Original Research

Circulating Exosomes and Ambient Air Pollution Exposure in COPD

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Running Head: Circulating Exosomes and Air Pollution in COPD

Abbreviations:
COPD: Chronic Obstructive Pulmonary Disease
PM10,2.5: particulate matters 10,2.5 micrometer
EVs: Extracellular vesicles
GOLD: Global Initiative for Obstructive Lung Disease
IFN-γ: Interferon gamma
IL-6: interleukin 6
TGF-β1: Transforming Growth Factor beta 1
BMI: Body Mass Index
FEV1: Forced Expiratory Volume in 1 second
TEM: Transmission Electron Microscopy

Keywords: chronic obstructive pulmonary disease; exosomes; particulate matter; ambient air pollution; inflammation;

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Note: *This article has an online supplement*
Abstract

Background: Chronic obstructive pulmonary disease (COPD) is characterized by progressive obstruction of airways due to chronic inflammation. Both genetic and environmental factors are risk factors for COPD. The most common cause of COPD is smoking. However, evidence suggests that 17 to 38% of COPD patients are non-smokers, so other factors like air pollution may also play a role.

Objective: The relationship between serum exosomes and exposure to PM 2.5 and 10 μm in the residing environment of COPD patients and healthy groups was investigated. Besides, the correlation between inflammatory cytokines level with exosome count was studied.

Methods: Peripheral blood samples were taken from 20 COPD patients without smoking and family history of COPD along with 20 non-smoker healthy controls. The serum exosomes were counted by flow cytometry using CD81 marker. The exposure to PM2.5 and 10 μm was measured in daily, weekly, and monthly intervals based on the longitudinal measurements of the monitoring stations and the correlation between exosome count and air pollutants was analyzed.

Results: The serum CD81+ exosome count in COPD patients was significantly elevated compare to the healthy controls and this was correlated with daily PM10 (P-value: 0.02) and monthly PM2.5 (P-value: 0.02) exposure. Although Interferon-γ levels of COPD patients were higher than healthy controls, there was no correlation between exosome count and cytokine level.

Conclusion: Considering the significant relationship between air pollutants and the count of serum exosomes demonstrated in the present study, air pollution might be a considerable risk factor in the progression of airway inflammation.

Keywords: Chronic Obstructive Pulmonary Disease; Exosomes; Particulate Matter; Ambient Air Pollution; Inflammation, Interferon-gamma
1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive inflammatory airway obstruction mainly caused by sustained exposure to environmental pollutants such as cigarette smoking, occupational smoke, and air pollutants, in genetically susceptible individuals (1). COPD is a leading cause of death among patients with chronic lung diseases. In 2019, the global prevalence of COPD among people aged 30–79 years was 10.3%, which means that nearly 400 million people are affected by this disease (2). The most common cause of COPD, with a prevalence of about 80% in adult patients, is smoking. However, evidence suggests that 17 to 38% of COPD patients are non-smokers, so other factors such as occupational and air pollution may also play a role (3).

Air pollution is one of the most challenging health concerns, particularly in developing countries, reported to be associated with almost 7 million premature deaths annually and inflammatory disease exacerbation (4, 5). Air pollution is defined as an excessive aggregation of particles, noxious gases, and vapors emitted from industrial and natural sources. The most monitored pollutants are particulate matters (PM), a combination of liquid and solid particles suspended in the air, known as PM10 and PM2.5 according to their size (6). There is also growing evidence of the implication of air pollution in the initiation and progression of respiratory diseases including COPD(7, 8). However, due to the lack of sufficient knowledge of molecular mechanisms that correlate the micro particles with inflammatory responses, no specific modality could be introduced for the prevention or treatment of air pollution-related COPD. Nonetheless, air pollutants have been shown to trigger the production of inflammatory mediators(9-11), and extracellular vesicles(12).

Extracellular vesicles (EVs) are membrane vesicles released from different types of cells and detected in various body fluids such as blood, urine, bronchoalveolar lavage, pleural effusion, and nasal secretions. EVs contained proteins, lipids, nucleic acids, and metabolites are supposed to be involved in cell-cell interactions(13). EVs are classified into three categories based on size, biogenesis, and secretory components: Exosomes, cellular Microvesicles/Microparticles and Apoptotic bodies. Exosomes are distinguished from other EV classes by their morphology, their endosomal origin (generated by early and late endosomes, and eventually, form multivesicular bodies) and small size, approximately 30–150 nm (14-16), while Microvesicles and Apoptotic
bodies are larger in size, > 100 nm and 1–5 μm, respectively (17). CD9, CD63, CD81, and CD82 peripheral membrane proteins (tetrasculins) are fused during release into the extracellular space and used as exosomal markers (18). Exosomes deliver cytokines such as Transforming growth factor beta (TGF-β), Tumor necrosis factor alpha (TNF-α), Interleukin 6 (IL-6), IL-8, and IL-10 to the recipient cells, leading to the development of chronic inflammation, oxidative stress, cell apoptosis, and decreased lung function with a pivotal role in the occurrence and progression of COPD (19). Among the cytokines mentioned above, IL-6 is an acute phase protein, a marker of the high systemic inflammation within an association of airway epithelium in COPD. Interferon-gamma (IFN-γ) enhances COPD by decreasing the number of Th2 cells and shifting the immune response toward COPD exacerbation. TGF-β directs numerous immune cell activities and promotes fibrotic remodeling of airways, which further diminished lung function in COPD.

According to the dynamic of the production and release of EVs and their variable contents, they might serve as biomarkers to monitor the inflammatory status of tissue in response to external stimulants. Therefore, extracellular vesicles, in particular exosomes, might be eligible candidates for investigating the effects of air pollution on inflammatory events in the respiratory system.

2. Patients and methods

2.1. Patients

Twenty non-smoker patients with COPD who were admitted to the Imam Khomeini Hospital (Tehran, Iran) were recruited. The diagnosis was approved by a specialist according to the Global Initiative for Obstructive Lung Disease (GOLD) criteria. The patients were middle-aged (46.5 ± 13.8 y/o). All patients were receiving anticholinergics, long-acting beta-agonists, and inhaled corticosteroids but none were under systemic immunosuppressive agents at the time of sampling. Twenty healthy age-matched (44.4 ± 12.6 y/o), non-smoker individuals with no clinical evidence of COPD were included as controls. None of the studied subjects had signs or symptoms of infectious, allergic, or autoimmune disorders at the time of sampling. All individuals gave informed consent. The study was approved by the Ethics Committee of Tehran University of medical sciences (EC: IR.TUMS.CHMC.REC.1397.045).

2.2. Isolation and illustration of Exosomes
a) The serum was separated from 5 CCs of fresh peripheral blood by centrifuging the sample at 2500 rpm for 5 minutes. Serum samples undergo the pre-purification stage by two-step centrifugation at 2000xg for 10 min (4 °C), followed by 10,000xg for 30 min (4 °C) before transferring to a 0.22 µm centrifugal filter (Sigma-Aldrich, MO, USA) for removing cell debris and particulate impurities from samples.

b) Exosomes were isolated from serum using the Exocib kit (Cibzist, Tehran, Iran)(20, 21). Based on the sedimentation method, according to the manufacturer's procedure, the collected supernatant was mixed with pre-heated reagent A (ratio 5:1), and vortexed for 5 min. Then, the mixture was incubated for 24 h at 4 °C. After vortexing for 1 min, the mixture was centrifuged at 3200×g for 40 min at 4 °C and the supernatant was discarded. The exosome pellet was resuspended in 500 µL of reagent B and stored at -80°C. Then isolated exosomes were first analyzed by Dynamic light scattering (DLS) to determine the distribution of the exosomes particle size. Then the morphological assessment of the isolated exosomes was performed using transmission electron microscopy (TEM). Briefly, exosomes were deposited onto Formvar-carbon coated grids for 20 min, blocked with 0.5% bovine serum albumin (BSA)/PBS for 10 min. Grids were fixed with 1% glutaraldehyde/PBS for 5 min, washed with dH2O, and contrasted using 0.5% uranyl acetate for 2 min. Excess stain was blotted off and grids were dried for ≥2 days. Observations were carried out using a JOEL 2100 electron microscope at 120 kV.

2.3. CD-81+ Exosomes counting

For counting the exosomes, a preferred counting of exosomes was performed using the flow cytometry technique(22, 23). In brief, 0.01 dilution of extracted samples were stained based on the manufacturer's procedure of Perfect-Count MicrospheresTM (Cytognos, Salamanca, Spain) which is a microbead-based single-platform system combined with monoclonal antibodies (MoAb) conjugated with fluorochrome.

Anti-CD81 conjugated antibodies (349505, BioLegend, Austria) were used to stain the exosomes due to the accumulation of CD81, a tetraspanin molecule used as exosomal markers, in small EVs. Exosome counts were calculated using conventional flow cytometry by the addition of a known amount of counting beads and the formula: MV/µl = (MV count/bead count) × final bead concentration. Flow cytometry was performed by FACSCalibur (BD FacsCalibur, Becton
Dickinson, USA) and data were analyzed with FlowJo v10.7 software (TreeStar Inc., Ashland, OR, USA).

### 2.4. PM2.5 and PM10 monitoring

Tehran, the capital and most populated city of Iran is located in a relatively limited area of the Alborz mountains in UTM 35° 34–35° 50' N and 51° 08–51° 37' E. The city is expanded in a total area of 730 km2 with around 9 million dwellers frequently facing metropolitan air pollution and dust storm. The heavy and steady traffic jams of Tehran and vehicle usage, along with substandard and poor-quality fuels being used and the poor air recycling because of the geographic location lead to particulate air pollution that reaches usually above the permissible levels. The concentration of airborne particles with an aerodynamic diameter of 2.5 mm (PM2.5) and 10 mm (PM10) are measured and recorded regularly (every hour) by 32 air quality monitoring stations for measuring PM2.5, and 19 stations for measuring PM10 (Tehran air quality control company). In the present study, we used the mean of the daily, weekly, and monthly values based on the longitudinal measurements of the monitoring stations. Each missed or unavailable data was modeled with GIS software and satellite information, and the correct values of conflict were obtained for each person. The residence area was defined as a place where the subject spends at least 10 hours during the daytime. The participants were residing in different districts of Tehran province.

### 2.5. ELISA

The ELISA tests were performed using Human IFN-γ (ZB-0105-H9648, ZellBio, Germany), Human IL-6 (ZB-0090-H9648, ZellBio, Germany), and Human TGF-β1 (ZB-0134-H9648, ZellBio, Germany) Kits according to the manufacturer's instructions with the sensitivity of 1.5ng/mL, 3.5pg/mL, and 5.11ng/mL, respectively. All samples were examined in duplicate. Absorbance was read using the Hiperion MPR4 ++ Microplate Reader (Medizintechnik GmbH & Co.KG Germany). The calibration curves were drawn to determine the concentration of the cytokines.

### 2.6. Statistical analysis

Data were displayed as mean ± standard deviation or mean ± standard error of the mean. Mann-Whitney test was used to compare the continuous values within the categorical variables. The correlation was determined as correlate bivariate and Pearson correlation coefficient (r). The
graphs were presented as box plots and dot plots. P-values less than 0.05 were considered significant (SPSS 26.0; SPSS Inc., Chicago, USA).

3. Results

3.1. Patients: The demographic, paraclinical, and clinical findings of the study are summarized in Table-1.

<table>
<thead>
<tr>
<th>Table-1: Demographic Characteristics of Study Population</th>
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<tbody>
<tr>
<td>Group</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>Age (year)</td>
</tr>
<tr>
<td>Gender ratio (M:F)</td>
</tr>
<tr>
<td>BMI (kg/m²) (Mean±SD)</td>
</tr>
<tr>
<td>COPD duration (year)</td>
</tr>
<tr>
<td>Disease severity:</td>
</tr>
<tr>
<td>Mild (FEV1&gt;80)</td>
</tr>
<tr>
<td>Moderate (FEV1=50-80)</td>
</tr>
<tr>
<td>Severe (FEV1=30-50)</td>
</tr>
<tr>
<td>Critical (FEV1&lt;30)</td>
</tr>
<tr>
<td>FEV1 (L) (Mean±SD)</td>
</tr>
<tr>
<td>FVC (L) (Mean±SD)</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
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<tr>
<td>CT scan findings:</td>
</tr>
</tbody>
</table>
3.2. Exosomes analysis

The size of exosomes in the dynamic light scattering (DLS) graph was presumed between 100-300 nm. The peak of the graph was 234.2±39.2 nm covering 99.7% of particles (Figure-1a). According to the literature, exosomes are typically 30–150 nm in diameter. However, we observed a slight increase in size in the DLS result, it is important to note that DLS measures the hydrodynamic diameter of particles, which can be larger than their actual physical diameter due to the presence of a hydration layer. Therefore, it's important to use other techniques, such as electron microscopy, to confirm the size and morphology of exosomes (figure 1b.). The results of the TEM in two scales of 50 and 100 nm indicated the size of the particles ~30-150 nm as well (Figure-1).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emphysema</td>
<td>47.6%</td>
</tr>
<tr>
<td>Vascular markings</td>
<td>33.3%</td>
</tr>
<tr>
<td>Cardiomegaly</td>
<td>19%</td>
</tr>
<tr>
<td>Pulmonary artery enlargement</td>
<td>19.1%</td>
</tr>
<tr>
<td>Right ventricular hypertrophy</td>
<td>9.5%</td>
</tr>
</tbody>
</table>

**BMI:** Body Mass Index, **FEV1:** Forced Expiratory Volume in 1 second, **FVC:** Forced Vital Capacity

Figure 1: Characterization of serum exosomes. a. Determination of exosomes’ size in DLS graph. b. The morphological assessment of the isolated exosomes by TEM in comparison to two scales of 100 and 50 nm. **DLS:** dynamic light scattering, **TEM:** Transmission electron microscopy
3.3. Higher Serum CD81+ exosome counts in COPD patients

The count of serum exosomes in the group of COPD patients was significantly higher than the healthy controls \([4789 \pm 960.2 \text{ vs. } 2284 \pm 367.8 (\text{Mean} \pm \text{SEM}) (P \text{ value: } 0.02)]\) (Figure-2).

![Figure-2](image-url)

**Figure-2:** a. A representative figure of flow cytometry exosome counts results. Gate a. contained internal controls microbeads A and B, the Gate b. contained the main analyzed population. b. Serum CD81+ exosome counts of COPD patients compared to the healthy controls (*: P-value: 0.02)

3.4. PM2.5 and PM10 exposures in patients and healthy controls

The mean daily exposure to PM2.5 and PM10 in COPD patients was slightly lower than healthy controls (93±7.9 and 84±4.2 Mean±SEM for PM2.5 and PM10, respectively); however, weekly exposure to PM10 was mildly higher in the patients group (77±5 vs. 69±7.1 Mean±SEM). Comparing the monthly exposure, the COPD patients had a significantly higher exposure to PM2.5 in the preceding month (53±4.1 vs. 41±4.0 Mean±SEM) (P-value:0.02) (Figure-3).
3.5. Direct correlation between exosome count and monthly PM2.5 and daily PM10 exposures in COPD patients

Peripheral blood exosome frequency in COPD patients showed a direct significant correlation with monthly exposure to PM2.5 with a Pearson correlation coefficient of 0.58 (CI 95%: 0.086 to 0.85 and P value: 0.02) (Figure 4a.). The result of correlation analysis in these variables was not
significant in the healthy group with a Pearson correlation coefficient of -0.005 (CI 95%: -0.44 to 0.43 and P value: 0.9) (Figure 4c.). In addition, serum exosome counts of COPD patients showed a direct significant association with PM10 exposure on the day of sampling with Pearson correlation coefficient of 0.61 (CI95%: 0.12 to 0.86 and P value: 0.02) (Figure 4b.). The result of correlation analysis in these variables was also not significant in healthy group with Pearson correlation coefficient of 0.28 (CI 95%: -0.18 to 0.64 and P value: 0.2) (Figure 4d.).

Figure-4: Direct correlation in patients group between a. serum exosome count and monthly exposure to PM2.5 (P value: 0.01) b. serum exosome count and daily exposure to PM10 (P value: 0.04). No correlation in healthy group between c. serum exosome count and monthly exposure to PM2.5 and d. serum exosome count and daily exposure to PM10. PM: Particulate Matter

3.6. Higher IFN-γ levels in COPD patients compared to the healthy controls

The serum level of IFN-γ was significantly higher in COPD patients than in the healthy controls [171.9 ± 126.7 vs. 63.7 ± 30.8 (P-value: 0.01)]. However, neither TGF-B1 (153.3 ± 17 vs. 163.6 ± 24) nor IL-6 (2.99 ± 0.4 vs. 2.94 ± 0.66) amounts showed a significant difference between the two groups (Figure-5).

Figure 5: a. IFN-γ levels in COPD patients compared to the healthy individuals (P-value: 0.01). b. and c. no significant difference between TGF-B1 and IL-6 concentrations between groups. IFN-γ; Interferon γ, IL-6; Interleukin 6, TGF-B1; Transforming growth factor beta1.

3.7. No correlation between exosome counts and cytokine levels
The correlation analysis showed no significant correlation between exosome counts and IFN-γ, TGF-B1, and IL-6 levels. There was also no statistically significant association between the concentration of air pollutants PM2.5 or PM10 and studied cytokines.

4. Discussion

Chronic obstructive pulmonary diseases are chronic inflammatory disorders leading to irreversible airway obstruction. COPD includes clinical subgroups such as asthma, bronchial hyperresponsiveness, and emphysema. They are presented with hyperinflation, cachexia, chronic bronchitis, frequent exacerbations, and systemic inflammation. COPD is considered the most common cause of death from respiratory disorders(24).

Air pollution is one of the most important health concerns in developing countries that causes millions of death per year and is estimated to become the third cause of death in the near future(25). Air pollutants include sulfur dioxide (SO2), nitrogen dioxide (NO2), carbon monoxide (CO), ozone (O3), methane, hydrogen chloride, aromatic hydrocarbons, dioxins, organic volatiles, particulate matters, etc. Particulate matters are classified into PM 0.1, 2.5 and 10 based on their diameter size (26). Air pollutants have been shown to be involved in the initiation, development and exacerbation of bronchopulmonary(6), cardiovascular(27), allergic(28) and autoimmune diseases(29). They can affect healthy populations but the hazard is greater for vulnerable individuals, such as the elderly, children, and those with chronic conditions e.g. asthma and emphysema (30). It has been shown that prolonged exposure to air pollutants increases the risk of developing COPD. There is also some evidence that exposure to particulate matter exacerbates COPD symptoms, thereby increasing the risk of death (31). Similarly, the present study showed a significant correlation between PM2.5 exposure in the preceding month of sampling and increased exosome counts in COPD patients. This finding might be suggestive of an association between chronic exposure to air pollutants and exacerbation of inflammatory conditions in these patients.

The role of exosomes in the pathogenesis of COPD has barely been studied(32); in addition, the results have generally been obtained from experimental models or in vitro studies. One research by Moon et al. on long lung epithelial cells showed that prolonged exposure to secondhand cigarette smoke could produce CCN1-rich exosomes, an extracellular matrix protein that plays an
important role in tissue repair and malformation(33). As a result, the exosomes concentrated with this protein may lead to the induction of paracrine CXCL8 production and subsequent recruitment of neutrophils and other inflammatory cells to the site, which can eventually enhance the process of fibrosis in the lungs. Indeed, microvesicles with proinflammatory function might be released by a broad range of respiratory cell types subsequent to exposure to various environmental factors such as microbes, cigarette smoke, and oxidative stress (34). These and other findings suggest the contribution of exosomes in sustained inflammation and airway remodeling in COPD. The present study also showed increased counts of serum exosomes in COPD patients. Moreover, the exosome count was significantly correlated with daily PM10 and monthly PM2.5 exposures as environmental triggers.

Besides, there have been some investigations about the association of exosome counts with inflammatory cytokines levels (35, 36). For instance, Tan et al. reported higher exosome counts in COPD patients compared to the healthy controls, they also demonstrated elevated levels of CRP, IL-6 and soluble Tumor Necrosis Factor Receptor 1 (sTNFR1) in COPD patients, which was correlated with the serum exosome counts. Furthermore, IL-6 and sTNFR1 levels in patients with acute exacerbation of COPD were higher than in stable patients (37). Similarly, our study demonstrated a higher number of exosomes in the blood of COPD patients compared to healthy individuals. Moreover, there was a significant association between the environmental pollutants PM10 and PM2.5 exposures in patients with the frequency of serum exosomes. There was also a significant difference between the serum levels of IFN-γ between patients and healthy individuals. However, we found no association between exosome counts and cytokine levels may be due to the administration of inhaled corticosteroids or high baseline amounts of IL-6 in healthy volunteers.

These findings suggest a correlation between air pollution and exosome release in COPD patients; therefore, it might be helpful to avoid polluted environments in order to reduce the severity of inflammation and exacerbation of the disease. Serum levels of circulating exosomes and their contents might serve as diagnostic and prognostic biomarkers in patient surveillance.

Nonetheless, the present study had some limitations, for instance, the sample size was fairly small. Indeed, since smoking is a considerable risk factor for developing COPD, finding patients without any history of active or passive smoking was challenging. In addition, due to the COVID-19 pandemic, the patients’ presence in the clinics was restricted. Therefore, the number of eligible
patients was lower than expected. The other limitation was the lack of information about the biological contents of the detected exosomes. Obviously, to discover the correlation of exosomes with chronic inflammation and disease pathogenesis, it is critical to investigate the probable cytokines, chemokines, nucleic acids, etc. In brief, the elevated number of exosomes in the serum of COPD patients and the correlation of exosome count with air pollutants urge further studies to reveal the mechanisms associating environmental factors with the inflammatory process in COPD.

**Declarations:**

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Conflict of interest: the authors declare no conflict of interest.

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Ethical approval: All the techniques carried out in the present study involving human participants were in accordance with the standards of the institutional research committee and with the Helsinki Declaration and its later amendments or comparable ethical standards. It has the ethical approval of the Tehran University of medical sciences (ethic Code: IR.TUMS.CHMC.REC.1397.045). Informed consent was obtained from all participants after describing the process and goals of the study.
References:

1. Silverman EK. Genetics of COPD. Annual review of physiology. 2020;82:413.


Figure 1

(a) Size Distribution by Number:

- Average (d nm): 181.3
- Peak 1: 261.2, 64.3
- Peak 2: 234.2, 39.7
- Peak 3: 0.000, 0.0

Result quality: Refer to quality report

(b) Images of samples

100 nm

Figure 2

(a) Graphical representation of data:

- Serum Exosomes count (dL)

(b) Comparison of COPD and Control groups

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>12,350</td>
<td>7,291</td>
</tr>
<tr>
<td>Median</td>
<td>9,850</td>
<td>4,930</td>
</tr>
<tr>
<td>Min</td>
<td>3,000</td>
<td>1,200</td>
</tr>
<tr>
<td>Max</td>
<td>30,000</td>
<td>12,000</td>
</tr>
</tbody>
</table>

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Figure 3

Figure 4
Figure 5
Online Supplement

Figure 1: a. the selected population are related to the A and B micro-beads as internal controls. b. FL2 histogram for selected beads population.