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The Effects of Particulate Matter on C57BL/6 Peritoneal and Alveolar Macrophages

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ABSTRACT

The presence of ambient particulate matter (PM) poses more dangers to human health than that of other common air pollutants such as Carbon dioxide (Co2) and ozone. Epidemiologic studies show a direct correlation between PM and the risk of respiratory and cardiovascular diseases. The immune system seems to play a critical role in the process of these diseases. The main goal of this study was to investigate the effect of Tehran particulate matter in two aerodynamic diameters (PM2.5 and PM10) on alveolar macrophages (AM) from C57/BL6 mice.

To evaluate the inflammatory effects of PMs, cultured alveolar, and peritoneal macrophages were treated with PM2.5 and PM10 (concentrations of 5 μg/mL and 10 μg/mL). Tumor necrosis factor-alpha (TNF-α) and IL-10 (representatives of inflammatory and anti-inflammatory cytokines, respectively) were assessed in the culture supernatant by ELISA. Expression of arginase and inducible nitric oxide synthase (*iNOS*) genes was carried out by quantitative real-time PCR. Different functional types of cultured alveolar macrophages (M1, M2) were also determined in this study.

Our results suggest that PM2.5 induces M1 inflammatory phenotype in comparison with PM10. We found Also, an increase in TNF-α and M1-related gene expression (iNOS), as well as a decrease in both IL-10 and M2 phenotype genes (Arginase). Moreover, a reduction in phagocytic capacity and increased apoptosis function of macrophage cells were detected.

PM2.5 as a major component in hydrocarbons has a considerable effect on polarizing the alveolar macrophages to an inflammatory phenotype and eliciting lung inflammation in mice.

Keywords: Inflammation; Macrophages; Particulate matter

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INTRODUCTION

Particulate matter (PM) is a complex mixture of dispersed solid particles and liquid droplets composing of heterogeneous components including acids, organic chemicals, metals, biological compounds, and dust particles.[1] PM is classified by its aerodynamic equivalent diameter into three major groups including PM10, PM2.5, and PM0.1.[1] Particle size is a critical parameter determining deposition site in the respiratory tract and adverse health effects. PM10 is trapped by mucus and cilia of the upper respiratory tract and cannot reach the deeper part of the lung.[1] PM2.5 can reach distal airways and alveolar regions where alveolar macrophages reside. Particles smaller than 0.1 μm can penetrate to the alveoli and translocate into the systemic circulation resulting in significant health problems on secondary organs and tissues.[1] Exposure to PM is responsible for serious adverse health effects in human beings including respiratory symptoms and decreased lung function, cardiovascular diseases and metabolic disorders.[1,2]

The exact mechanism underlying PM adverse health effects has not been indicated yet. One of the suggested mechanisms declares that inhaled PMs induce oxidative stress and cause elevated reactive oxygen species (ROS) production that can have cytotoxic effect on cells and trigger inflammatory responses.[1,2] Alveolar macrophages (AM) represent major population of immune system cells keeping the lung alveoli clear by removing pathogens and environmental agents.[3] Clearing pathogens and particulates without inducing inflammatory responses that might disrupt gas exchange in the lung is a critical role of AMs.[4] In steady-state AMs modulate immune responses and limit inflammation by receptors and cytokine/chemokine mediated interaction with lung cells and microenvironment.[3] In vivo and in vitro studies show that alveolar macrophages in response to air pollution shift to inflammatory phenotype and produce inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), IL-1, IL-6, and IL-8 after PM exposure[5,6,7,8].

Although studies focus on the association between air pollution and cardiovascular and respiratory diseases, there have been few studies to examine the potential effect of air pollution on the digestive system.

Inhalation of particles is the main route of human exposure to air pollution but human contact to the particulate matter by ingestion intake too. Particulate matter contaminates water supplies and foods and enters the gastrointestinal system. Furthermore, PM which is trapped in the upper respiratory tract mucus enters the gut by ingestion.[9] Epidemiologic studies show an association between air pollution and gastrointestinal cancers. Additionally, emergency room visits, for appendicitis, increased during air pollution episodes. Furthermore, inflammatory gut disease, for instance, inflammatory bowel disease is more prevalent in developed and industrialized countries. A recent study in the United Kingdom investigated the incidence of ulcerative colitis and Crohn's disease was higher in children and young adults of polluted regions.[10] Studies link these observations to dysregulation of the systemic and gut immune system. Several animal studies suggest that air pollution exposure might drive intestinal inflammatory response via increasing gut permeability and impact on intestinal microbiota. It is well known that chronic inflammation has a crucial role in the pathogenesis of digestive system disorders such as inflammatory bowel disease, obesity, and diabetes mellitus type II.[9] Peritoneal macrophages are tissue resident-cells found in the peritoneal cavity. They are the main leukocyte population in the abdominal cavity with high phagocytic roles and defense mechanisms against microorganisms and particles in the intestine. The function of peritoneal macrophages has not been determined exactly but it might contribute to clear foreign pathogens and materials which pass across the gut tubes.[11,12]

The present study was designed to reveal the level of inflammatory cytokine the (TNF-α) and immunoregulatory cytokines such as (IL-10) produced by C57/BL6 AMs and peritoneal macrophages exposed to urban particle matter of Tehran. The level of IL-10 and TNF-α in the supernatant of cultured alveolar and peritoneal macrophages was measured, to determine the inflammatory potential of particles. Also, the expression of M1 (*iNOS*) and M2 (*Arg-1*) related gene was studied following stimulation with particles. We also determined the impact of PM on apoptosis and phagocytosis ability of peritoneal macrophages.

MATERIALS AND METHODS

Particle Collection and Preparation

PM collector filters of different stations were gifted from the Tehran air quality standard agency.

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Paper rolls were cut separately (2.5 μm and 10 μm) in small pieces from airborne particle filtered spots and soaked in deionized water for 10 minutes. In the next step, Particulate matter was collected by shaking of filters for 5 min in ultrapure water by sonication.[13] Filter suspension passed through mesh with a different pore size of 100, 80, and 40 microns. Then Particle suspension was added to 15 microns Polypropylene tube and centrifuged for 20 min at 12000 rpm to obtain particles pellet. The supernatant was removed. To have sufficient material, PM pellets were pooled. Particle dried at room temperatures under lamina flow. Dry particles were weighed and weighted particles were suspended in Phosphate Buffer Saline at 5 μg/mL and 10 μg/mL concentration and stored at 4°C until use.

Harvested particles (2.5 & 10 μm) were suspended in RPMI medium at concentrations of 5 μg/mL and 10 μg/mL, sonicated, and then added to alveolar and peritoneal macrophage culture of C57BL/6 inbred mice.[14]

Animals

Male C57/BL6 mice of eight weeks old were purchased from the Iran Pasteur institute animal resource center and were kept in the immunology department animal room following the principles outlined in the ‘‘Guide for the Care and Use of Laboratory Animals’’ issued by the National Institutes of Health (NIH). These mice weighted 20±4 grand 6-8 weeks old. A schematic of this study’s procedures has been shown in Figure 1.

Ethics

This study was approved by the committee on the ethical handling of Research Animal of Shahid Beheshti Medical Science University. The work was also approved and supported by Vice Chancellor for Research. All procedures followed were following the ethical standards of the responsible committee on animal experimentation (institutional and national) and with the: IR.SBMU.MSP.REC.1396.571 Approved Ethical Code. All institutional and national guidelines for the care and use of laboratory animals were done.

Isolation of Alveolar Macrophage

To collect bronchoalveolar lavage fluid (BALF), Mice were euthanized with isoflurane, bronchus was punctured by thin intravenous cannula attached to 1ml syringe, and cold RPMI was perfused into the lung, after 1-2 minutes RPMI was aspirated.[15] To increase the total number of cells, BALF collected from ten mice was pooled in a 10 mL conical centrifuge tube and centrifuged for 10 min at 400×*g*, 4°C.[16] Cell pellet was suspended in 10 Ml RPMI and counted in a hemacytometer. The final viability was determined with the trypan blue dye exclusion test (up to 95%).

Peritoneal Macrophage Isolation

Four male C57BL/6 mice of eight weeks old were sacrificed by cervical dislocation. To harvest peritoneal macrophages, the abdomen skin of mice was cut to expose the abdominal region. The peritoneal cavity was lavaged with 5 mL cold RPMI medium. After 4-5 times lavages, harvested cells were centrifuged at 400 g for 10 min at 4°C. Then RPMI medium supplemented with 10% fetal bovine serum was added to the cell pellet and enriched for future cultivation.

Cell Culture and Particles Exposure

Alveolar Macrophages were cultured in RPMI-1640 (Biowest, France). The medium supplemented with 10% fetal bovine serum (FBS, Gibco-USA), at a density of 8×10^5 cells/well in 6 wells plates at 37°C in a 5% CO2 containing incubator. Alveolar macrophages were allowed to attach to the culture bed; non-adherent cells were removed after 4 hours. Macrophages were treated with PM2.5 and PM10 separately prepared in RPMI medium at a concentration of 5 μg/mL and 10 μg/mL.

An appropriate concentration for the assay was determined using the MTT test. Stimulation time was 6, 12, and 24 hours. Alveolar macrophages culture without particles was used as a control group.

Peritoneal macrophages were cultured in 1×10^6 cells per well for cytokine assay and 4×10^5 cells per well for phagocytosis and apoptosis assay.

Cultured cells were incubated with PM2.5 and PM10 (5 μg/mL & 10 μg/mL) for 24 h at 37°C in a humidified atmosphere containing 5% CO2.

Measurement of TNF-α and IL10

Supernatants of mouse alveolar macrophage culture were collected after 6, 12, and 24 hours incubation and centrifuged and stored at -80°C until analysis of cytokines. Also, supernatants of peritoneal macrophage culture were harvested after 24 hours of exposure. TNF-α and IL-10 measurements were performed using Enzyme-linked Immunosorbent Assay (ELISA)

according to the ELISA kit protocol (Invitrogen Thermo Fisher Scientific, USA).

RNA Extraction and Quantitative Real-time PCR

Alveolar macrophages were collected in RNase/DNase free tube 6 hours after stimulation by PM2.5 and PM10 at the concentration of 5 µg/mL and 10 µg/mL and kept in -80°C freezers until mRNA extraction. Total RNA was extracted from AMs using the RNeasy micro kit (Qiagen,, Germany). The concentration and purity of extracted RNA were determined by optical density measurement using a nanodrop spectrophotometer at 260/280 nm, only samples with an A260/280>1.8 were used for further analysis to ensure RNA quality. cDNA was synthesized using cDNA synthesis Kit Yekta Tajhiz Azma.

Gene expression analysis was carried out by SYBR green real-time PCR and ABI 7500 fast real-time PCR system. GAPDH was used as an internal control and the expression level of each target gene was normalized to that of GAPDH using the $2^{-\Delta\Delta Ct}$ method. All results were analyzed by the Relative expression software tool REST software.

Primers used for the reactions were listed in Table 1.

Table 1. Primer sequences for real-time

PCR Primer name	Sequence
GAPDH (forward)	Forward: AACTTTGGCATTGTGGAAGG
GAPDH (reverse)	Reverse: GGATGCAGGGATGATGTTCT
Arg-1 (forward)	Forward: GGAACCCAGAGAGAGCATGA
Arg-1 (reverse)	Reverse: TTTTTCCAGCAGACCAGCTT
iNOS (forward)	Forward: TTGGAGCGAGTTGTGGATTG
iNOS (reverse)	Reverse: GTGAGGGCTTGGCTGAGTGA

Phagocytosis Assay

The phagocytosis ability of macrophages was investigated using *Saccharomyces cerevisiae* yeast uptake**.** (Inactivated yeast *S. cerevisiae* C NCM I-3856 (referenced as IY) is a primary grown dried whole yeast, obtained by drum drying of *S. cerevisiae* CNCM I-3856. (Centro *Servizi* Stabulario Interdipartimentale, BIOSTAB. Italy).

After cell exposure to PM2.5 and PM10 for 24 h, treated macrophages and control group were challenged with *Saccharomyces cerevisiaes* and incubated for 30 min at 37°C in a 5% CO2 atmosphere. After the end of the culture, phagocytic cells were fixed with methanol and then stained with Giemsa dye. The number of phagocytized *Saccharomyces cerevisiaes* was determined by counting at least 100 macrophages, and results were expressed by calculating the percentage of cells that have phagocytized yeast particles.

Apoptosis Assay

After 24h treatment with PM, cultured cells in 6 well plate were centrifuged at 1000 rpm for 5 minutes, and then 100 µL EB/AO dye mix (Acridine orange Ethidium Bromide; Zigma, USA) was added to each well, and cells were viewed and counted under fluorescent microscope and number of apoptotic cells was recorded in 100 total cells.

Statistical Analysis

The statistical analysis was performed by SPSS (version 21.0). All values are given as mean±standard error of the mean (S.E.M.). Data were statistically analyzed by Kruskal Wallis followed by Mann Whitney u test. A *p*-value of<0.05 was considered statistically significant.

Study Design

We summarized the various steps and design of the experience in this schematic scheme (Figure 1).

RESULTS

Effect of Particles on Peritoneal Macrophages

The level of TNF-α in the supernatant of peritoneal macrophages exposed to both particles (PM2.5 and PM10) were significantly increased in comparison to control (p=0.001). The cytokine release induced by the fine fractions was notably higher compared to the cytokine release induced by the coarse fractions (p=0.015). We also evaluated the level of IL-10 produced by peritoneal macrophages after 24 h treatment with PM2.5 and PM10. Our results show that fine particles reduce IL-10 production after 24 h incubation compared to the control group (p=0.034) and PM10 treated group

(p=0.05). We observed no difference in the release of IL-10 between the control group and the PM10 treated group. The differences were statistically significant for both cytokines (Figure 2).

Effect of Particles on Alveolar Macrophages

To investigate the pro-inflammatory potential of PM10 and PM2.5 particles, AMs were exposed to these particles at 10 µg/mL concentration for 6, 12, and 24 h. Particles in both sizes induced a high level of TNF-α released after 6, 12, and 24 hours of incubation (p=0.001). The greatest TNF-α cytokine production by AM was induced in response to PM2.5 (p=0.001). After 6 and 12h of incubation, the level of TNF-α was significantly higher in the PM2.5 treated group compared to the PM10 treated group (p=0.021). While Statistics analysis showed no significant difference between PM2.5 treated and PM10 treated after 24 h of incubation. Generally, the TNF-α release peaked at 6h after incubation with both particles.

The results indicate that the production of IL-10 by AMs has significantly decreased following 6 and 12 h PM2.5 & PM10) exposure (($p=0.034$ *and* $p=0.050$ respectively). The level of IL-10 did not change significantly after 24 h incubation with PM2.5 and PM10 in comparison to the control group. The data also showed that the PM2.5 particle was more potent than the PM10 particle in decreasing the level of IL-10 ($p=0.034$). IL-10 level reached the minimum amount after 6h incubation with PM2.5 and after 10 h for PM10 particle (Figure 3).

Effect of PM Exposure on Alveolar Macrophage Polarization

We performed a real-time PCR assay to determine the effect of particulate matter on the expressions of M1 related gene (*iNOS*) and M2 related gene (*Arg-1*). As our results defined, particles induced the most inflammatory effects after 6h incubation time. So we investigated the effects of particulate matter on gene expression at 5 µg/mL and 10 µg/mL concentrations after 6 h incubation.

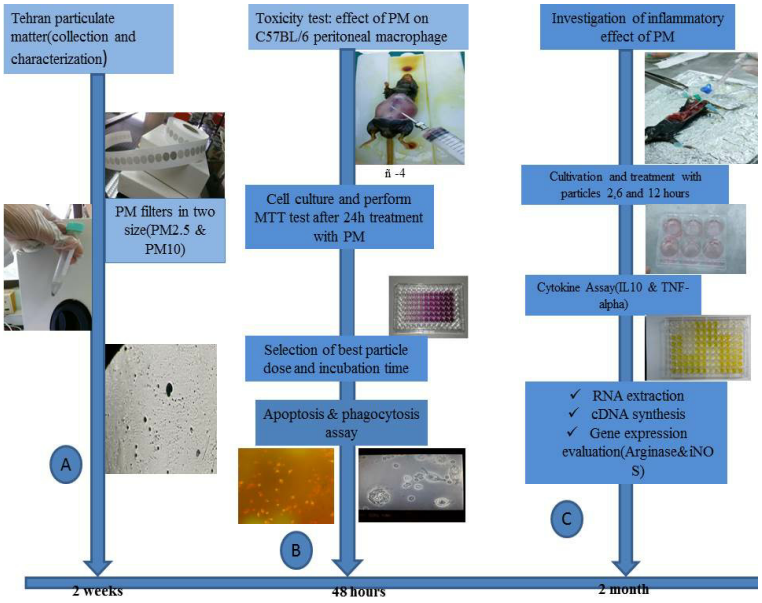

Figure 1: Different stages of experimental designs: *in vitro* and *ex vivo* steps. A; PM collector filters processed to water-insoluble suspends and organized in a different size. B; After harvesting of peritoneal lavaged cells from C57BL/6 mice, toxicity effects of PMs were defined and appropriate dose determined and incubation time by MTT assay. In this stage, phagocytosis ability and vitality changes were determined after the cultivation of cells treated by PMs. C; Inflammatory responses of alveolar lavaged cells treated with PMs detected by cytokine assay (ELISA)and finally M1M2 polarization determined by Arginase and iNos gene expression.

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Our results showed a significant reduction in ARG1 mRNA expression in both sizes (PM2.5 & PM10) and both concentrations were compared with the control group (p=0.007).

Although the difference in ARG1 mRNA expression was observed in PM2.5 treated group compared to the PM10 treated group, this difference was not statistically significant (Figure 4). Also, no statistical differences were observed in both particle doses.

Up-regulation of iNOS mRNA expression in PM2.5 treated cells was more impressive than PM10 treated group. The results showed that this increase was dose-dependent. (p=0.005) (Figure 4).

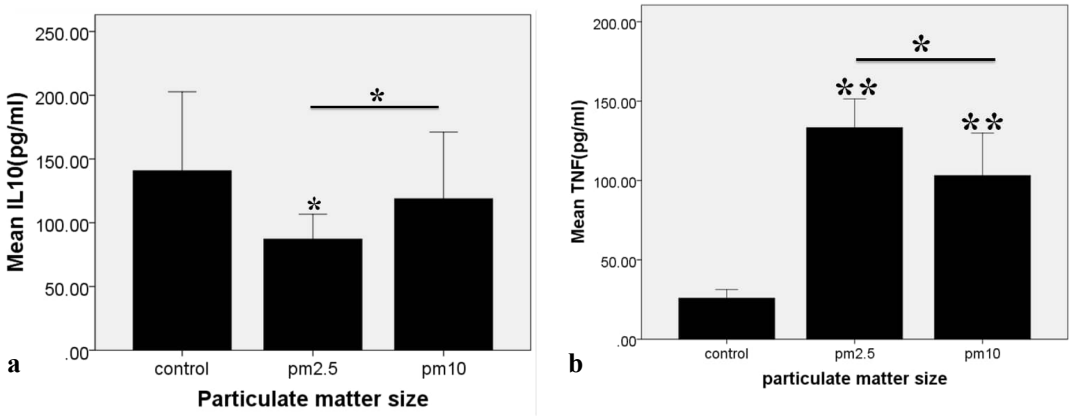


Figure 2. Effect of Particulate matter (PM2.5 & PM10) on IL-10 (a) and TNF-α (b) production in peritoneal macrophages. The cells were incubated in the presence of 10 µg/mL of the particles. Values are means±/SEM. of three experiments assayed in duplicate. Values with an asterisk are significantly different from untreated control cells. * (p<0.05), ** (p<0.01)

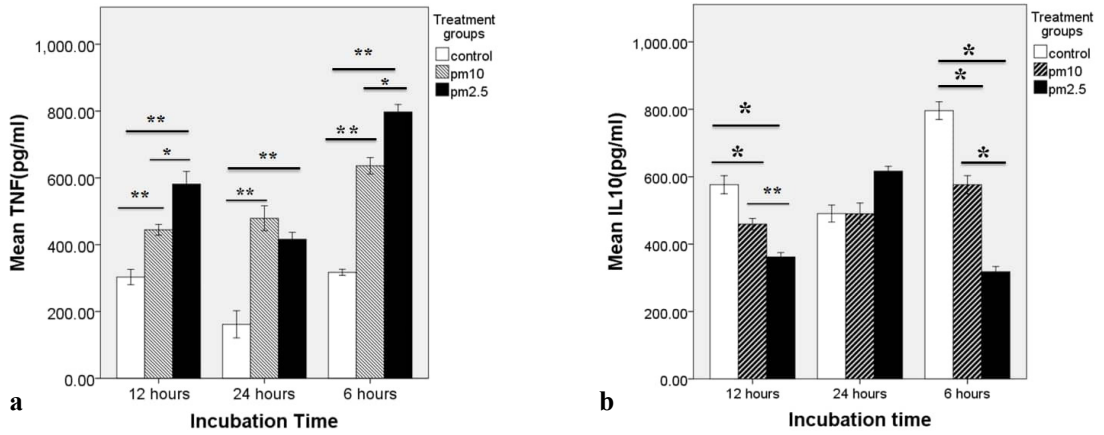


Figure 3. Cytokine production by alveolar macrophages in response to particulate matters (PM2.5 and PM10). Murine isolated alveolar macrophages were exposed to 10 µg/mL of particles for 6, 12, and 24 h. Cell culture supernatant was examined for cytokine production; TNF-α (a) and IL-10 (b). The bars represent mean±SEM of three experiments assayed in duplicate.* p<0.05, **p<0.01

Particle Exposure Effects on Cell Death and Phagocytosis Ability

To establish PM effects on cell apoptosis, peritoneal macrophages following 24 h incubation with PM were stained with Ethidium Bromide/Acridine Orange and cell morphology was assessed under a fluorescent microscope. The number of apoptotic cells was counted and results were recorded. Our results showed that PM treated with particles showed morphological features of apoptosis characterized by nuclear pyknosis and fragmentation. The percentage of the apoptotic cell was significantly higher in PM treated group compared to the control group (p=0.050 in PM2.5 treated group & p=0.046 in PM10 treated group). However, no significant changes were found between PM2.5 treated group and PM10 treated group (Figure 5). Phagocytosis activity was assessed by calculating the percent of macrophages that internalized yeasts.

As Figure 5 demonstrates, the phagocytic function of peritoneal macrophage has been decreased after particle treatment. However both particles (PM2.5 & PM10) decreased the phagocytic ability of peritoneal macrophages, the fine fractions did not induce any significant change (Figure 6).

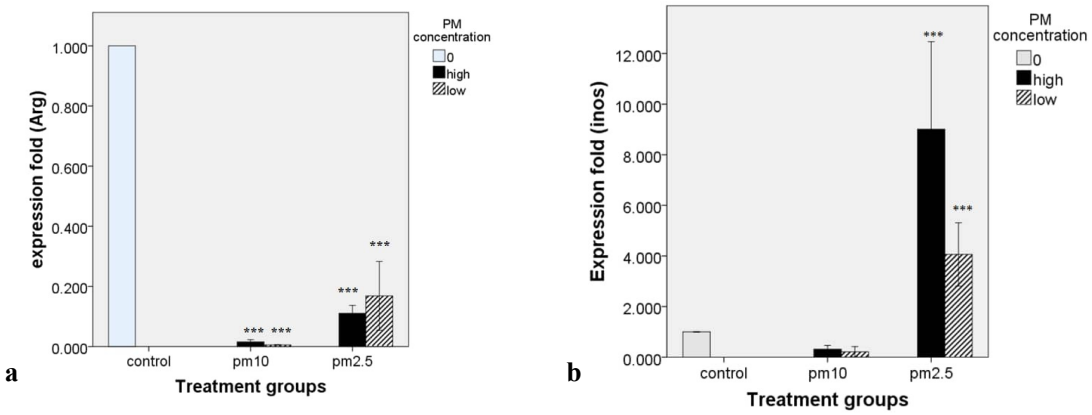
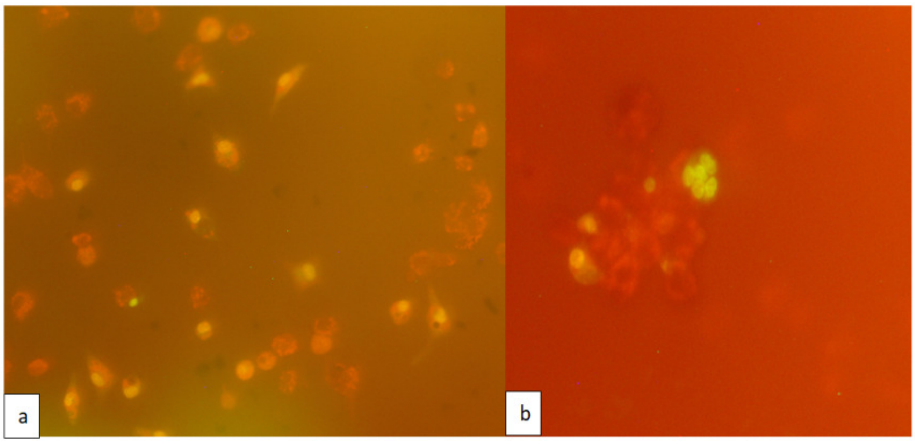


Figure 4. Quantitative RT-PCR was used to measure mRNA levels of M1 and M2 related genes *Arg-1(a)* and *iNOS(b)*. mRNA expressions in the alveolar macrophages were determined by real-time PCR following PM2.5 and PM10 exposure for 6 h in two concentrations; high (10 µg/mL) and low (5 µg/mL). Experiments were done more than twice. Data were shown as mean±SD (N=3). ***p<0.001



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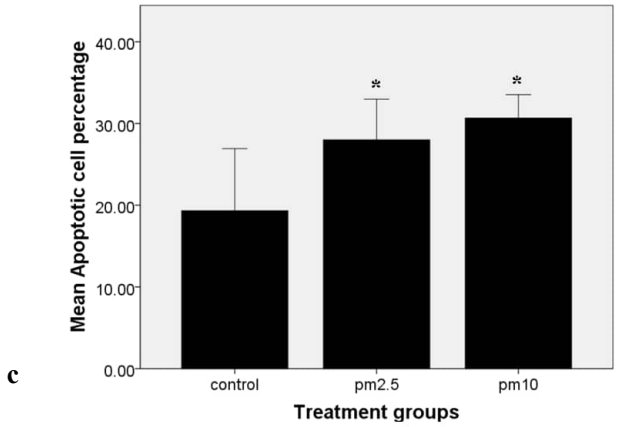

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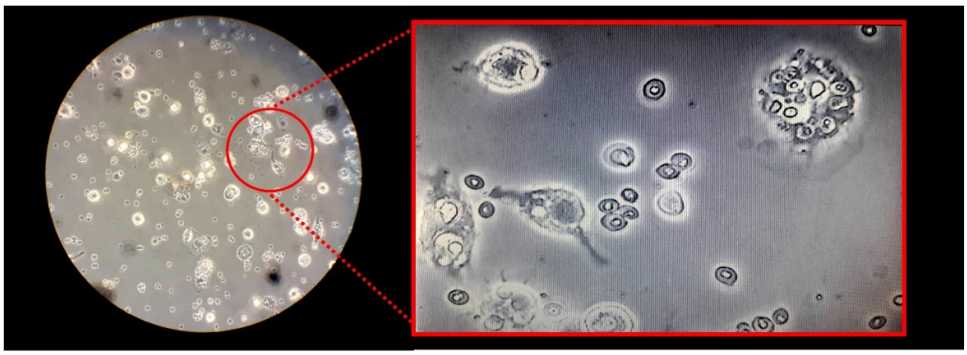
Figure 5. Effect of particulate matter on cell death. Peritoneal macrophages were cultured by PM2.5 and PM10 particles. After 24 hours, the number of apoptotic cells and live cells were assessed; using Ethidium Bromide/Acridine orange dye. a. non-apoptotic cells with light green condensed nuclei. b. the number of cells with pyknotic nuclei was increased after PM treatment. c. PM2.5 and PM10 increased cell Apoptosis in comparison to the control group (p<0.05). Experiments were done more than twice. Data were shown as mean±SD (N=3). * p<0.05



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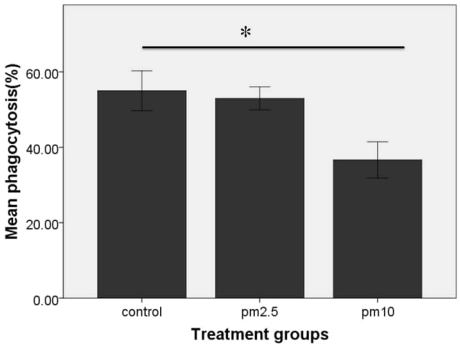

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Figure 6. Microscopic photos of cultured macrophages and *saccharomyces cerevisiae*: (a) Magnification×20 (b) Magnification×100. (c) The total number of phagocyted yeast was decreased after 24-hours treatment. Only PM10 particles decreased phagocytosis ability of peritoneal macrophage statistically significant (p=0.050). Experiments were done more than twice. Data were shown as mean±SD (N=3). * p<0.05

DISCUSSION

Previous epidemiologic studies have shown a direct correlation between high levels of particulate matter increases the risk of respiratory and cardiovascular diseases.[17,18] Although the exact mechanism involving in adverse health effect of urban particles is not clear yet, Several studies suggest that during PM exposure, immune system responses, play a central role in respiratory dysfunction and the lung enters the inflammatory phase.[5,18] PM components such as transient metals can trigger inflammation by inducing ROS production. In addition the organic component of PM especially endotoxin is recognized by toll-like receptors (TLRs) and leads to immune responses.[6,17]

Our results showed that particulate matter causes a functional change in macrophage cells, including cytokine production, gene expression, suppressing phagocytosis ability, and increasing apoptosis. Studies suggest that organic components such as Lipopolysaccharides (LPS) and fungal spores adsorbed onto the particles and after treating particles with endotoxin-inhibitor antibiotics, the inflammatory effect of PM was reduced.[13] The exact amount of endotoxin in particle composition has not been identified yet.

Additionally, transition metals fraction of particles was supposed to affect the inflammatory function and induces apoptosis. Several authors reported a reduction of the inflammatory effect of the particle after treatment using metal chelators.[7,19] Heavy metals can activate oxidative stress reactions in immune and non-immune cells. Oxidative stress trigger inflammatory cascade including pro-inflammatory cytokines production and expression in inflammatory-related genes like *iNOS*[2]. These actions were supposed to promote through an increase in the level of transcriptional factor, nuclear factor kappa-light-chain-enhancer of activated B cells (Nuclear factor-kappa B/ NF-kB,) followed by increasing the level of TNF-α and iNOS gene expression.[20]

It seems that particulate matter can affect alveolar macrophage polarization; macrophages can be classified on basis of function: the M1 and M2-like phenotype, the balance of these two phenotypes is the primary factor in organ hemostasis.[21] M1 macrophages are characterized by pro-inflammatory cytokines production and promoting Th1 immunity, on the contrary, M2 macrophages are closely related to type II immune response and immune-modulatory activity which inhibit inflammation.[22]

It has been reported that particle matters would most likely polarize the M1-phenotype of alveolar macrophages.[23,24] We investigated this hypothesis using exposed C57BL/6 peritoneal and alveolar macrophages to PM2.5 and PM10, then TNF-α and IL-10 were measured for M1 and M2 polarization, respectively. We further examined the molecular change in macrophage polarization related genes after PM exposure.

Our study identified a significant increase in the release of TNF-α after the treatment of alveolar and peritoneal macrophages with urban particles (PM2.5 & PM10) in comparison with untreated macrophages. This finding is consistent with previous reports.[19] TNF-α is a potent inflammatory mediator of the innate immune system that triggers inflammatory reactions, including induction of other cytokine production, activation, or expression of adhesion molecules. These reactions resulted in the migration of other immune system cells to injured organs or tissue. Furthermore, TNF-α can enter the bloodstream and perform systemic effects. TNF-α is responsible for several additional functions linked to lipid metabolism, coagulation, insulin resistance, and endothelial function. On the other hand, it can explain one of the possible mechanisms underlying systemic effects of the particle through increasing inflammatory cytokines especially TNF-α.[25]

In this study, it was found that smaller particle (PM2.5) induces higher levels of TNF-α cytokines compared to larger particles (PM10). However, several studies reported a higher inflammatory potential of PM10 in inducing immune responses to the presence of endotoxin rich compounds.[7,26] However pieces of evidence are conflicting due to differences in size, composition, and sources of particles, geographic region, and season. Bekki et al found that PM2.5 collected from China residential areas strongly induces inflammatory responses.[27] More ever Tehran PM2.5 analysis showed a high amount of organic carbons and PAH (Poly Aromatic Hydrocarbons) components. PM component analysis reported high levels of organic matter in PM2.5 composition. Arhami et al investigated the component of PM2.5 particles of Tehran in 2014-2015, after collection of PM from filters; the composition of PM was estimated by CMC models. Arhami et al study showed that PM

concentration in Tehran is about 33 μg/m^3. This study showed that organic matter (OM) was the dominant component of PM2.5.Consisting of: Organic matter (35%), dust (25%), non-sea salt sulfate (11%), EC (9%), 8 ammonium (5%), nitrate (2%), OE (1%), and sea salt (1%).[28]

On the other hand, Polyaromatic Hydrocarbons (PAH) which is frequent in the organic fraction of PM, were considered as a ligand for Aryl hydrocarbons receptor (Ahr) which resulted in high expression of inflammatory cytokines e.g. TNF-α and IL-8. Additionally smaller particles (PM2.5) have more sites of interaction with the immune system receptors due to the vaster surface.[29,30]

We also demonstrated urban particle treatment resulted in a reduction of IL-10 production (as a marker of M2 polarization). This cytokine plays an important role in the control of alveolar macrophage activity, restrict inflammation in the lung environment, and also inhibit polarization of lymphocyte to Th1 phenotype.[4,31] In this study we showed that PM2.5 particles had a greater effect on reducing the level of IL-10 compared to PM10 particles. Numbers of studies show that IL-10 production decreased following PM exposure.[24] Some studies have not found any alteration in IL-10 production after PM exposure.[32]

Lung epithelial cells are the main source of IL-10 in steady states. Alveolar macrophages express IL-10 receptors and produce IL-10 which can affect this cell by autocrine function. This cytokine plays an important role in the control of alveolar macrophage activity in the lung environment and polarization of lymphocyte toTh2 phenotype. Indeed IL-10 is a pivotal cytokine in the lung environment.

In the next step to confirm the roles of PM in M1 macrophage polarization, we measured the molecular hallmarks of M1 and M2 macrophages after exposure to PM. Th1-associated cytokines such as TNF-α and IFN-γ promote M1 differentiation, characteristic with increased iNOS/Arg-1 ratio. In contrast, Th2 cytokines such as IL-4 or IL-13 results in STAT6-phosphorylation and M2 differentiation with high expression of arginase.[22,32] The metabolism of L-arginine by the nitric oxide synthase isozymes (iNOS) to nitric oxide (NO) plays a major role in organ homeostasis. Unlike, the arginase isozymes compete with the iNOS by converting L-arginine into L-ornithine and urea. INOS/ arginase ratio is related to macrophage polarization.[22,32] Previous studies were shown that particulate pollution, resulting in higher arginase expression in murine models of asthma and causes exacerbation of airways responsiveness. We analyzed the iNOS/Arg1 mRNA expression in macrophage treatment with PM, and our results showed that ambient particles induced the expression of iNOS, the prototypic the M1 marker, whereas the expression of the M2 marker, Arg1, was decreased. Since our data are in agreement with previous studies, so it can be argued that PM polarized the macrophage toward the M1 phenotype.

The other possible mechanism of urban PM influencing the immune system can be explained by their interaction with scavenger receptors (SR) on alveolar macrophages.[5,33] Upon exposure to airborne particles, macrophages are activated and produce pro-inflammatory cytokines and undergo apoptosis or programmed cell death. It has been demonstrated that particle matters can induce apoptosis in macrophage cells by interacting with SR.[5,33] So we tested this hypothesis that inhaled PM contributes to the apoptosis of macrophages using acridine orange/ethidium bromide staining. In our study, the apoptotic death of macrophages increased after 24 hours of treatment with air pollution in comparison with control. According to previous studies, it can be concluded that particle matters are responsible for programmed cell death by induction of high levels of oxidative stress and the NF-kB pathway.[34] Metals which are present in PM composition induce oxidative stress condition and NFkB production.[35] High levels of oxidative stress mediators affect mitochondria permeability, followed by destruction in the electron transport chain and cell apoptosis and necrosis.[3,7] Also several studies suggested that PM increases caspase-mediated cell death by inducing p53 phosphorylation and increase caspase levels.[5,34,36]

Alveolar macrophages provide the first line of immune defense in the lung and their role in phagocytosis of apoptotic cells (efferocytosis) is critical for lung homeostasis.[5] We found that PM10 particles cause functional impairments of the phagocytosis capacities of *saccharomyces cerevisiae* by macrophages. However, this result was not seen in peritoneal macrophage exposed to PM2.5. This finding is consistent with previous reports concerning macrophages phagocytosis reduction after particle treatment, additional studies have shown that phagocytosis of bacteria and yeast cells is reduced by

human alveolar macrophages and mice after treatment with air pollution particles.[37,38]

We evaluated the PM exposure effect on peritoneal macrophages. For years immunology studies focus on peritoneal macrophages because they are the most available immune cells in animal models, which easily reach by peritoneal lavage in the high cellular count.

However; the application of primary alveolar macrophage cultures in experimental studies is difficult due to the instability of their phenotype and function ex vivo.[39] The number of AMs obtained by BAL is low; therefore a high number of mice should be sacrificed. We decided to use peritoneal macrophages in some steps of our experiment.

Generally, our study just established macrophage behavior in cell culture condition, how these particles change immune cells phenotype in the lung environment is more complicated, due to the presence of epithelial and endothelial cells and their interaction with Alveolar macrophages. Epithelial cells by expressing molecules such as CD-200 ligand and secretion of regulatory molecules including TGF-beta and IL-10 which receptors are present in the Alveolar macrophages control the immune system in the lung.[4]

All these inflammatory functions may be transient and followed by a returning phase to M2 macrophages. If the progression million continues to the inflammatory phase, leads to triggering immune dysregulation in the lung. As similar in vivo studies showed exacerbation of emphysema and allergic reactions post PM installation in a long period (60 days).[40]

Therefore, in human urban life on polluted days, the days of starting the contacts, the inflammatory phase started and then followed by a shift to M2 phenotype again. These alveolar macrophages' polarization plays an important role in a long period which can result in multi-organ dysfunction. Studies suggest various factors determining this molecular shift especially sex and hormones such as estrogen intend continuing inflammatory conditions. However immune responses to urban particles are determined by several factors not identified yet, future in vivo studies will complete this in vitro data.

In summary, the present study demonstrated that particulate matter collected from west of Tehran during winter days with a high level of organic carbon (OC) causes inflammatory cytokine production, inflammatory gene expression, induction of apoptosis, and reduction of phagocytosis activity in macrophage cells. Generally, the fine particle was more potent in inducing inflammatory condition in macrophage cells, while coarse particles cytotoxicity effect and inhibition of phagocytosis was more obvious. Since this fraction of PM can be harmful to human health.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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