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Research Article

Particulate matter exposure aggravates osteoarthritis severity

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Several diseases have been linked to particulate matter (PM) exposure. Outdoor activities, such as road running or jogging, are popular aerobic exercises due to few participatory limitations. Osteoarthritis (OA) is a progressive degenerative joint disease, usually observed at age 40, and not noticed before pain or diagnosis. Although exercise has health benefits, it is unclear whether outdoor jogging in higher PM (standard reference material 1649b, SRM 1649b) concentration environments could affect OA development or severity. Hence, a PM exposure monosodium iodoacetate (MIA)-induced OA animal jogged model was established for investigation. Results showed that high doses of PM (5 mg) significantly increased pro-inflammatory factors such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and IL-6, and M1 macrophages in the lung region, also obtained in systemic IL-6 and TNF-α expressions in this MIA-OA rat model. Moreover, levels of osteocalcin, cartilage oligomeric matrix protein (COMP), and N-telopeptides of type I collagen were especially influenced in MIA+PM groups. Morphological and structural changes of the knee joint were detected by micro-computed tomography images (micro-CT) and immunohistochemistry. MIA + PM rats exhibited severe bone density decrease, cartilage wear, and structure damages, accompanied by lower levels of physical activity, than the sham group and groups receiving MIA or PM alone. The findings suggest that the severity of OA could be promoted by PM exposure with a PM concentration effect via systemic inflammatory mechanisms. To the best of our knowledge, this is the first study to provide direct effects of PM exposure on OA severity.

Introduction

Following rapid urban development and industrialization, the air pollution problem has worsened. The pollutants of air pollution include gaseous and particulate matter (PM). Recently, several diseases have been reported to be associated with PM exposure. Therefore, the impacts of PM on human health and the strategies for reducing PM production have become a main issue of concern to the public, government, and scientists [1]. PM exposure has been demonstrated as a major risk factor associated with pulmonary dysfunction, cardiovascular disorder, hepatic fibrogenesis, skin diseases, allergy, cancer, and even neurological disorders [2–7]. PM is a complex mixture, which includes polyaromatic hydrocarbons, organic toxins, metals, and biological materials [8–10]. Hence, the pathogenicity of PM is dependent on their size and components, because the size determines the routes and regions of PM exposure, and these components influence PM toxicity in the human body. In general, the size of PM could be identified as coarse

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particles (particle diameter of 10 μm or less, PM10), fine particles (2.5 μm or less, PM2.5), and ultrafine particles (0.1 μm or less, UFP) [11]. Furthermore, it is well known that smog consists primarily of higher concentrations of fine particles [12]. Generally, toxicity is negatively associated with particle size [11,13]. Consequently, from the perspective of cellular biology and biochemistry, PM can produce reactive oxygen species (ROS) and influence pro-inflammatory cytokine release, such as tumor necrosis factor α (TNF-α), interleukin-1, and -6 (IL-1 and IL-6), triggering accelerated coagulation, increasing immune and endocrine system activities, and contributing to neurotoxicity [14–19] to cause abnormal cellular and systemic conditions.

Aerobic exercise has been suggested as an effective non-pharmacological method for improving health and quality of life, and it also has benefits for reducing the risk of some diseases, including hypertension and obesity [20,21]. Road running is an outdoor and popular aerobic sport due to its fewer limitations (such as age, gender, and professional or amateur athlete.) on participation. However, according to a report by the State of Global Air 2018, 95% of the global population live in areas that exceed the World Health Organization (WHO) air pollution limitation [22]. Although, the level of PM2.5 is lower in developed countries, the levels of PM2.5 and PM10 were high in China and southern Taiwan (created domestically and blown over from China) especially during the winter [23,24]. This implies that prolonged outdoor activities in those of higher air pollution areas may increase the risk of health hazards. Moreover, osteoarthritis (OA) is a type of progressive degenerative joint disease that results from cartilage, synovium, and subchondral bone damage [25]. OA is usually onset after the age of 40, and its prevalence is associated with age [25,26]. Besides age, several factors have been reported to be associated with OA progression, such as gender, obesity, occupation, and sports activities [26]. However, no strong evidence to support that aerobic exercise (including road running) causes OA under normal conditions exists. However, higher impact contact, torsional loads, and overuse increase OA risk [26–29]. In general, the public is unaware that individuals may have OA before significant symptoms or diagnosis, which may cause people to ignore their own joint condition and over-exercise. Unlike rheumatoid arthritis (RA), OA has no effective drugs for treatment, and joint replacement is ultimately the only option to restore ability and alleviate pain [25]. Pro-inflammatory factors (especially, TNF-α, IL-1, and IL-6) have been suggested as associated with OA due to their elevated levels in the synovial fluid and membrane, subchondral bone, and cartilage of the patients [30].

A previous study has suggested that 30 min of outdoor aerobic exercise in dirty city air increases the inhalation level of pollutants [31]. Most studies on health impacts of PM have focused on respiratory and cardiovascular diseases. However, the impact of PM exposure in the lung on OA severity in the knee joint is unknown. In the current study, a PM exposure monosodium iodoacetate (MIA)-induced OA animal model was established to investigate the effect of a single high dose PM exposure in the lung on knee joint OA development or severity. And forced jogging for animals enhanced the effect of MIA on OA. Furthermore, changes in macrophage phenotypes, cytokines in the lung and plasma, OA biomarkers, joint structures, and behaviors were investigated.

Materials and methods

Animals

Male LEW/SsNNarl rats 10 week-old (National Laboratory Animal Center, Taipei, Taiwan), weighing 400–500 grams, were acclimatized to a room with a controlled temperature (25°C) and humidity (50 ± 10%) with a 12-h day–night cycle (light on 07:00–19:00) for 24 h before the experiments. After surgery, the rats were kept individually in separate cages and provided with food (LabDiet 5053, PMI Nutrition International, St. Louis, MO, U.S.A.) and water *ad libitum*. All animal studies were performed in the Chang Gung Memorial Hospital animal room. All animal experiments designed and performed were followed the Chang Gung Memorial Hospital Animal Core ethical guidelines. The animal studies have been approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (IACUC number: 2018062802).

Procedures of OA inducement and intratracheal PM instillation surgery

A method of intratracheal instillation of PM (urban air standard reference material 1649b, SRM 1649b, National Institute of Standards and Technology, U.S.A.) upon MIA-induced OA was performed on the rats. Briefly, the rats were removed from their home cage and injected with concentrations (2 mg) of intra-articular MIA (I2512, Sigma, U.S.A.) into the right hind knee. After 7 days, rats were anesthetized with a 1:1 mixture of Zoletil 50 and Rompun 2% (1 ml/kg), the necks of the rats were shaved, and the surgical area was sterilized with 75% alcohol. A vertical 5-mm incision was made, and the trachea was exposed. The anterior wall of the trachea between the second and third tracheal cartilage rings was punctured using an insulin syringe at a 45° angle to avoid damaging the posterior wall. PM was thawed at room temperature (25°C) and diluted in sterile PBS to final concentrations of 2–10 mg/100

μl. The concentrations of PM were considered from previous studies [23,32–35] and the intermediate range of doses (2–10 mg/kg) were selected to test in the current study. Briefly, a suspension containing 50 μl of PM (1–5 mg/rat, approximately 2–10 mg/kg) in sterile PBS was slowly instilled intratracheally, followed by 50 μl of clean air. The surgical site was disinfected with povidone-iodine after surgery.

Jogging exercise and assessment of physical activity level

After a 4-week post-surgical recovery, all surgical rats in each group and the rats in the control group were subjected to daily forced jogging exercises. The distance of the forced jogging exercise was set as 300 meters, 3 days per week, for 3 months. For keeping the animals to be forced jogging, the electrical stimulator (Ugo Basile S.R.L., Italy), intensity was set at 3 Hz, 0.5 mA, was acted when the animal stopped moving. The electrical stimulant numbers (counts/day) required to finish the jogging course were routinely recorded to assess the physical activity levels (indirectly to estimate the OA severity). The experimental groups were separated as follows: (1) Group 1: Sham group; (2) Group 2: PM 5 mg only; (3) Group 3: MIA 2 mg only; (4) Group 4: MIA (2 mg) + PM 1 mg; (5) Group 5: MIA (2 mg) + PM 2 mg; and (6) Group 6: MIA (2 mg) + PM 5 mg.

Collection and preparation of blood samples, lung tissues and knee joint tissues

Blood samples (1 ml) were collected from rat tail veins at 16 weeks. After centrifugation at 5000×***g*** for 5 min at 4°C, the top plasma layer was transferred to a new tube and stored at −80°C. Then rats were killed with a 1:1 mixture of Zoletil 50 and Rompun 2% (3 ml/kg), the lung and knee joint tissues were also taken and stored at −80°C.

Analysis of micro-CT image for OA

Bones were fixed in 4% paraformaldehyde solution for 48 h. Following this process, the bones were transferred to 70% ethanol and stored at 4°C. The microarchitecture of the proximal trabecular bone and midshaft cortical bone of the tibia was analyzed by micro-CT-18 μm scans using the manufacturer's software.

Analysis of cytokine and OA biomarkers in rat blood plasma

The collected plasma from rats was used to identify expression levels of cytokines and OA biomarkers. Levels of IL-6, TNF-α, IL-1b, osteocalcin, cartilage oligomeric matrix protein (COMP), and N-Telopeptides of Type I Collagen (NTX-I) in the plasma were measured using rat IL-6 (#437107, Biolegend, San Diego, CA, U.S.A.), TNF-α (#438207, Biolegend, San Diego, CA, U.S.A.), IL-1 (EK0393, ScienCell Research Laboratories, Carlsbad, CA, U.S.A.), osteocalcin (#AF-12F1, Immunodiagnostic Systems, Boldon, U.K.), COMP (#CSB-E13833r, Cusabio, Houston, TX, U.S.A.), and NTX-I (#CSB-E09243r, Cusabio, U.S.A.) enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions.

Western blot analysis of lung and knee joint tissues

Rat lung tissues were homogenized and lysed in a protein extraction reagent (Tissue protein extraction reagent, Pierce, Rockford, U.S.A.). Western blot analysis was performed as described in our previous study [36]. Briefly, equal amounts of the protein were separated by SDS/polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred on to a polyvinylidene fluoride (PVDF) membrane. Commercially available primary antibodies (concentration: 1:1000) against IL-1β (ab9722, Abcam, Cambridge, U.K.), TNF-α (ab6671, Abcam, U.K.), IL-6 (bs-0782R, Bioss, Woburn, MA, U.S.A.), CD68 (ab125212, Abcam, U.K.), CD206 (60143-lg, Proteintech, Rosemont, IL, U.S.A.), CD80 (ab215166, Abcam, U.K.), and β-actin were used. After extensive washing, appropriate secondary antibodies were used in the experiments, including anti-rabbit and anti-mouse IgG antibodies conjugated to horseradish peroxidase (concentration: 1:10000) (Biolegend, U.S.A.). The signals were measured using the enhanced chemiluminescence (ECL) detection kit and detected using Hyperfilm. The signals on Hyperfilm were scanned, quantified, and normalized to β-actin.

Toluidine Blue staining and immunohistochemistry

Serial paraffin sections of knee joint (3 μm) and lung tissues (5 μm) were used for Toluidine Blue staining and immunohistochemical staining. For immunohistochemical staining, the IL-1β, IL-6, TNF-α, CD68, CD80 and CD206 signals were detected in lung tissues, and TNF-α in bone. The tissue sections were individually incubated with anti-IL-1β (200 dilution; ab9722; Abcam, Cambridge, U.K.), anti-IL-6 (200 dilution; bs-0782R, Bioss, U.S.A.), anti-TNF-α (100 dilution; ab6671; Abcam, U.K.), anti-CD68 (100 dilution; ab125212, Abcam, U.K.), anti-CD80 (100

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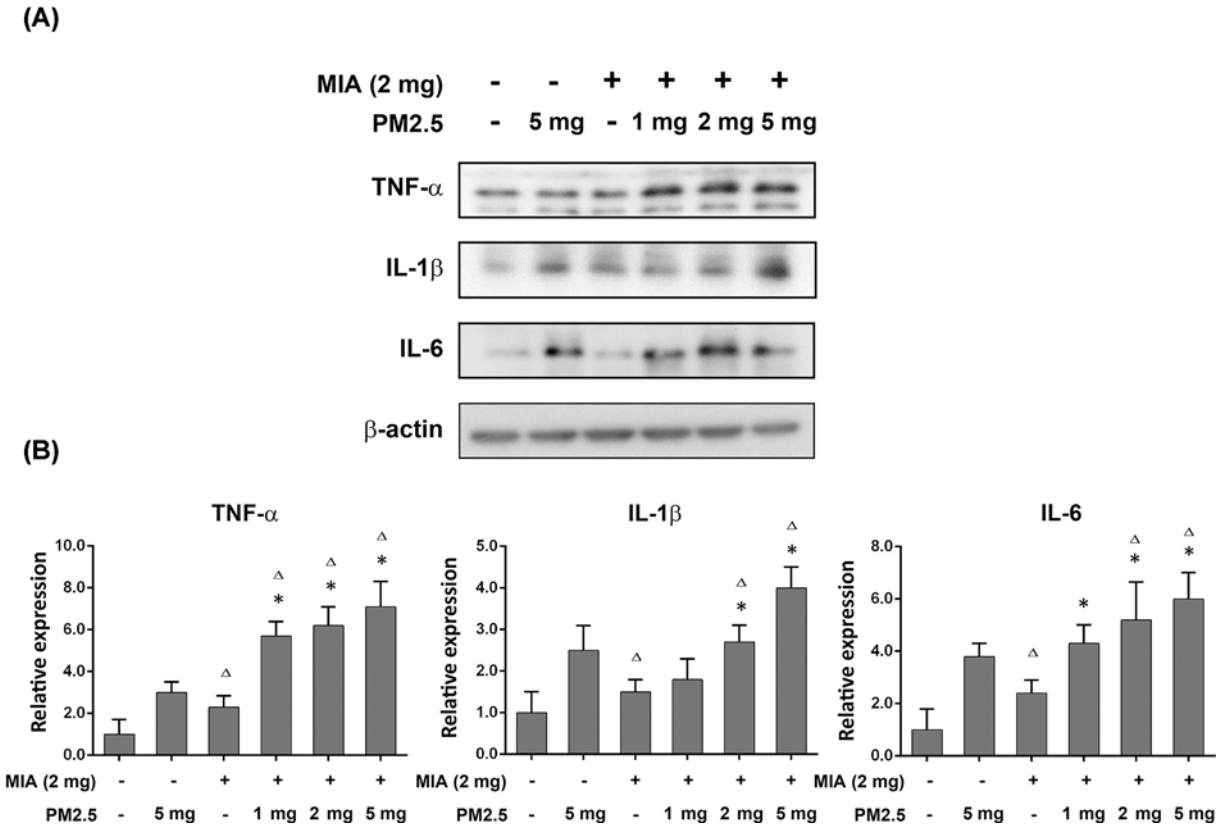

Figure 1. Expression of pro-inflammatory factors in lung lysates of all treated groups

(**A**) Representative Western blot analysis of TNF-α, IL-1β, and IL-6 in different groups. β-actin was used as an internal control. (**B**) Densitometric quantification of Western blot analysis for all tested proteins normalized to β-actin (n=5 for each group). *, $P<0.05$, for results compared with group receiving MIA only; $^{\Delta}$, $P<0.05$, for results compared with group receiving PM only.

dilution; ab215166; Abcam, U.K.), and anti-CD206 (100 dilution; 60143-lg; Proteintech, Rosemont, IL, U.S.A.) antibodies overnight at 4°C according to the experimental designs, followed by the Polink-2 Plus HRP Mouse/Rabbit with DAB Kit (GBI LABS, U.S.A.) to obtain the labeled signals. All stained slides were digitized using a 3D HISTECH Panoramic SCAN. Staining scores of signals of TNF-α, IL-6, IL-1β, CD68, CD80, and CD206 in lung were counted, the numbers of positive cell on four random 200 μm × 200 μm fields (approximately 200–300 cells) of one image of each rat. And the staining scores of TNF-α in knee joint sections were evaluated by multiplying the percentage of positive cells (P) by the staining intensity score (I), as proposed by Krajewska et al. [37] and Shen et al. [38].

Data analyses and statistics

The results were expressed as the mean ± standard deviation (SD). All data were evaluated and graphed with Microsoft Excel software (Microsoft, Redmond, WA, U.S.A.). The results were analyzed using GraphPad Prism software (v5, GraphPad, San Diego, CA, U.S.A.), with one-way ANOVA, followed by the post-hoc Tukey's multiple comparison test. A P-value <0.05 was considered significant.

Results

Cytokine expression in lung lysate of PM-treated, MIA-induced OA rat

To determine cytokine changes following PM exposure in the lung, immunoblotting and immunocytochemistry (IHC) were utilized to assay in the PM-treated, MIA-induced OA rat model. As shown in Figure 1A, PM (5 mg) or MIA (2 mg) not only significantly increased TNF-α expression in the lung lysate as compared with the control. A synergistic effect was obtained in the MIA-induced OA rat that was exposed to PM. In addition, TNF-α levels showed a concentration-dependent increase in PM co-treated with MIA. IL-1β and IL-6 also showed a similar pattern to TNF-α (Figure 1B). Interestingly, the IL-6 level was noticeably increased in the PM only group (Figure 1B).

Furthermore, the IHC results of lung were consistent with the immunoblotting findings (Figure 2). The PM (5 mg) with MIA group showed higher TNF-α, IL-1β, and IL-6 expression levels than PM and MIA only. These results indicate that PM co-exposure with MIA promoted inflammatory factor or cytokine expression in the lung in a PM dose-dependent manner.

Distribution of macrophage types in lung lysate of PM-OA animals

It is well known that macrophages play an important role in responding to invasive pathogens, including PM. Moreover, the polarization ratio of macrophage phenotypes may be affected by PM. In general, macrophages can be separated into M1 (pro-inflammatory role) or M2 phenotypes (anti-inflammatory role) [39,40]. As shown in Figure 3, the total number of macrophages, detected by CD68 signals, was significantly increased in the PM (5 mg) with MIA group. Moreover, evaluation of macrophage types was conducted to understand the macrophage polarization in the PM-OA model. M1-type macrophages (CD80 signals) were increased with PM concentration, while M2 macrophages (CD206 signals) were decreased in groups that received both MIA and PM, as compared with the sham group. Furthermore, IHC results of the lung tissues were consistent with immunoblotting findings (Figure 4). This indicates that macrophage polarization could be affected by PM and MIA co-exposure, trending toward the M1 type.

Cytokine expression in plasma of PM-OA animals

The effects of PM and MIA-induced OA may cause systemic changes to cytokines. Following the findings of cytokine changes in the lung region, we further investigated the levels of cytokines in the circulatory system to confirm this hypothesis. As shown in Figure 5, the levels of TNF-α and IL-6 were synergistically and significantly increased in the PM (5 mg) with MIA group as compared with the PM (11.6 *vs*. 2.37 pg/ml for TNF-α, 38.42 *vs* 7.89 pg/ml for IL-6) or MIA (11.6 *vs*. 2.18 pg/ml for TNF-α, 38.42 *vs* 3.16 pg/ml for IL-6) only groups. Furthermore, the PM combined MIA groups showed a PM concentration-dependent increase both in TNF-α (PM 1 *vs*. 2 *vs*. 5 mg, 3.08 *vs*. 5.83 *vs*. 11.6 pg/ml) and IL-6 (PM 1 *vs*. 2 *vs*. 5 mg, 4.21 *vs*. 12.32 *vs*. 38.42 pg/ml) expressions. This result indicates that the cytokines induced by MIA and PM were located in the lung region and plasma. This suggests that MIA and PM may have a systemic impact.

OA biomarker changes in plasma of PM-OA animals

The OA biomarkers—osteocalcin, cartilage oligomeric protein (COMP), and NTX-I—were measured in the plasma to correlate to the knee joint structure in this animal model. Figure 6A showed the osteocalcin was decreased in MIA treated rats (166.2 ng/ml) and PM exposure (206.7 ng/ml) was slightly reduced as compared with the control (265.7 ng/ml). A significant decrease was found in MIA co-treated with PM (5 mg) (90.5 ng/ml) relative to control (265.7 ng/ml), MIA (166.2 ng/ml), and PM only (206.7 ng/ml). Decreases in osteocalcin were also found in a PM concentration-dependent manner. As shown in Figure 6B, expressions of COMP were increased after PM (3.5 ng/ml) and MIA (3.61 ng/ml) administration, and the group that received both MIA and PM (5 mg) (5.36 ng/ml) demonstrated the highest COMP expression as compared with control (1.35 ng/ml) in all tested groups. Additionally, another OA biomarker NTX-I showed a similar pattern to COMP. PM (15.97 nM BCE) or MIA treatment only (17.02 nM BCE) increased expression of NTX-1 relative to controls (8.53 nM BCE), and a notable increase was observed in the PM (5 mg) co-treated with MIA group (25.11 nM BCE) (Figure 6C). This result indicates that the OA-related biomarker could be influenced by PM or MIA, and a marked effect occurred in the PM co-treated with MIA administration.

Structural changes in knee joint region of PM-OA animals

Furthermore, we examined changes in the knee joint region after a jogging exercise with the PM, MIA, or PM co-treated with MIA administration. As shown in Figure 7, the control and PM-treated rats showed no marked changes in structure formation according to the coronal view of micro-computed tomography images (micro-CT). Knee joint structure in the MIA-treated groups showed slight damages to the cartilage of the medial knee joint in the MIA group. In the PM co-treated with MIA group, there were mild, moderate, and severe structural disruptions with increasing PM concentrations. Additionally, 3-D reconstruction images clearly showed cartilage destruction of medial knee compartment and in the degrees of cartilage wear in the tested groups. The images presented the most severe cartilage wear in the high dose of PM (5 mg) group of the MIA-induced OA knee joint (Figure 7B). PM only caused slight wear, which was less than other groups. Moreover, the severity of damage in the knee joint was associated with PM exposure concentrations in MIA co-treated rats. Bone mineral density (BMD) was also measured by micro-CT as shown in Figure 7C, and BMD levels were both decreased significantly in the trabecular and condyle of

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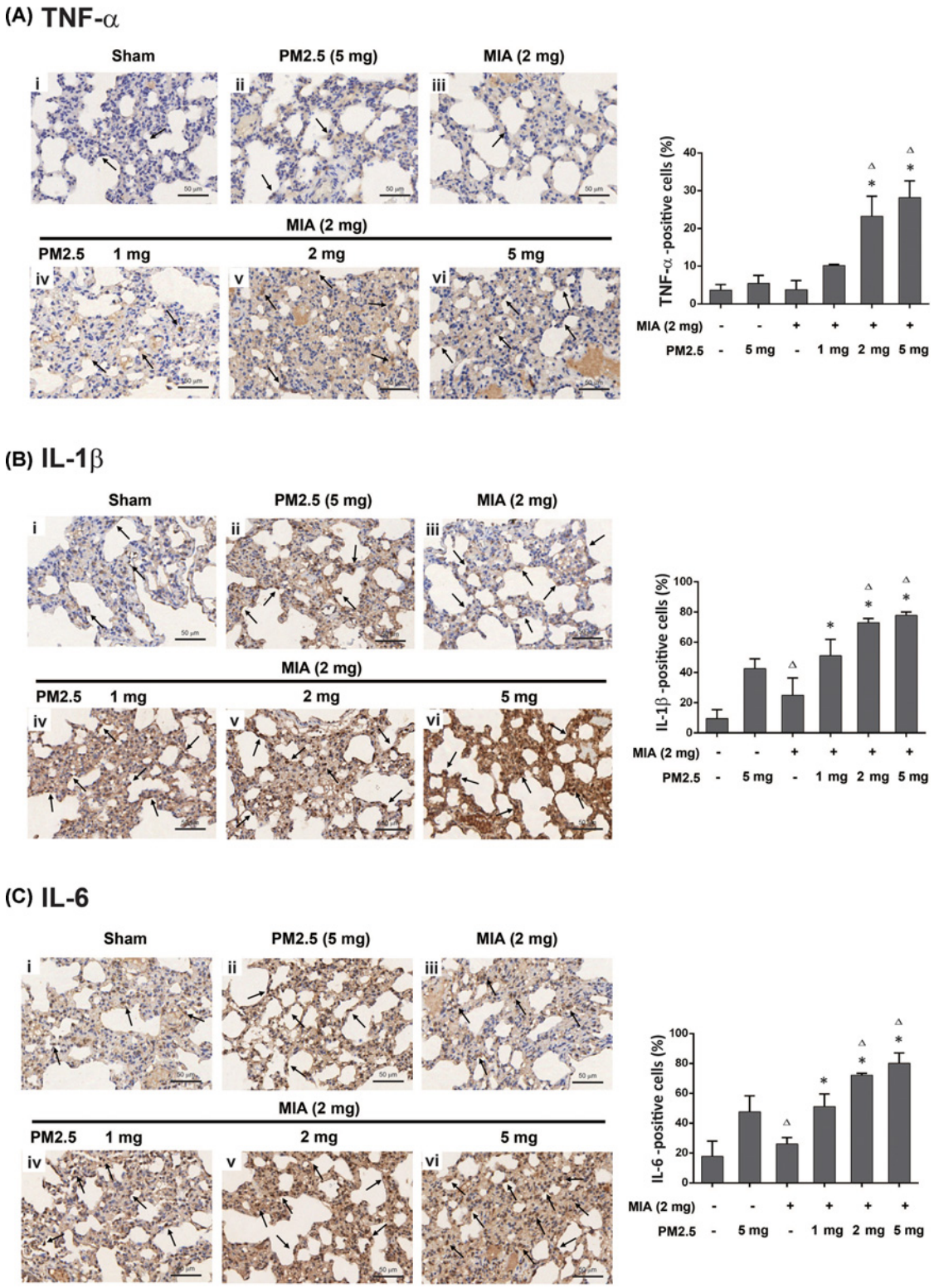

Figure 2. Histological evaluation of pro-inflammatory factor staining in lung tissues

(**A**) TNF-α, (**B**) IL-1β, and (**C**) IL-6. Black arrow indicates positive staining. A synergistic effect was obtained in the MIA-induced OA rat receiving PM. In addition, TNF-α levels showed a concentration-dependent increase in PM co-treated with MIA. IL-1β and IL-6 also showed similar patterns to TNF-α. Quantification for histological images of TNF-α, IL-1β, and IL-6 was scored as the percentage of average positive cells. *, $P<0.05$, for results compared with the group receiving MIA only; $^{\Delta}$, $P<0.05$, for results compared with the group receiving PM only.

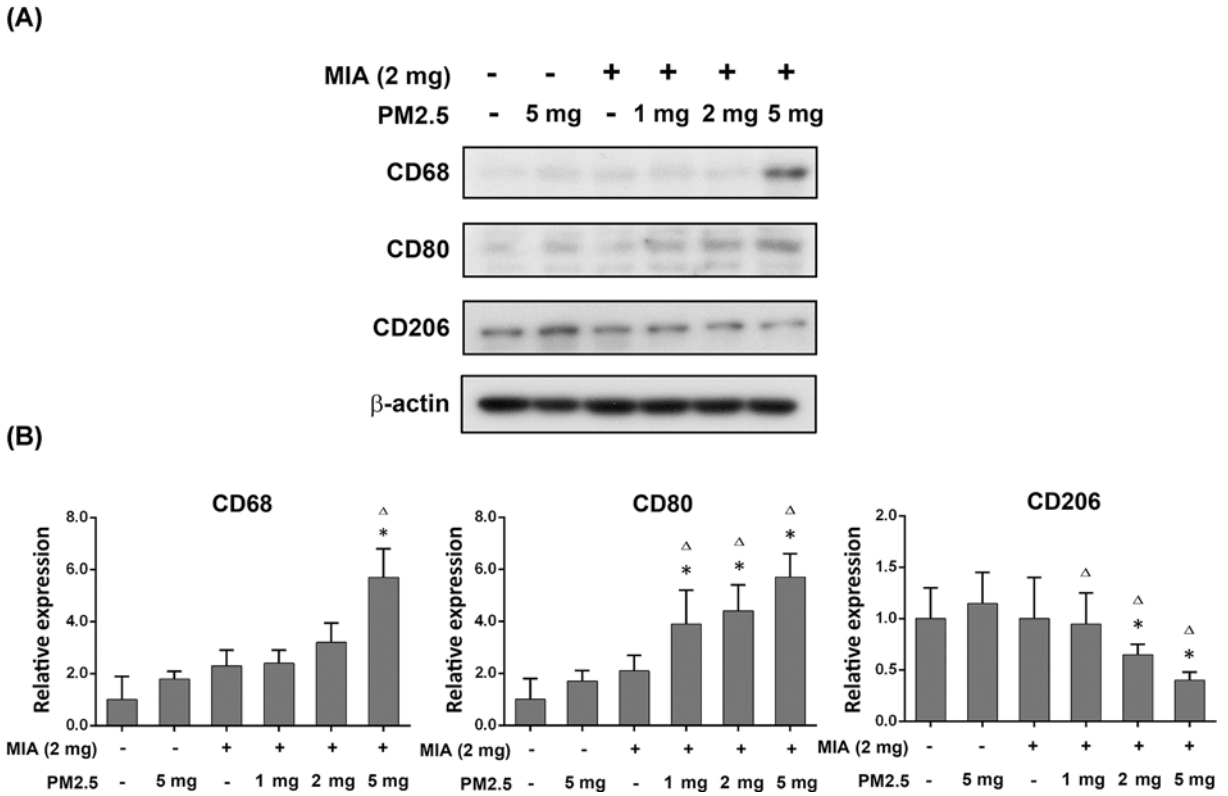

Figure 3. Expression of macrophage-related protein in lung lysates of all treated groups

(**A**) Representative Western blot analysis of CD68, CD80, and CD206 in different groups. β-actin was used as an internal control. (**B**) Densitometric quantification of Western blot analysis for all tested proteins normalized to β-actin (n=5 for each group). *, $P<0.05$, for results compared with the group receiving MIA only; $^{\Delta}$, $P<0.05$, for results compared with the group receiving PM only.

the tibia in treated rats that received both MIA and PM (5 mg) as compared with the MIA only group. However, no significant changes were detected between the MIA only and PM only groups. In terms of differences between groups, BMD of the experimental groups was decreased compared with the sham group. Furthermore, the microarchitecture of the knee joint was sectioned and stained by Toluidine Blue and TNF-α and the results were consistent with the micro-CT image findings (Figure 8). The Toluidine Blue staining images showed the cartilage matrix loss and the abnormal repair and changes of bone remodeling on the contour of the articular surface in the MIA only group. Furthermore, those of damages were more severity with a dose dependent manner in the PM + MIA groups (Figure 8A). In addition, the expression of TNF-α was also significantly increased in the MIA only group, and a dose-dependent manner increases in PM 1–5 mg with MIA groups were obtained. Furthermore, the electrical stimulant numbers were used to indirectly estimate the OA severity. The 'Results' showed the highest in the PM (5 mg) + MIA group, which may represent difficulties in animal movements that require an electronic stimulant to force jogging (Figure 9). These results suggested that levels of physical activity assessed by forced jogging in all treatment groups were closely correlated with the levels of OA damages aggravated by PM in MIA-induced OA rats.

Discussion

The epidemiological studies have demonstrated that exposure to air PM is associated with several chronic inflammatory diseases, including pulmonary dysfunction, cardiovascular disorder, skin diseases, allergy, and cancer [2–7]. Recently, a mouse study suggested that chronic PM2.5 exposure (1 h daily, 5 days/week, 3 months) caused the genotoxic and epigenotoxic effects on DNA of the liver, kidney, and lung [41]. This indicates PM2.5 exposure could cause long-term impacts on health. In current study, a high-dose PM exposure animal model was used to attempt to investigate the relationship of PM and OA. Results show the high dose of PM (SRM 1649b) exacerbated the OA severity through circulation of inflammatory cytokines secreted by the lung. The Standard Reference Material® 1649b is a kind of PM with organic constituents (such as polycyclic aromatic hydrocarbons and polychlorinated biphenyl),

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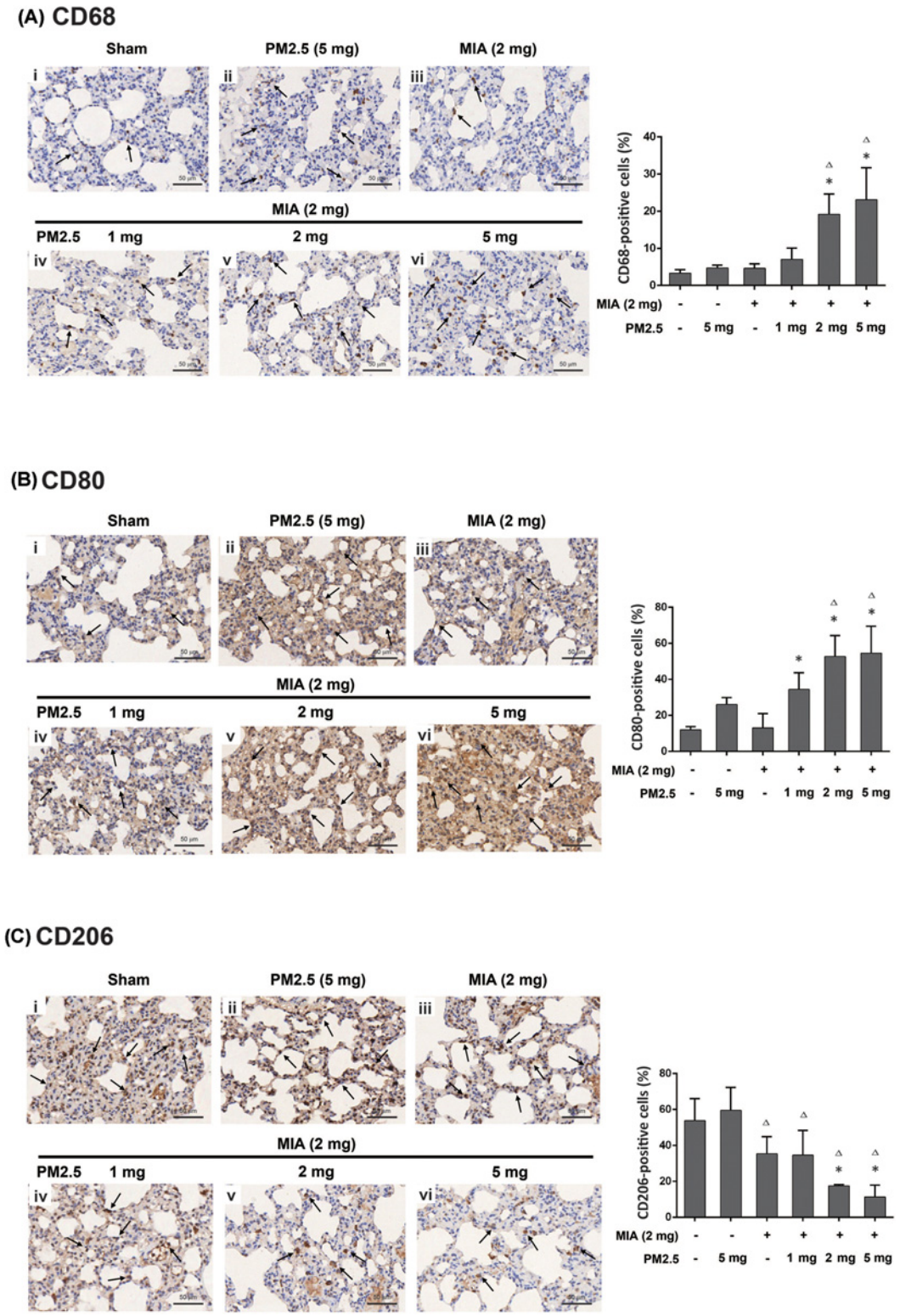

Figure 4. Histological evaluation of macrophage-related protein staining in lung tissues

(**A**) CD68, (**B**) CD80, and (**C**) CD206. Black arrow indicates positive staining. The total number of CD68-positive macrophages was significantly increased in the PM (5 mg) with MIA group. CD80-positive M1 type macrophages increased with PM concentration, while CD206-positive M2 macrophages decreased in groups receiving both MIA and PM, when compared with the sham group. Quantification for histological images of CD68, CD80, and CD206 was scored as the percentage of average positive cells. *, $P<0.05$, for results compared with the group receiving MIA only; $^{\Delta}$, $P<0.05$, for results compared with the group receiving PM only.

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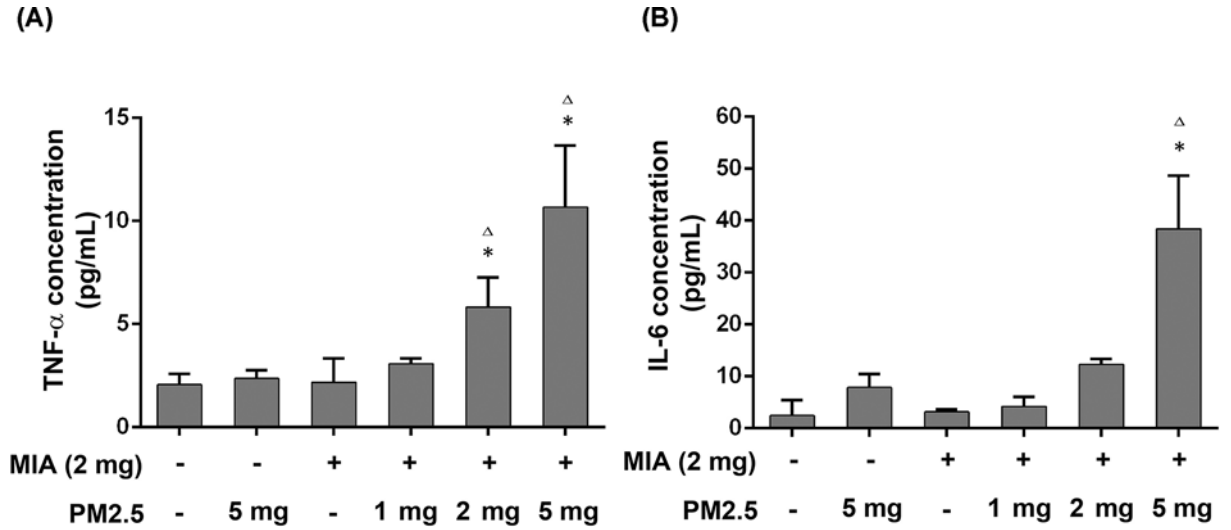

Figure 5. Cytokine levels in blood plasma of MIA-induced OA rat with PM exposure

The concentrations of plasma TNF-α (**A**) and IL-6 (**B**) in different treated groups were evaluated after jogging exercise. All data are expressed as mean $\pm$ SEM (n=5 for each group). *, $P<0.05$, for results compared with the group receiving MIA only; $^{\Delta}$, $P<0.05$, for results compared with the group receiving PM only.

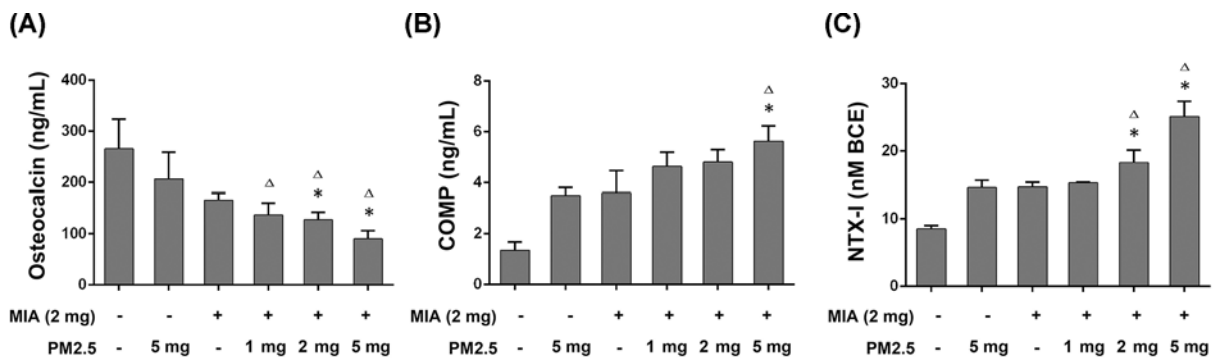

Figure 6. OA biomarker levels in blood plasma of MIA-induced OA rat with PM exposure

The concentrations of plasma osteocalcin (**A**), COMP (**B**), and NTX-I (**C**) in different treatment groups were evaluated after jogging exercise. All data are expressed as mean $\pm$ SEM (n=5 for each group). *, $P<0.05$, for results compared with the group receiving MIA only; $^{\Delta}$, $P<0.05$, for results compared with the group receiving PM only.

which was certified by the National Institute of Standards and Technology. Moreover, according to the certificate, SRM 1649b were collected from Washington, DC area in 1976 and 1977 and passed through 63 μm (230 mesh). Hence, the PM (SRM 1649b) includes different sizes of particles (coarse, fine particles, and ultrafine particles). In the current study, SRM 1649b were selected to contribute the effects of damage attributable to urban dust on the body. PM could be breathed in and arrive to the lower respiratory tract, and PM would be phagocytosed by alveolar macrophages to subsequently cause local inflammation [42,43]. Macrophages are important immune responders for detecting, engulfing, and destroying invasive pathogens, apoptotic cells, and PM [13,44]. PM has been shown to induce macrophage polarization to the M1 and M2 phenotypes [45,46]. In general, two phenotypes of macrophages are activated by different stimulants. M1-type macrophages play microbicidal and pro-inflammatory roles, while M2-type play suppressor, immunity adaption, and anti-inflammatory roles [39,40]. Zhao et al. [45] suggested that short-term PM exposure could affect the polarization balance of M1- and M2-type macrophages, trending toward the M1 type. The M1 type could be enhanced by PM via ROS activation, while M2 type polarization was suppressed by mTOR-dependent pathways. However, expression levels of M1 or M2 were not significantly changed in the lung region of PM-exposed rats in the current study, which may be due to single exposure and measurements after 4 months. Similar results were found for cytokine expression in the lung. Several pro-inflammatory factors are released from

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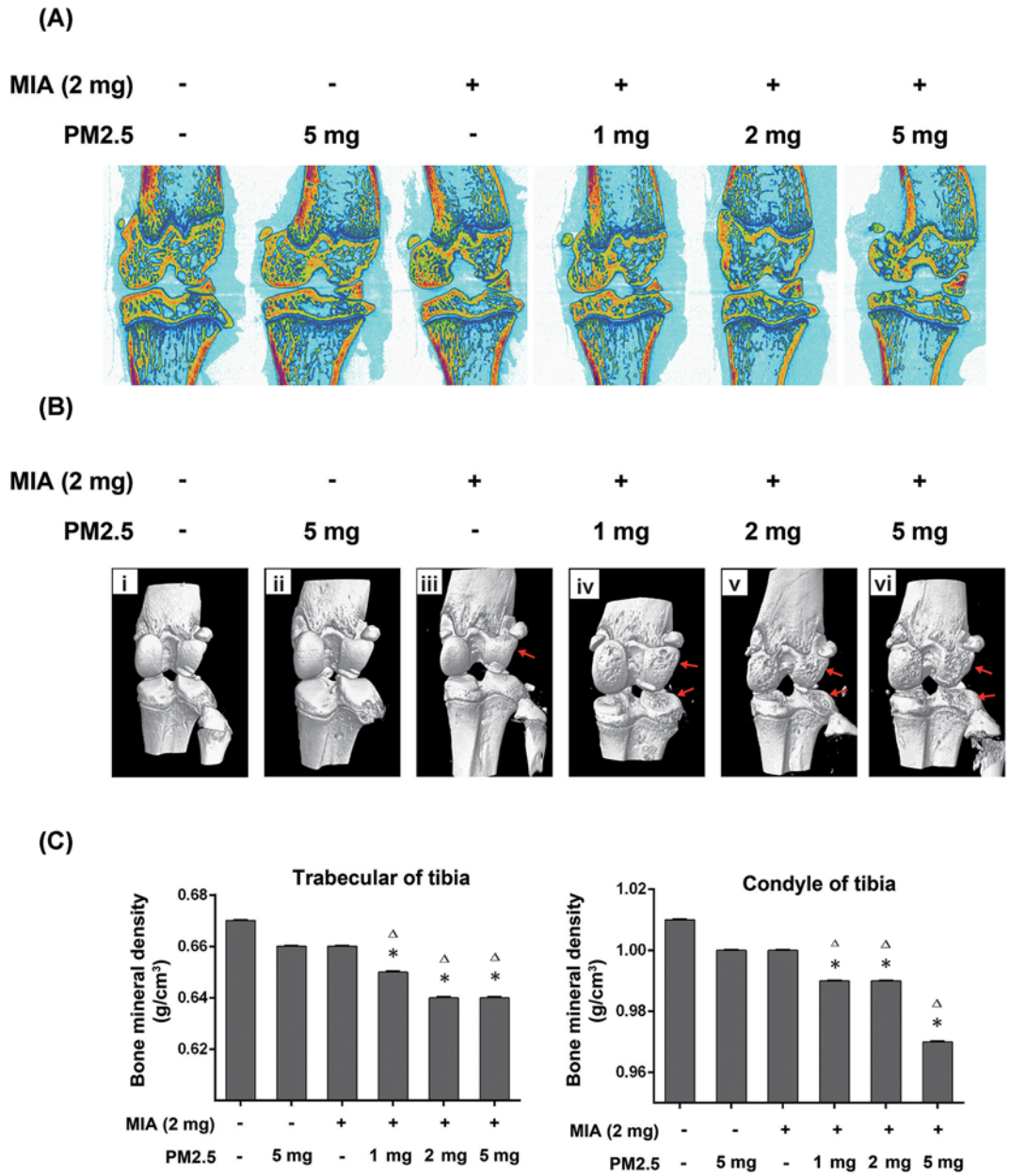

Figure 7. micro-CT

The groups of micro-CT images were separated into (i) Sham group; (ii) PM 5 mg only; (iii) MIA 2 mg only; (iv) MIA (2 mg) + PM (1 mg); (v) MIA (2 mg) + PM (2 mg); (vi) MIA (2 mg) + PM (5 mg). (**A**) Coronal view. (**B**) 3-D reconstruction images showing cartilage destruction of medial knee compartment and the level of destruction in the groups. Red arrows indicated wear of articular surface. (**C**) The mean BMD in the groups. (i) Trabecular of tibia BMD, (ii) Condyle of tibia BMD.

macrophages, such as ILs and TNF-α [13,47]. Previous article suggested that different sizes of particles may through different regulated pathways to affect the alveolar macrophages, such as coarse particles are via endotoxin-toll-like receptor (TLR) 4 pathway; while the fine and ultrafine particles are transition metals and/or polyaromatic hydrocarbons to generate ROS to activate innate immune responses [13]. Due to the PM (SRM 1649b) has a wide range of particle sizes (including coarse, fine and ultrafine), it may imply several cytokines, such as IL-4, IL-8, IL-10 and IL-12 could be released, not only the TNF-α, IL-1β, and IL-6. In the present study, the expressions of TNF-α, IL-1β, and IL-6 were first selected to assay. PM (5 mg) exposure caused the pro-inflammatory factors TNF-α, IL-1β, and especially IL-6 to be slightly elevated in the lung region. However, there were no notable changes at 4 months after the last

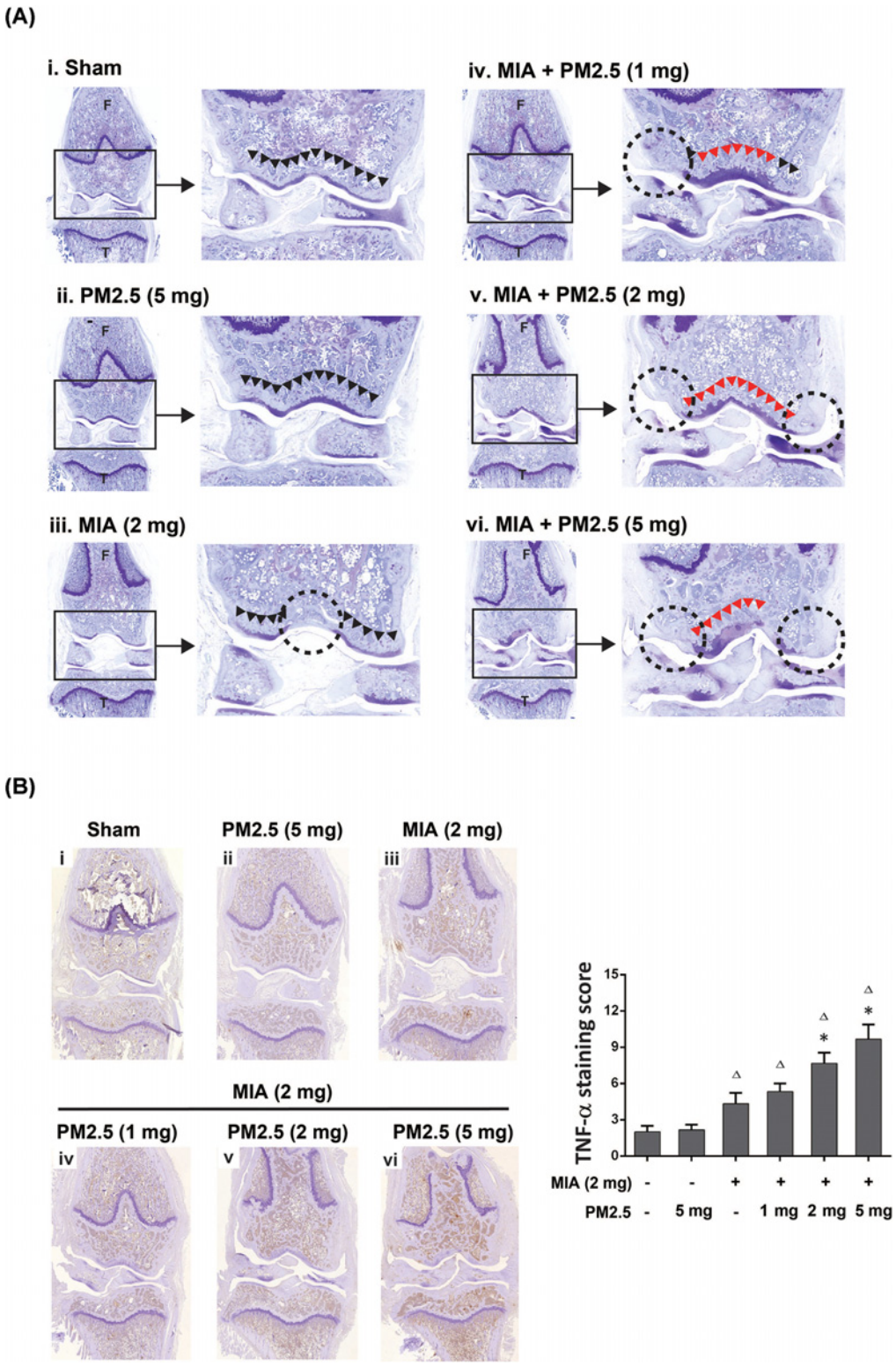

Figure 8. Histologic analysis of knee joint in rat OA model with PM exposure

(**A**) Toluidine blue staining of knee joints areas of the MIA-induced OA rats (i–iv). The details of selected regions of image were amplified to show on the square (a–f). Black triangle indicated intact cartilage; red triangle means abnormal repair and changes in bone remodeling on articular surface. Dash cycle showed cartilage matrix loss. (**B**) TNF-α expressions on knee joint region. The TNF-α signals were scored and showed as a bar graph. F, femur; T, tibia. *, $P<0.05$, for results compared with the group receiving MIA only; $^{\Delta}$, $P<0.05$, for results compared with the group receiving PM only.

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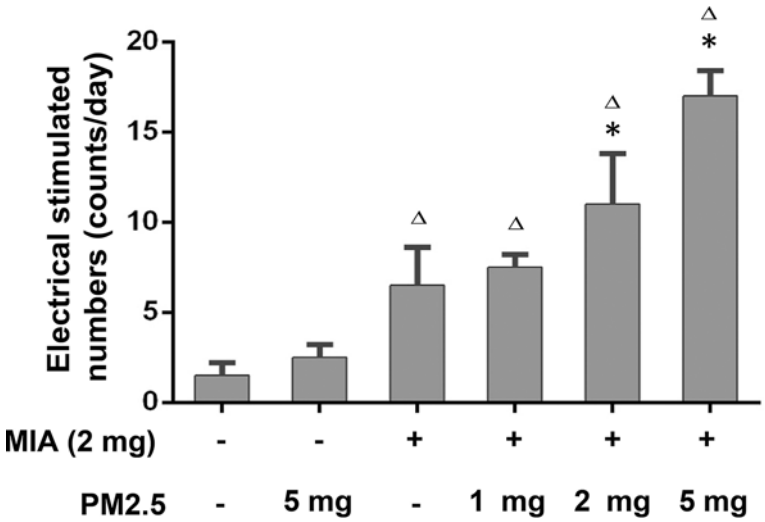

Figure 9. Assessment of force jogging performance in all treated groups after jogging exercise

The electrical stimulant numbers required to finish the jogging course (300 meters) were routinely recorded in all six groups. All data are expressed as mean ± SEM (n=5 for each group). *, $P<0.05$, for results compared with the group receiving MIA only; $^{\Delta}$, $P<0.05$, for results compared with the group receiving PM only.

PM exposure. Further, significant synergistic effects of PM exposure and MIA-induced OA were obtained in M1-type macrophage expression and all of pro-inflammatory factors we tested in this study, while M2-type macrophages were decreased. This may imply that there is a co-regulated mechanism in the immune responses of PM and MIA-induced OA to prolong the response time and degree.

MIA induces OA through metalloproteinase (MMP) activation, suppressing proteoglycan synthesis and resulting in cartilage necrosis [48]. And our Toluidine Blue staining results were also presented the phenomena of cartilage matrix loss, abnormal repair, and bone remodeling changes on the contour of the articular surface after MIA administration (Figure 8). MIA-induced OA is a useful animal model because animal movement activity is associated with the severity of OA, due to pain production and cartilage and bone damages [49]. However, surgery on the anterior cruciate ligament transaction or meniscectomy could also induce OA, yet not as stable as MIA [50]. OA is a type of age-related progressive degenerative joint diseases, which is usually observed over 40 years old and causes pain due to cartilage, synovium, and subchondral bone damage near the joint [25,26]. Age, gender, occupation, obesity, and sports have been reported as risk factors for OA occurrence and development [26]. The levels of pro-inflammatory factors TNF-α, IL-1, IL-6, IL-15, and IL-18, etc. in the synovial fluids and serum have been linked to OA patients [30,51–53]. Moreover, these pro-inflammation factors may play an important role for OA pathogenesis by indirectly regulating adipocytes to release adiponectin and leptin [30]. Leptin has been reported to increase the cartilage collagen degradation-promoting factors metalloproteinase MMP9 and MMP13 and increase IL-1 and IL-6 expression in the synovial fluid under the OA condition [54]. Activated synoviocytes, mononuclear cells, and articular cartilage itself could stimulate IL-1 and TNF-α release to up-regulate MMP expression, subsequently causing cartilage erosion and synovial inflammation [55]. IL-1β and TNF-α have been considered as two key cytokines involved in the pathogenesis of OA [56]. IL-1β and TNF-α were secreted by the same cells in the joint, and both could be detected showing a similar response pattern in synovial fluid, synovial membrane, cartilage, and subchondral bone layer in OA [56]. IL-1β was not be detected due to limitation of the ELISA kit in the current study; however, the TNF-α result may indirectly reflect the expression pattern of IL-1β. IL-6 has important functions in human joint inflammatory diseases and is considered a biomarker to reflect OA severity [57]. Mori et al. [58] suggested the IL-1β and TNF-α could stimulate IL-6 expression in inflammatory arthritis. Furthermore, IL-6 could promote the acute stage of inflammation to chronic and play a promoter role in bone resorption and osteoclast formation under pathological conditions [51]. Previous studies suggested IL-6 as a multiple driver for acute and chronic inflammation both in OA and RA development [51,58]. High levels of IL-6 activity could be found in synovial fluid and serum of inflammatory arthropathies, such as OA and RA [30,51–53]. Furthermore, these studies also suggested that the concentrations of IL-6 were higher in knee OA patients' plasma and showed a positive correlation with OA severity. In a current view on OA, IL-6 is considered to be the key cytokine for OA due to IL-6-induced changes in the subchondral bone layer. This

effect is largely based on synergism with IL-1β and TNF-α to promote osteoclast activities during bone resorption [56].

Moreover, PM could cause systemic inflamation and influence cytokine levels in the circulation [18,19]. Some pro-inflammatory cytokines, such as TNF-α and IL-6, were detected in the joint region and plasma or serum, and they are linked to OA [56,59,60]. The expressions of TNF-α and IL-6 in plama and the TNF-α in the knee joint in this study are in accord with those of previously studies. Hence, the co-adjustment effects of the immune responses of PM and MIA-induced OA are possibly through the circulation to prolong and enhance the immune responses. Our systemic results of pro-inflammatory factors seem to respond as IL-6 and TNF-α expressions were observed in the plasma of PM (5 mg) + MIA rats. Hence, the main hypothesis in the present study was that PM produced pulmonary inflammation with systemic release of macrophage-driven cytokines, which may influence remote MIA-induced OA end points. According to the cytokine and macrophage expression changes in the lung of PM-exposed OA rats, OA may cause lung inflammation. However, there are still lacking the stronger evidence to support the view that OA could cause lung inflammation. Recently, a possible mechanism has been proposed which may be used to explain our finding. The low-grade inflammation caused by the abnormal conditions of body (obesity, hypertension, etc.) and aging which released inflammatory factors to blood, thus initiation and/or perpetuating OA processes. The cells of OA in turn release inflammatory factors into joint cavity and finally into the circulation. Those of inflammatory factors released by OA cells may induce or acceleration the low-grade inflammation-induced chronic diseases (Alzheimer disease, stroke and myocardial infarction) [61]. The causal relationship of PM and OA causing pro-inflammatory factor increases still requires further investigation.

Biomarkers, such as osteocalcin, COMP, and cross-linked NTX-I, have been linked to the prognostic and evolution factors of OA [62–64]. In agreement with previous reports that studied OA [62–64], our study found decreases in osteocalcin, an osteoblast-secreted specific protein that plays a regulatory function in the rate of mineral maturation during bone formation [65], in the MIA-treated, PM-treated, and especially the MIA co-treated with PM groups. Furthermore, contrasting patterns were obtained for the COMP and NTX-I expression, indicating significant decreases in the PM co-treated with MIA groups. COMP is an extracellular matrix protein primarily present in cartilage and related to cartilage turnover [66]. NTX-I, a bone resorption marker, contributes when osteoclasts regulate bone resorption via breaking down the type I collagen comprised matrix and removing minerals from the bone [67,68]. Those of biomarkers changes in this study showed an imbalance between bone formation and bone resorption in the MIA-induced OA model, and PM exposure promoted bone resorption processes rather than formation, subsequently aggravating the OA severity.

Micro-CT images could improve our understanding of the microarchitecture of OA-mediated trabecular bone alteration patterns [50]. Specifically, 3-D reconstruction images of micro-CT showed clear morphological changes in the medial knee joint in the current study. The BMD was also significantly decreased in rats that received both MIA and PM compared with MIA only. Furthermore, our jogging exercise data directly reflected the bone and cartilage stage via the movement activities. The behavioral activities of jogging recorded by numbers of electrical stimulants reflected the micro-CT images and immunohistochemistry findings, in which the most severe damage to the knee joint was in the PM (5 mg)-exposed, MIA-induced OA rats. Our current results did not support that PM could induce OA occurrence. Up this point, our current finding seems to agree with those of a previous study by Kang et al. [69], which suggested that OA prevalence was not associated with direct or indirect exposure to smoke. A systematic review presented a contrary finding, which suggests that higher prevalence of OA among COPD individuals [70]. Whereas, it is a limitation of single PM exposure animal model in our study, the real conclusions are need further investigations with others animal experiments, such as chronic PM exposure or evaluated on the spontaneous OA older rats. Moreover, changes in OA biomarkers in PM-exposed rats and identical BMD values with MIA only warrant further investigation. Interesting, the results from macrophage polarization in the lung, pro-inflammatory factor expressions in the lung, plasma and knee joint, structural changes from IHC and micro-CT in the knee joint, OA biomarker expressions and behaviors all suggest an association with PM exposure concentrations. This indicates that the doses of PM exposure are a key factor for OA severity under the jogging condition.

Although, the present study has yielded finding on the relationship of PM exposure and OA severity, its design is not without flaws. We do not deny the limitations of the current study, especially the effects of PM single high dose exposure or chronic low dose exposure, and also the acumination dosages of PM exposed. Previously, public health studies suggested that chronic exposure with PM2.5 had stronger effects on hospitalization (respiratory, cardiac, and stroke admissions rate) compared with short-term exposure in New England [71] and also got a larger effect of chronic exposure with PM2.5 on preterm birth in Beijing, China [72]. Those findings may imply that the chronic exposure could cause larger effects on health than short exposure to PM under the non-lethal doses range. However, these of

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public health studies still could not fully answer the dosage questions, and further researches should be pursued to ensure the real effects of single high and chronic accumulated PM exposure on OA severity or development.

In conclusion, our results confirmed and extended the results of previous studies of pro-inflammatory factor changes under PM exposure and OA conditions. We compared three concentrations of PM exposure in the MIA-induced OA animal model. The study revealed that a single high concentration of PM exposure could cause long-time effects on the OA severity, which may function through mechanisms of systemic inflammation. This is the first evidence to support that PM exposure might have contributed to the severity of OA.

Clinical perspectives

- Exercise has health benefits, but it is unclear whether outdoor jogging in environments with higher PM concentration could affect OA development or severity.
- In the current study, results from macrophage polarization in the lung, pro-inflammatory factors (TNF-α, IL-6, and IL-1β) expressions in the lung and plasma, structural changes from IHC and micro-CT in the knee joint, OA biomarkers (osteocalcin, COMP and NTX-I) expressions and jogging behaviors all suggest an association with PM exposure concentrations. This indicates that the doses of PM exposure are a key factor for OA severity under the jogging condition.
- A single high concentration of PM exposure could cause long-time effects on the OA severity; the present study supports that PM exposure might have contributed to the final outcome of severity of OA.

Author Contribution

K.-T.P., Y.-C.C. and C.-W.L. performed the experiments and wrote the manuscript. P.-C.C., M.-H.C., H.-N.S. and P.-J.C supervised the data of Western blotting, immunohistochemistry, ELISA behavioral, images, and oversaw analysis of the results. K.-T.P., Y.-C.C., J.-F.L. and C.-W.L. helped with writing the manuscript. K.-T.P., P.-C.C. and C.-W.L. designed the experimental protocols, supervised all experiments, interpreted the results. All authors have read and approved the final manuscript.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

BCE, bone collagen equivalent; BMD, bone mineral density; COMP, cartilage oligomeric matrix protein; ELISA, enzyme-linked immunosorbent assay; IHC, immunocytochemistry; IL, interleukin; MIA, monosodium iodoacetate; micro-CT, micro-computed tomography image; MMP, metalloproteinase; NTX-I, cross-linked N-telopeptide of type I collagen; OA, osteoarthritis; PM, particulate matter; RA, rheumatoid arthritis; ROS, reactive oxygen species; SRM 1649b, Standard Reference Material 1649b; TNF-α, tumor necrosis factor α.

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