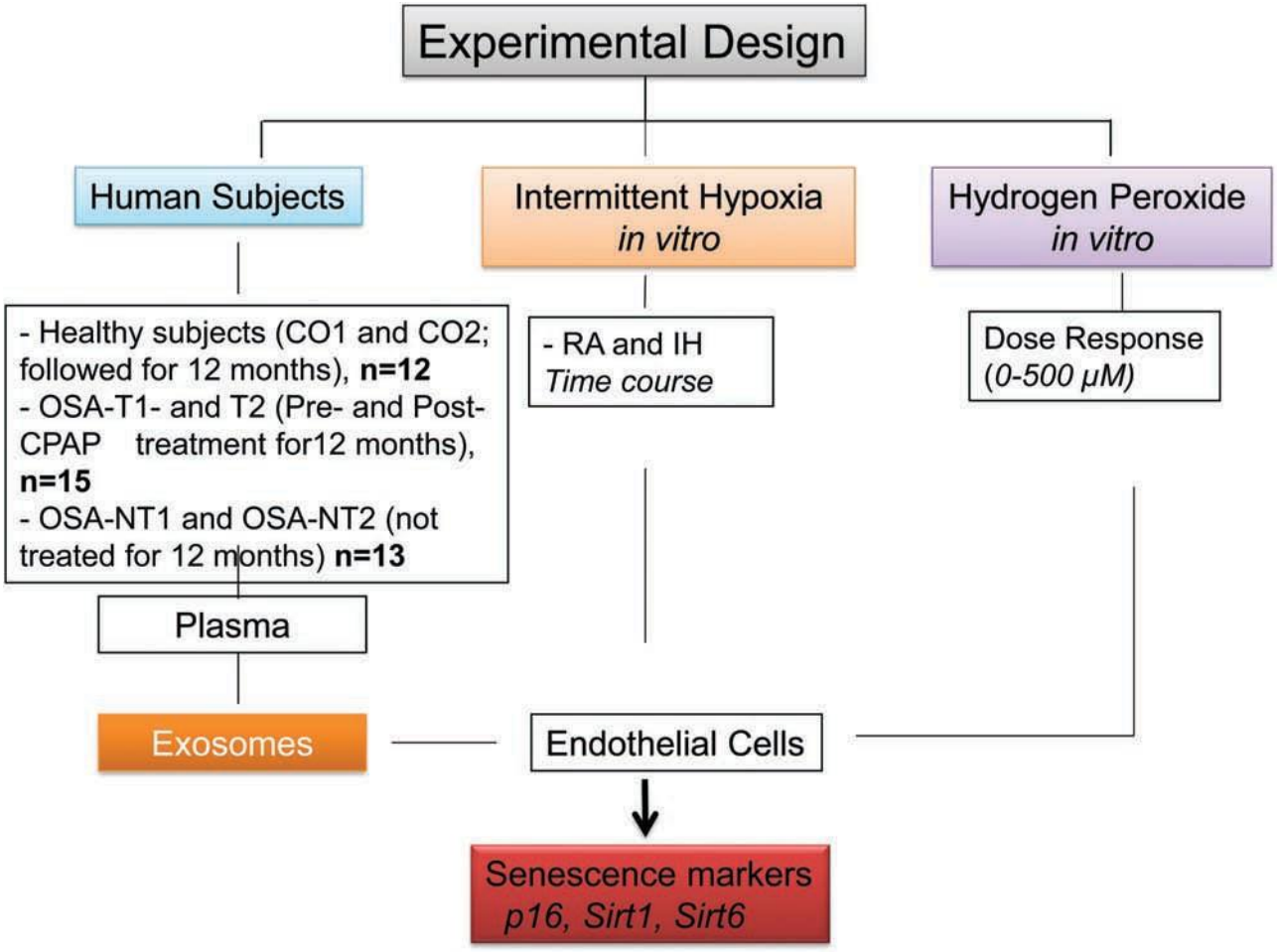

Figure 2. Schematic diagram illustrating subject recruitment and experimental design. Plasma samples were isolated from each subject twice in 1-year interval including CO, OSA-T, and OSA-NT.

Table 1. Characteristics of healthy controls before and after 12 months, and OSA subjects before and following adherent CPAP treatment for 12 months

	CO (n = 12)		OSA-NT (n = 13)		OSA-T (n = 15)	
	CO1	CO2	OSA-NT1	OSA-NT2	OSA-T1	OSA-T2
Age	36.17 ± 5.37	37.17 ± 5.37	38.36 ± 8.27	39.36 ± 8.27	41.71 ± 8.27	42.38 ± 7.0
BMI, kg/m^2	27.30 ± 2.61	27.28 ± 2.23	28.46 ± 2.85	28.47 ±2.26	30.11 ± 0.59	32.25 ± 3.41
Cigarette, smokers/heavy smokers/ mean packs/year	3/3/20		3/3/8		4/4/10	
AHI, events/hour	1.57 ± 1.18	2.25 ± 1.09*	32.87 ± 17.33	31.20 ± 12.67	70.13 ± 16.77*	2.71 ± 2.05**
Triglycerides (mg/dL)	109.57 ± 49.58	111.21 ± 32.04	160.93 ± 56.57	170.31 ± 92.97	222.54 ±74. 86	148.59 ± 78.76**
Total cholesterol (mg/dL)	212.93 ± 45.25	189.13 ± 29.33	216.8 ± 36.69	213.75 ± 45.41	234.77 ± 20.48	212.88 ± 33.87*
HDL cholesterol (mg/dL)	49.78 ± 8.39	48.4 ± 7.08	51.67 ± 10.47	44.56 ± 7.63	47.15 ± 10.76	42.82 ± 7.83*
LDL cholesterol (mg/dL)	141.36 ± 38.45	118.47 ± 28.27	139..21 ± 31.53	134.67 ± 32.53	135.69 ± 13.87	151.64 ± 33.96
Glucose (ng/mL)	91.71 ± 10.89	91.27 ± 9.88	104.13 ± 58.84	103.75 ± 12.76	101.62 ± 11.76	94.59 ± 14.48*
SysBP	122. 64 ± 9.19	119. 54 ± 8.66	125.79 ± 10.90	123.57 ± 12.86	126.75 ± 10.57	125.12 ± 15.55
DyBP	73.93 ± 8.38	71.85 ± 9.48	77.07 ± 9.83	78.57 ± 8.76	82.31 ± 8.53	73.63 ± 10.15*
SpO_2 during wake (%)	94.67 ± 0.98	94.55 ± 1.18	93.42 ± 1.50	94.70 ± 1.60	90.50 ± 2.87	94.70 ± 1.60**

*(p value <0.05); **(p value < 0.01).

HDL = high-density lipoprotein; LDL = low-density lipoprotein.

Considering the protective role of sirtuins in endothelial cell senescence [18], we examined changes in *SIRT1* and *SIRT6* expression in naïve endothelial cells treated with exosomes from the six study groups (Figure 4). RT-PCR analyses revealed that compared to CO1 or CO2, mRNA levels of *SIRT1* and *SIRT6* decreased after addition of exosomes from OSA-T1, OSA-NT1,

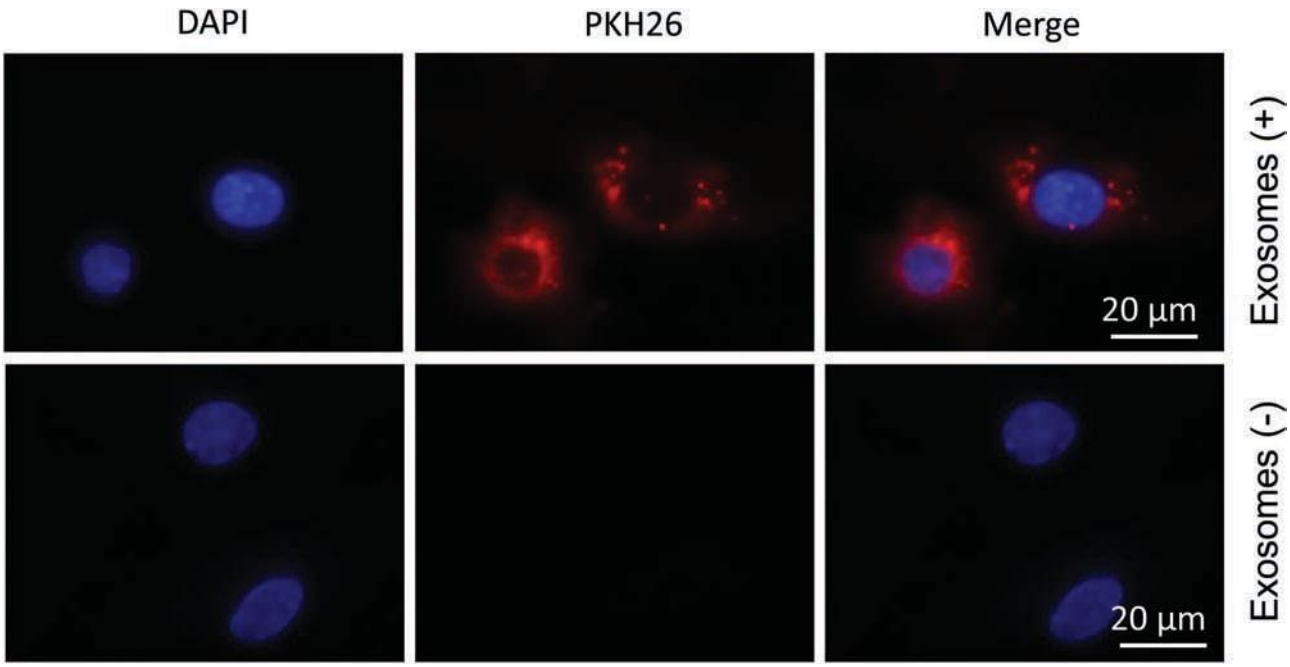

Figure 3. Uptake of fluorescently labeled exosomes by human microvascular endothelialcells-dermal (HMVEC-d). Confluent cell monolayers were grown on 12 cover slips for 24 hours in appropriate medium containing 10% FBS, after which cells were washed with medium containing depleted FBS (System Biosciences). Exosomes were isolated from plasma and labeled with the PKH26 Red Fluorescent (lipophilic). To remove unbound dyes, samples were filtered through a microspin column G-25 (Sigma-Aldrich). The labeled exosomes were added to human HMVEC-d cells for 24 hours in a cell culture incubator at 37°C. The cells were washed, and nuclei (blue) were stained with DAPI. Representative images of labeled exosomes applied into a naïve HMVEC-d cells grown on coverslips for 24 hours, and images of PKH26 exosome uptake were measured using confocal laser scanning microscopy, $n = 8$, scale bar in 10 µm. As controls, no exosomes were used, but PKH26 was added.

and OSA-NT2 (Figure 4). Furthermore, despite improvements in *SIRT1* and *SIRT6* expression in OSA-T2, the latter still exhibited significant reductions when compared to CO2 ($p = 0.01$; Figure 4).

Effect of H_2O_2 and IH on senescence markers

To further expand on the role of oxidative stress and IH in endothelial senescence, we exposed endothelial cells to H_2O_2 (0–500 µM), and to a previously reported IH profile [45] for 1–6 days (Figures 4 and 5). Cells exposed to H_2O_2 showed dose-dependent significant increases in p16, which peaked at 80 µM ($p = 0.001$) and corresponding decreases in *SIRT1* ($n = 8$; $p = 0.01$), and *SIRT6* ($n = 8$; $p = 0.02$) (Figure 3). We also found significant increases of positively labeled cells for SA-β-Gal/DAPI ratio with optimal H_2O_2 concentration (80 µM; $p = 0.003$; Figure 5). At higher concentrations of 150–500 µM H_2O_2, excessive cell death resulted in spurious decreases in cell senescence markers.

Endothelial cells exposed to IH for 3 days showed significant increases in p16 expression compared to RA ($n = 8$; $p = 0.004$; Figure 4). The number of SA-β-Gal positive cells was significantly higher in cells exposed to IH for 3 days (11.8 ± 0.53) versus RA (10 ± 0.61; $n = 8$; $p = 0.02$). However, cells exposed to IH for longer periods of time showed reductions in SA-β-Gal positive cells that were attributable to cell death. Similarly, IH exposures for 3 days resulted in significant decreases in SIRT1 and SIRT6 expression ($n = 8$; $p = 0.03$ and $p = 0.01$, respectively, vs. RA).

Discussion

This study provides initial evidence that circulating exosomes derived from untreated patients with OSA induce significant increases in endothelial cell senescence markers with reciprocal decreases in sirtuin expression, which are only partially reversed upon long-term adherent CPAP treatment. Furthermore, two different paradigms that encompass constitutive perturbations associated with OSA, namely IH and oxidative stress, elicit similar responses further reinforcing the conceptual framework that OSA-induced accelerated senescence underlies, at least in part, the cardiovascular morbidity associated with this common condition [46, 47]. Therefore, OSA might either directly or indirectly via exosomes trigger or exacerbate cellular aging, possibly via oxidative stress-related pathways.

Before we address the potential implications of our findings, several methodological issues deserve comment. First, we carefully identified overweight to mildly obese patients to minimize as much as possible the potential impact of obesity on our results [48]. Secondly, we matched as best as possible all subjects, and even if the overall severity of the patients with OSA-T was higher, both OSA-T and OSA-NT were in the severe category, while the BMI of CO was within the range of the OSA groups. Thus, it is unlikely that the significant differences between the effects of exosomes from untreated OSA patients and CO can be exclusively explained by obesity-related contributions, and furthermore, there were no significant differences in the senescence-promoting effects of exosomes from OSA-T1 and OSA-NT1. Third, all subjects were followed for 12 months after their initial recruitment to ascertain that changes in exosome function were specifically related to adherent CPAP therapy rather than just time-related effects. We should mention that the changes in the levels of triglycerides, cholesterol, and dBP could partially account for the improvements in senescence markers found in the group of patients with OSA treated with CPAP; however, this explanation is speculative at

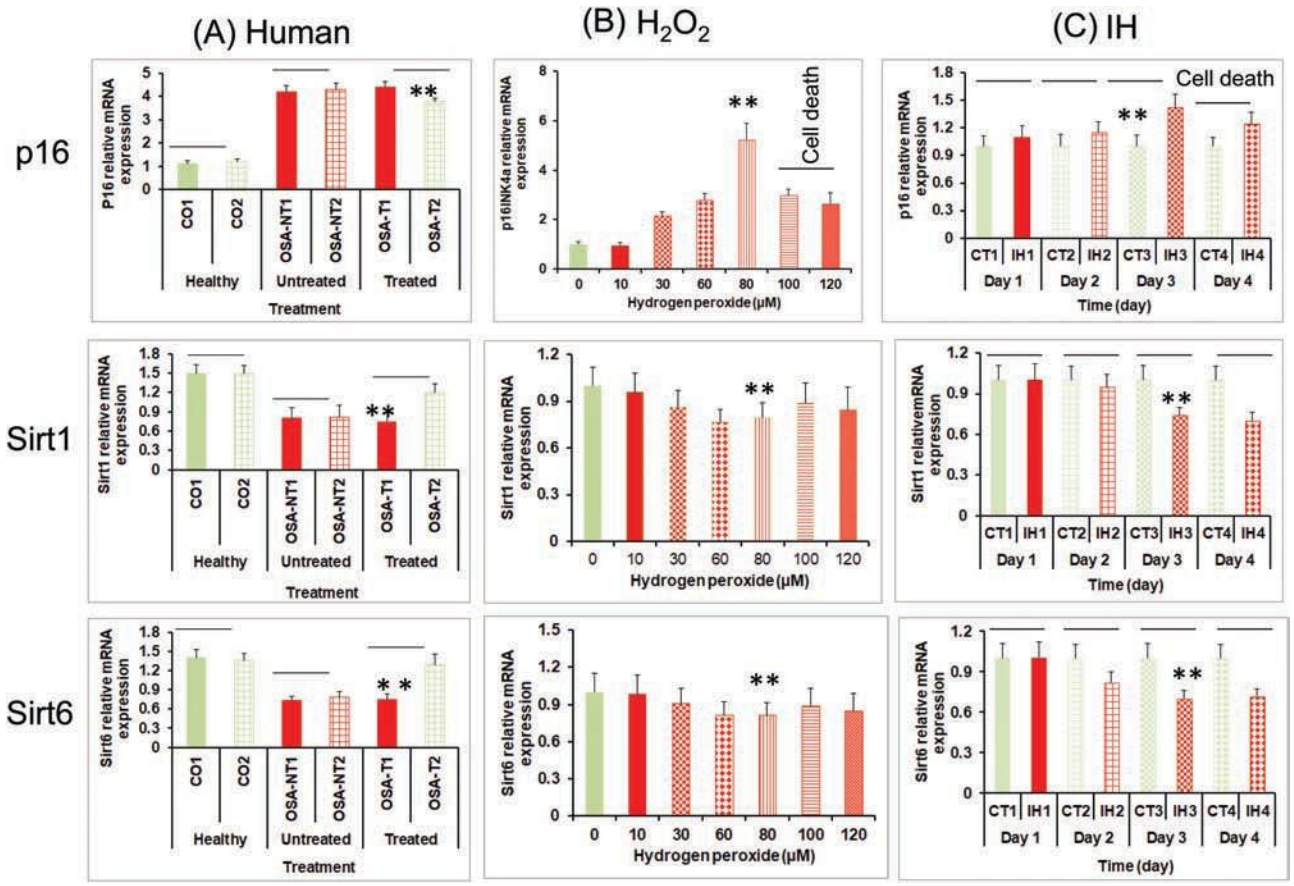

Figure 4. Effects of exosomes derived from CO, OSA-NT, and OSA-T at 1-year interval and applied into naïve human endothelial cells (HMVEC-d). Similarly, HMVEC-d exposed to IH as well as cells treated with H_2O_2 (dose-response). qRT-PCR analysis was utilized to investigate the expression of senescence genes (p16, *SIRT1*, and *SIRT6*). Panel (A) shows mean mRNA expression of senescence markers from experiments involving plasma-derived exosomes. Panel (B) shows changes in mRNA expression of senescence markers following in vitro exposures to IH (1%–21% O_2) or to RA (21%–21% O_2) for 1–6 days (n = 8/condition). Panel (C) shows mean changes in mRNA expression of senescence markers following H_2O_2 treatment (n = 8/treatment). * Indicates statistical significance, $p < 0.05$, and ** indicates statistical significance, $p < 0.01$.

this point. We should also point out that all six groups were always concurrently evaluated for any of the measures of interest to prevent potential differences related to target endothelial cell passage, potential variances in medium from batch to batch, or any other intrinsic variability factors associated with in vitro experiments. We should also mention that it was necessary to evaluate the course of the control group over time (i.e. not only during the recruitment initial visit, but also after 1-year follow-up) to ascertain that the changes or lack thereof in the other groups were not a function of time but rather reflected real findings. Accumulation of senescent cells with age has been observed in the context of cardiovascular diseases [49], and oxidative stress induced by H_2O_2 induces premature senescence in human and mouse cells [50–52], such that our findings expand the senescence-inducing effect of both IH and H_2O_2 to human endothelial cells. Furthermore, cell senescence involves chromatin remodeling, endoplasmic reticulum stress, autophagy, and increased β-galactosidase activity that can be readily detected by histochemical staining of cells grown in culture or in vivo tissue sections [53, 54]. In addition, EVs secreted from senescent cells have unique characteristics and contribute to modulating the phenotype of naïve recipient cells by inducing accelerated senescence, inflammation, and cancer progression in tumoral cells [55–58]. Here, we used equivalent numbers of carefully characterized plasma exosomes based on their distinctive expression of tetraspanins at their membrane surface, as well as by using EM. We have previously examined exosomes in patients with OSA in both children and adults, and shown that exosomes trigger signaling pathways by transferring their cargos into the target endothelial cells, ultimately changing their phenotypes [28, 44, 59, 60]. Present data show that exosomes derived from OSA patients increase the expression of the senescence markers in p16, and β-galactosidase along with reciprocal decreases in *SIRT1* and *SIRT6*, and that adherent treatment with CPAP reduces these effects, but does not completely abrogate them when compared to the effects of exosomes originating from CO subjects. Considering that adherence was deemed per convention to encompass a proportion of the sleep duration rather than its totality, it remains unclear whether applying more stringent adherence criteria would have resulted in more prominent trends toward normalization of the exosomal effects on naïve endothelial cells. We should point out that p16 increases are viewed as robust reporters of aging in most mammalian tissues [61]. Stress-induced senescence works mainly through the activation of the cyclin-dependent kinase inhibitor and tumor suppressor protein CDKN2A (also known as p16INK4a) through Cyclin D/Cdk4,6 inhibition, which results in the inactivation of the E2F transcription factor and target genes

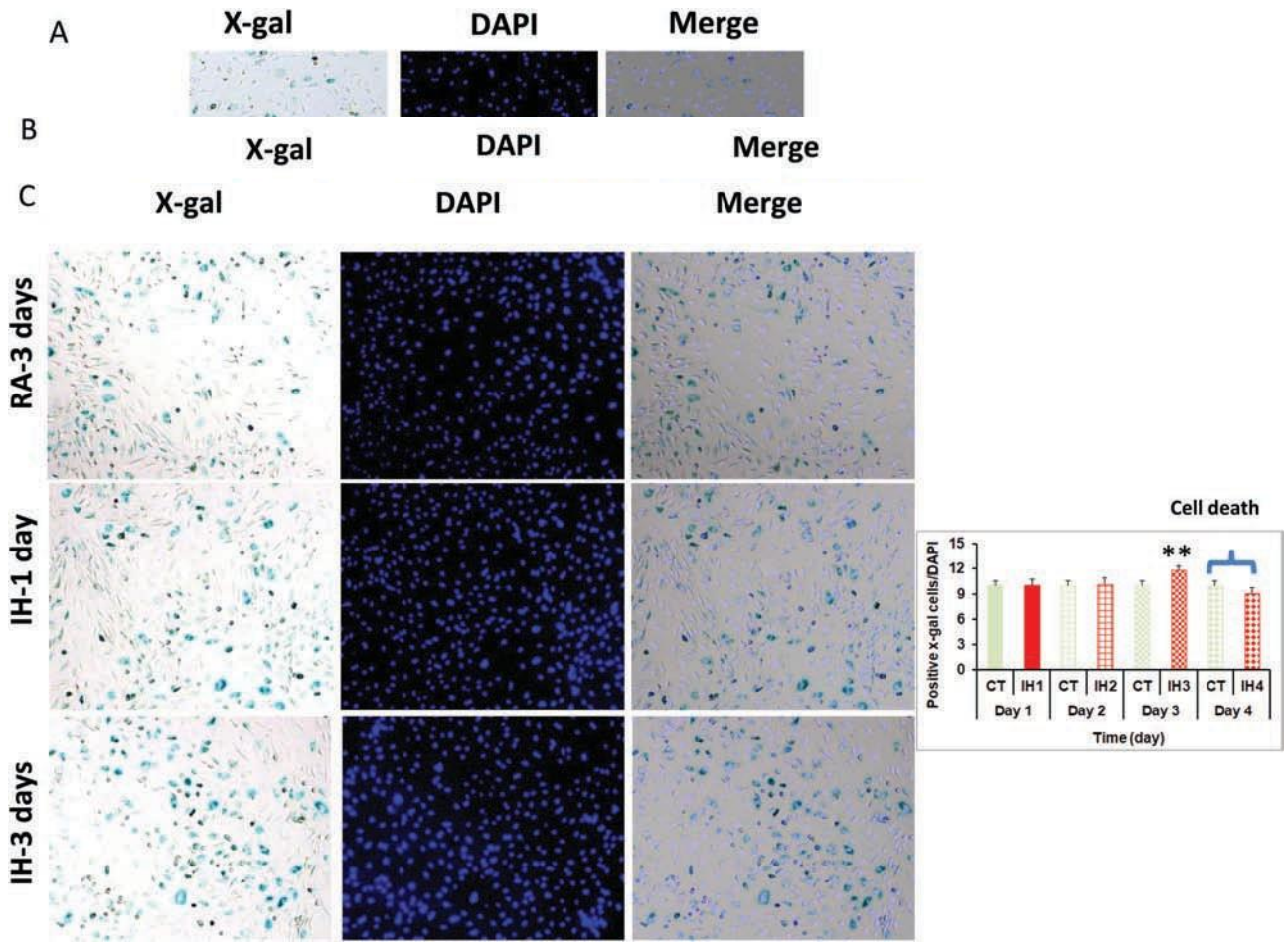

Figure 5. Changes in senescence-associated expression of β-galactosidase (SA-β-Gal) activity induced exosomes derived from CO, OSA-NT, and OSA-T at 1-year interval and applied into naïve human endothelial cells (HMVEC-d). Similarly, HMVEC-d exposed to IH as well as cells treated with H_2O_2 (dose-response). At least five fields of vision were counted. The number of β-galactosidase stained cells versus unstained cells was counted and used to calculate a final average ratio of the number of stained to unstained cells in each well. * Indicates statistical significance, $p < 0.05$, and ** indicates statistical significance, $p < 0.01$.

[62], leading to increased expression of p16 [63]. In this study, the link between senescence and oxidative stress, which would require measuring reactive oxygen species (ROS) signaling following treatment of naïve endothelial cells with exosomes derived from OSA patients, was not determined, primarily due to constraints related to the availability of sufficient exosomes to conduct such studies.

Endothelial cells play critical roles in the regulation of vascular functions, and therefore increased senescence of these cells will adversely impact on vascular aging and related diseases [64]. Stresses such as hypoxia dramatically increase their replication rate without inducing much denudation, suggesting that in the context of either short-term or mild stress, the replication or regeneration of the adjacent endothelial cells may be sufficient to replace the impaired or dysfunctional endothelial cells, or to cover the segments where endothelial cells are denudated [65, 66]. It is now well accepted that OSA can result in endothelial dysfunction, and ultimately lead to arterial disease [8, 9]. Thus, the current findings support the concept that increased senescence of endothelial cells either directly or via exosome-mediated effects contributes to vascular aging and age-related vascular diseases, particularly atherosclerosis and hypertension [67]. At this point, we do not know which components of the exosomal cargo are playing a role in the induction of endothelial senescence. We hypothesize that exosomal miRNAs are likely to mediate major components of such functional signals to naïve endothelial cells.

Data from several studies have shown that CPAP treatment might improve endothelial function in patients with OSA [68–71], likely by reducing oxidative stress [22], but also by reducing OSA-associated genomic instability, epigenetic alterations, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, and altered intercellular communication [35].

A growing evidence suggests that elevated sirtuin activity can have beneficial effects on aging and aging-related diseases in mammals [72, 73]. These actions may be related to sirtuin-mediated modulation of DNA and metabolic damage [72]. A critical role of *SIRT1* in preventing endothelial cells from replicative senescence or stress-induced premature senescence has been reported recently [74]. Conversely, *SIRT1* expression and activity decrease with age in the vasculature, which is likely to contribute to endothelial dysfunction [75]. We also included assessments of SIRT6 in our studies because in addition to Sirt1, this protein is well characterized for its protective roles against inflammation, vascular aging, heart disease, and atherosclerotic plaque development [76]. At the vascular level, the

physiological functions of SIRT1 and SIRT6 in the control of the cellular redox state are mediated by deacetylation of multiple targets, including histones, transcription factors (FOXO, NF-kB, p53, and Nrf2), and enzymes involved in the vascular protection [77]. SIRT6 plays beneficial roles in heart failure and in the control of cardiac fibrosis, a pathological condition critical in the development of heart failure [78]. SIRT6 negatively regulates cardiac fibroblast differentiation into myofibroblasts, its depletion increases cardiac fibroblast proliferation and extracellular matrix deposition, and upregulates focal adhesion-related genes and fibrosis-related genes through NF-kB signaling [78]. Several studies reported that SIRT6 involvement in vascular protection is predicated on observation indicating that SIRT6 deficiency raises the expression of endothelial pro-inflammatory cytokines and increases NF-kB transcriptional activity [77, 79]. The protective effect of SIRT6 against premature endothelial senescence is exerted through a fine control of the expression of intercellular adhesion molecule-1 (ICAM-1), PAI-1, p21Cip1/Waf1, and eNOS [80]. Accordingly, studies also show that pharmacological activation of SIRT1 significantly improves endothelial function in aged mice [81]. In current experiments, SIRT1 and SIRT6 were downregulated by exosomes from untreated OSA patients, as well as cells exposed to IH or to low dose H_2O_2, with significant improvements after 1-year adherent CPAP treatment, but not after 1 year without receiving any treatment. Thus, it is conceivable that OSA patient stratification conducted by relying on the properties of circulating exosomes as reporter assays of underlying vascular morbidity may contribute improved patient initial assessment and monitoring during treatment under a management umbrella aimed at personalized medicine. Indeed, accurate detection of the magnitude of senescence-promoting activity in each OSA patient and their response to therapy might halt or slow down the progression of cellular and molecular changes contributing to aging progression and age-related disorders (Figure 6).

Overall, we cannot conclude that exosomes are directly and causally involved in eliciting an oxidative stress in endothelium without measuring ROS. Unfortunately, due to the restricted volume of plasma samples for exosome isolation in those groups, such additional experiments were precluded. In light of our current observations showing that exosomes from untreated OSA patients have effects on SIRT1 and SIRT6 expression that are similar to those of IH or H_2O_2, it is possible that some of the effects of exosomes are mediated by increasing oxidative stress. However, measures of oxidative stress were not specifically assessed.

Since previous studies have shown that OSA and its associated IH induce oxidative stress, we evaluated in parallel whether H_2O_2 and IH would induce senescence changes in endothelial cells, such as to infer that (1) there is a direct effect of IH and H_2O_2 on senescence and (2) that exosomes from OSA patients induce senescence possibly via induction of similar oxidative stress processes. Second, we were intent on exploring whether there could be common pathogenic pathways in the presence or absence of exosomes. Based on current findings, it is likely that circulating exosomes contribute to senescence of endothelial cells as induced by direct exosome cargo effects that are either additive or synergistic with the effects of the disease via alternative pathways. The important finding is therefore that circulating exosomes contribute to the senescence of endothelium in OSA, and are amenable to improvements after treatment of OSA with adherent CPAP. Thus, it is highly likely that OSA, a systemic disease which activates a multiplicity of pathophysiological pathways, will exert its detrimental end-organ effects via a large number of mechanisms. Among those mechanisms, exosomes and their functional cargo appear to be implicated.

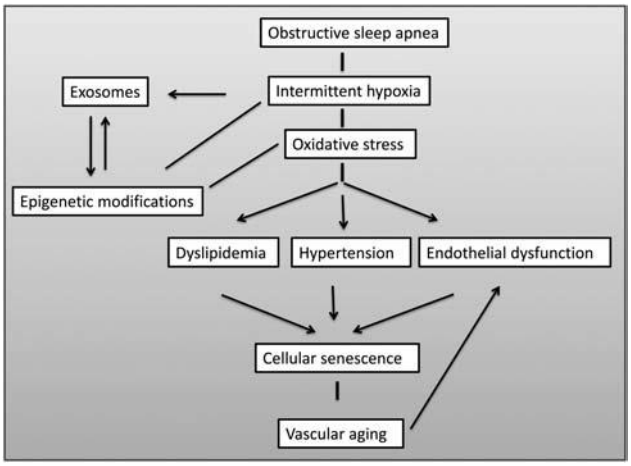

Figure 6. Hypothetical pathway illustrating how exosomes released by intermitted hypoxia (IH) during sleep in patients with OSA can affect endothelial cells and induce cellular senescence and vascular aging.

Conclusions

Cellular senescence is complex biological phenomenon and common cellular stresses yield senescent cells that accumulate in various tissues over time and may contribute to tissue dysfunction. Under the conceptual framework that OSA-induced morbidities are mediated by accelerated cellular senescence, we have shown here that exosomes derived from OSA patients induce senescence markers in vitro on naïve human endothelial cells, a process that is at least partially reversible, and possibly related to increased oxidative stress. Identification of critical elements within exosomal cargo in OSA patients underlying the accelerated senescence may enable development of therapeutic strategies. Alternatively, more precise phenotyping of exosome senescence-inducing properties among patients being diagnosed with OSA may provide accurate reporting of OSA disease activity and response to treatment, thereby improving individualized outcomes.

Supplementary Material

Supplementary material is available at *SLEEP* online.

Table S1. Inclusion and exclusion criteria used in the EPIOSA study.

Figure S1. Exosome characterization and quantification. Panel (A) shows the size distribution of exosomes as assessed by electron microscopy. Panel (B) is a representative image of flow cytometry for exosomes as derived from the study subjects, and showing forward scatter versus FITC intensity for beads with no exosomes (negative), and beads with exosomes (positive). Flow cytometry analysis of purified exosomes following isolation with magnetic beads coated with anti-CD63, CD31, HLA-G, and Rab5b, using FACS analysis (FACSCalibur). The Exo-Flow magnetic stand

for exosome separation and FACS analysis showing the absence of exosomes (negative, blue color) and the presence of exosomes (positive, red color) beads are displayed on the FACS plot. The FITC flow cytometric intensities are then plotted versus the number of exosome particles input into the flow reaction. Panel (C) shows quantification of absolute exosome counts in plasma, illustrating that the in vitro studies performed and reported included equivalent numbers of exosomes for each experiment.

Funding

Supported by NIH grants HL130984 (LK-G) and HL140548 (DG), and by Instituto Carlos III, Spanish Ministry of Health grants PI12/2175 and PI15/1940 (JMM).

Author Contributions

AK, JMM, LK-G, and DG conceived the study; AK, DS, and ZQ performed the experiments; AK, LK-G, and DG contributed to data analysis and interpretation; AK and DG contributed to drafting the manuscript; all authors reviewed and approved the final version of the manuscript.

Conflict of interest statement. None declared.

References

1. Marin JM, *et al*. Long-term cardiovascular outcomes in men with obstructive sleep apnoea-hypopnoea with or without treatment with continuous positive airway pressure: an observational study. *Lancet*. 2005;**365**(9464):1046–1053.
2. Mokhlesi B, *et al*. The effect of sex and age on the comorbidity burden of OSA: an observational analysis from a large nationwide US health claims database. *Eur Respir J*. 2016;**47**(4):1162–1169.
3. Marshall NS, *et al*. Sleep apnea and 20-year follow-up for all-cause mortality, stroke, and cancer incidence and mortality in the Busselton Health Study cohort. *J Clin Sleep Med*. 2014;**10**(4):355–362.
4. Gaines J, *et al*. Obstructive sleep apnea and the metabolic syndrome: the road to clinically-meaningful phenotyping, improved prognosis, and personalized treatment. *Sleep Med Rev*. 2018;**42**:211–219.
5. Farré N, *et al*. Intermittent hypoxia mimicking sleep apnea increases passive stiffness of myocardial extracellular matrix. A Multiscale Study. *Front Physiol*. 2018;**9**:1143.
6. Shpilsky D, *et al*. Association between ideal cardiovascular health and markers of subclinical cardiovascular disease. *Clin Cardiol*. 2018;**41**(12):1593–1599.
7. Kapur VK. Obstructive sleep apnea: diagnosis, epidemiology, and economics. *Respir Care*. 2010;**55**(9):1155–1167.
8. Ayers L, *et al*. Elevated levels of endothelial cell-derived microparticles following short-term withdrawal of continuous positive airway pressure in patients with obstructive sleep apnea: data from a randomized controlled trial. *Respiration*. 2013;**85**(6):478–485.
9. Feng J, *et al*. Endothelial mechanisms of endothelial dysfunction in patients with obstructive sleep apnea. *Sleep Breath*. 2012;**16**(2):283–294.
10. Kato M, *et al*. Impairment of endothelium-dependent vasodilation of resistance vessels in patients with obstructive sleep apnea. *Circulation*. 2000;**102**(21):2607–2610.
11. Liao JK. Linking endothelial dysfunction with endothelial cell activation. *J Clin Invest*. 2013;**123**(2):540–541.
12. Zhang F, *et al*. Polysomnographic correlates of endothelial function in children with obstructive sleep apnea. *Sleep Med*. 2018;**52**:45–50.
13. Imes CC, *et al*. Correlates of endothelial function in older adults with untreated obstructive sleep apnea and cardiovascular disease. *J Cardiovasc Nurs*. 2019;**34**(1):E1–E7.
14. van Deursen JM. The role of senescent cells in ageing. *Nature*. 2014;**509**(7501):439–446.
15. Sharpless NE, *et al*. Forging a signature of in vivo senescence. *Nat Rev Cancer*. 2015;**15**(7):397–408.
16. Childs BG, *et al*. Cellular senescence in aging and age-related disease: from mechanisms to therapy. *Nat Med*. 2015;**21**(12):1424–1435.
17. Katsuumi G, *et al*. Vascular senescence in cardiovascular and metabolic diseases. *Front Cardiovasc Med*. 2018;**5**:18.
18. Ungvari Z, *et al*. Mechanisms of vascular aging. *Circ Res*. 2018;**123**(7):849–867.
19. Guarner V, *et al*. Low-grade systemic inflammation connects aging, metabolic syndrome and cardiovascular disease. *Interdiscip Top Gerontol*. 2015;**40**:99–106.
20. Lee AK, *et al*. Severe hypoglycaemia, mild cognitive impairment, dementia and brain volumes in older adults with type 2 diabetes: the Atherosclerosis Risk in Communities (ARIC) cohort study. *Diabetologia*. 2018;**61**(9):1956–1965.
21. Baar MP, *et al*. Targeted apoptosis of senescent cells restores tissue homeostasis in response to chemotoxicity and aging. *Cell*. 2017;**169**(1):132–147.e16.
22. Conti V, *et al*. Aging-related changes in oxidative stress response of human endothelial cells. *Aging Clin Exp Res*. 2015;**27**(4):547–553.
23. Shalia KK, *et al*. Circulating levels of cell adhesion molecules in hypertension. *Indian J Clin Biochem*. 2009;**24**(4):388–397.
24. Vallance P. Importance of asymmetrical dimethylarginine in cardiovascular risk. *Lancet*. 2001;**358**(9299):2096–2097.
25. Yáñez-Mó M, *et al*. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles*. 2015;**4**:27066.
26. Khalyfa A, *et al*. Plasma exosomes disrupt the blood-brain barrier in children with obstructive sleep apnea and neurocognitive deficits. *Am J Respir Crit Care Med*. 2018;**197**(8):1073–1076.
27. Khalyfa A, *et al*. Sleep-disordered breathing, circulating exosomes, and insulin sensitivity in adipocytes. *Int J Obes (Lond)*. 2018;**42**(6):1127–1139.
28. Khalyfa A, *et al*. Exosome and macrophage crosstalk in sleep-disordered breathing-induced metabolic dysfunction. *Int J Mol Sci*. 2018;**19**(11):E3383.
29. Khalyfa A, *et al*. Circulating exosomes in obstructive sleep apnea as phenotypic biomarkers and mechanistic messengers of end-organ morbidity. *Respir Physiol Neurobiol*. 2018;**256**:143–156.
30. Lee Y, *et al*. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum Mol Genet*. 2012;**21**(R1):R125–R134.
31. Bellingham SA, *et al*. Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? *Front Physiol*. 2012;**3**:124.
32. Khalyfa A, *et al*. Circulating plasma extracellular microvesicle microRNA cargo and endothelial dysfunction in children with obstructive sleep apnea. *Am J Respir Crit Care Med*. 2016;**194**(9):1116–1126.

Downloaded from https://academic.oup.com/sleep/article/43/2/zsz217/5573413 by guest on 05 January 2025

33. Janiszewski M, *et al.* Platelet-derived exosomes of septic individuals possess proapoptotic NAD(P)H oxidase activity: a novel vascular redox pathway. *Crit Care Med.* 2004;**32**(3):818–825.
34. Azevedo LC, *et al.* Platelet-derived exosomes from septic shock patients induce myocardial dysfunction. *Crit Care.* 2007;**11**(6):R120.
35. Gaspar LS, *et al.* Obstructive sleep apnea and hallmarks of aging. *Trends Mol Med.* 2017;**23**(8):675–692.
36. Tempaku PF, *et al.* The paradigm of obstructive sleep apnea in aging: interactions with telomere length. *Sleep Med.* 2018;**48**:155–156.
37. Halliwell B. Reactive oxygen species and the central nervous system. *J Neurochem.* 1992;**59**(5):1609–1623.
38. Siqueira IR, *et al.* Total antioxidant capacity is impaired in different structures from aged rat brain. *Int J Dev Neurosci.* 2005;**23**(8):663–671.
39. Marin JM, *et al.* Epigenetics modifications and subclinical atherosclerosis in obstructive sleep apnea: the EPIOSA study. *BMC Pulm Med.* 2014;**14**:114.
40. Berry RB, *et al.*; American Academy of Sleep Medicine. Rules for scoring respiratory events in sleep: update of the 2007 AASM manual for the scoring of sleep and associated events. Deliberations of the sleep apnea definitions Task Force of the American Academy of Sleep Medicine. *J Clin Sleep Med.* 2012;**8**(5):597–619.
41. Masa JF, *et al.* Alternative methods of titrating continuous positive airway pressure: a large multicenter study. *Am J Respir Crit Care Med.* 2004;**170**(11):1218–1224.
42. Khalyfa A, *et al.* Circulating plasma extracellular microvesicle miRNA cargo and endothelial dysfunction in OSA children. *Am J. Resp Crit Care Med.* 2016;194(9):1116–1126.
43. Lötvall J, *et al.* Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the international society for extracellular vesicles. *J Extracell Vesicles.* 2014;**3**:26913.
44. Bhattacharjee R, *et al.* Exosomal cargo properties, endothelial function and treatment of obesity hypoventilation syndrome: a Proof of Concept Study. *J Clin Sleep Med.* 2018;**14**(5):797–807.
45. Gozal E, *et al.* Mild sustained and intermittent hypoxia induce apoptosis in PC-12 cells via different mechanisms. *Am J Physiol Cell Physiol.* 2005;**288**(3):C535–C542.
46. Heinzer R, *et al.* Prevalence of sleep-disordered breathing in the general population: the HypnoLaus study. *Lancet Respir Med.* 2015;**3**(4):310–318.
47. Drager LF, *et al.*; INCOSACT Initiative (International Collaboration of Sleep Apnea Cardiovascular Trialists). Sleep apnea and cardiovascular disease: lessons from recent trials and need for team science. *Circulation.* 2017;**136**(19):1840–1850.
48. Burton DGA, *et al.* Obesity and type-2 diabetes as inducers of premature cellular senescence and ageing. *Biogerontology.* 2018;**19**(6):447–459.
49. Muñoz-Espín D, *et al.* Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell Biol.* 2014;**15**(7):482–496.
50. Chen Q, *et al.* Oxidative DNA damage and senescence of human diploid fibroblast cells. *Proc Natl Acad Sci USA.* 1995;**92**(10):4337–4341.
51. Parrinello S, *et al.* Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol.* 2003;**5**(8):741–747.
52. Sharma V, *et al.* Oxidative stress at low levels can induce clustered DNA lesions leading to NHEJ mediated mutations. *Oncotarget.* 2016;**7**(18):25377–25390.
53. Dimri GP, *et al.* A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA.* 1995;**92**(20):9363–9367.
54. Zajic LB, *et al.* Comparison of proliferative and immunomodulatory potential of adipose-derived mesenchymal stem cells from young and geriatric cats. *J Feline Med Surg.* 2017;**19**(10):1096–1102.
55. Takasugi M. Emerging roles of extracellular vesicles in cellular senescence and aging. *Aging Cell.* 2018;17.
56. Kadota T, *et al.* Emerging role of extracellular vesicles as a senescence-associated secretory phenotype: insights into the pathophysiology of lung diseases. *Mol Aspects Med.* 2018;**60**:92–103.
57. Takasugi M, *et al.* Small extracellular vesicles secreted from senescent cells promote cancer cell proliferation through EphA2. *Nat Commun.* 2017;**8**:15729.
58. Robbins PD. Extracellular vesicles and aging. *Stem Cell Investig.* 2017;**4**:98.
59. Khalyfa A, *et al.* Circulating microRNAs as potential biomarkers of endothelial dysfunction in obese children. *Chest.* 2016;**149**(3):786–800.
60. Khalyfa A, *et al.* Effect on intermittent hypoxia on plasma exosomal micro RNA signature and endothelial function in healthy adults. *Sleep.* 2016;**39**(12):2077–2090.
61. Krishnamurthy J, *et al.* Ink4a/Arf expression is a biomarker of aging. *J Clin Invest.* 2004;**114**(9):1299–1307.
62. Collado M, *et al.* Cellular senescence in cancer and aging. *Cell.* 2007;**130**(2):223–233.
63. Ohtani N, *et al.* The p16INK4a-RB pathway: molecular link between cellular senescence and tumor suppression. *J Med Invest.* 2004;**51**(3–4):146–153.
64. Minamino T, *et al.* Vascular cell senescence and vascular aging. *J Mol Cell Cardiol.* 2004;**36**(2):175–183.
65. Langille BL, *et al.* Injury and repair of endothelium at sites of flow disturbances near abdominal aortic coarctations in rabbits. *Arteriosclerosis.* 1986;**6**(2):146–154.
66. Cines DB, *et al.* Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood.* 1998;**91**(10):3527–3561.
67. Wong BW, *et al.* Endothelial cell metabolism in health and disease: impact of hypoxia. *EMBO J.* 2017;**36**(15):2187–2203.
68. Ip MS, *et al.* Endothelial function in obstructive sleep apnea and response to treatment. *Am J Respir Crit Care Med.* 2004;**169**(3):348–353.
69. Butt M, *et al.* Myocardial perfusion by myocardial contrast echocardiography and endothelial dysfunction in obstructive sleep apnea. *Hypertension.* 2011;**58**(3):417–424.
70. Ciccone MM, *et al.* Reversibility of the endothelial dysfunction after CPAP therapy in OSAS patients. *Int J Cardiol.* 2012;**158**(3):383–386.
71. Pengo MF, *et al.* The effect of positive and negative message framing on short term continuous positive airway pressure compliance in patients with obstructive sleep apnea. *J Thorac Dis.* 2018;**10**(Suppl 1):S160–S169.
72. Herranz D, *et al.* Impact of Sirt1 on mammalian aging. *Aging (Albany NY).* 2010;**2**(6):315–316.
73. Herskovits AZ, *et al.* SIRT1 in neurodevelopment and brain senescence. *Neuron.* 2014;**81**(3):471–483.
74. Zu Y, *et al.* SIRT1 promotes proliferation and prevents senescence through targeting LKB1 in primary porcine aortic endothelial cells. *Circ Res.* 2010;**106**(8):1384–1393.
75. Donato AJ, *et al.* SIRT-1 and vascular endothelial dysfunction with ageing in mice and humans. *J Physiol.* 2011;**589**(Pt 18):4545–4554.

76. D'Onofrio N, *et al*. SIRT1 and SIRT6 signaling pathways in cardiovascular disease protection. *Antioxid Redox Signal*. 2018;**28**(8):711–732.
77. D'Onofrio N, *et al*. Ergothioneine oxidation in the protection against high-glucose induced endothelial senescence: involvement of SIRT1 and SIRT6. *Free Radic Biol Med*. 2016;**96**:211–222.
78. Tian K, *et al*. Sirtuin-6 inhibits cardiac fibroblasts differentiation into myofibroblasts via inactivation of nuclear factor κB signaling. *Transl Res*. 2015;**165**(3): 374–386.
79. Balestrieri ML, *et al*. Response to comment on Balestrieri *et al*. Sirtuin 6 expression and inflammatory activity in diabetic atherosclerotic plaques: effects of incretin treatment. Diabetes 2015;64:1395-1406. *Diabetes*. 2015;**64**(5):e6.
80. Cardus A, *et al*. SIRT6 protects human endothelial cells from DNA damage, telomere dysfunction, and senescence. *Cardiovasc Res*. 2013;**97**(3):571–579.
81. Gano LB, *et al*. The SIRT1 activator SRT1720 reverses vascular endothelial dysfunction, excessive superoxide production, and inflammation with aging in mice. *Am J Physiol Heart Circ Physiol*. 2014;**307**(12):H1754–H1763.