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PM2.5 inducing myocardial fibrosis mediated by Ang II/ERK1/2/TGF-β_1 signaling pathway in mice model

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Abstract

Objects: To discuss the influence of PM2.5 on myocardial fibrosis and related mechanism.

Methods: PM2.5 particles were prepared into different concentrations of solution to drip into the mice's trachea twice each week. The mice were divided into five groups, Blank control group (C group), NS control group (J group), high dose group (G group, 10 mg/kg), medium dose group (Z group, 5 mg/kg), and 1ow dose group (D group, 2.5 mg/kg). After 6 weeks, the myocardial fibrosis was observed by HE and Masson staining. The expression of Ang II, ERK1/2, and TGF-β_1 was examined by Western Blotting (WB) and Real time PCR (RT-PCR).

Results: The higher dose PM2.5 was administrated, the worse the myocardial fibrosis was in PM2.5 groups. The expression of Ang II, ERK1/2, and TGF-β_1 was increased in higher dose groups in protein and mRNA level.

Conclusion: 1. PM2.5 induced the cardiac fibrosis. 2. PM2.5 dripped into trachea in mice model activated the expression of Ang II, ERK1/2, and TGF-β_1. The activation of renin-angiotensin system (RAS) was suggested to participate in the cardiac fibrosis induced by PM2.5.

Keywords

Cardiac fibrosis, RAS, PM2.5, pollution

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Background

While Modern society industrializing, more scientists are focused on the atmosphere pollution related with human's health. According to aerodynamic diameter, particulate matter 2.5 (PM2.5) is referred to atmospheric particles with diameter less than or equal to 2.5 μm. The effect of PM on human body depends on particle size, which is related to its aerodynamic diameter (AD). Most PM10 particles range from 2.5 to 10 μm, while PM2.5 is ⩽2.5 μM. PM2.5 particles may enter the blood through the alveoli, causing adverse effects on health.[1–3] Compared to PM10, PM2.5 as smaller particle and larger specific surface area has greater impact on the cardiovascular system.[4,5] PM2.5 entering the respiratory system, would cause inflammatory reaction and oxidative stress in the lung even the whole body. It affects the coagulation system, the autonomic nerve function, the vascular endothelium, and the vasomotor function.[6–10] The above reaction related with PM2.5 inhalation might activate cardiac stress response. Earlier study has proved that 6 h after exposure to PM2.5, the mRNA expression of inflammation related genes such as TNF-α, TNF-β, IL-6, and IL-8 increased. IL-6 can react quickly to air pollution and increase the production of C-reactive protein (CRP).[11] According to another report, ERK1/2 signaling pathway was indicated to involve in oxidative stress and inflammatory response induced by PM2.5.[12] PM2.5 may increase the levels of VCAM-1 and ICAM-1 to aggravate the adhesion between monocytes and endothelial cells through ERK/Akt/NF-κb axis.[13] As was known to us, ERK/Akt/NF-κ b axis also

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participated in cardiac fibrosis. In our study, the effect of PM2.5 inhalation on cardiac was investigated in mice model. Furthermore, its related mechanism was also discussed primarily in our work.

Methods

Particulate matter extraction

The samples were collected daily using multichannel particle composition monitors (PCM) from Binhai New Area Environmental Protection Bureau. In one channel, the air passes through a WINS impactor to remove particles greater than 2.5 mm. Finally, the remaining PM2.5 fraction is collected on glass fiber filter membrane. After that, it was cut into small pieces and put them into sterile ultra pure water in a 250 ml beaker. After staying low-temperature ultrasound and shaking gently every 5 min for 1 h, it was mixed as the suspension. The membrane was washed three times and the suspension was collected into the tubes. Then, the suspension was filtered with six layers of gauze, freezed, and dried at low temperature. After all that, the PM2.5 particles were separated from the fiber successfully. And it was weighed and stored in the refrigerator at −20°C. Before experiment, the suspension was mixed again with ultrasonic vibration and prepared into different concentrations of PM2.5 particles.

Preparation of the mouse model

We obtained experimental animal use approval by the Experimental Animal Administration Committee of Tianjin Medical University and Tianjin Municipal Commission for Experimental Animal Control. For this study, thirty SD male rats weighing between 270 and 300 g, from the animal laboratory of Tianjin Medical University, were randomly assigned into three experimental groups and two control groups, six in each group. They were shame group (C group), NS control group (J group), high dose group (G group, 10 mg/kg), medium dose group (Z group, 5 mg/kg), and 1ow dose group (D group, 2.5 mg/kg) respectively. The rats of N group and experimental groups were anesthetized with 5% chloral hydrate and dripped normal saline and different concentrations of solution into their trachea twice each week respectively. After 6 weeks, all the rats were killed to gain their left ventricular tissues determined.

Histology

Small portions of the left ventricle were excised and fixed for histological analysis. Tissue fibrosis was evaluated by Masson's trichrome staining. Microscopic images were analyzed with Motic Images Advanced 3.2 software. Connective tissue was identified according to its color. And a percentage of the fibrotic tissue area was recorded to express the degree of fibrosis. The analysis was performed by a pathologist unaware of the experiment.

Total RNA preparation and quantitative analysis by real-time reverse transcription polymerase chain reaction

After the pathological studies, the left atrium was cut off and used for molecular biological studies. Specimen were rapidly frozen in liquid nitrogen and stored separately at −80°C for further analysis. One aliquot of each tissue sample was used to investigate the mRNA expression of Ang II, ERK1, and TGF-β_1. In brief, 100 mg of tissue was homogenized in 1 ml of TRIzol reagent (Gibco BRL, Life Technology) extracted with chloroform and precipitated in isopropyl alcohol. Total RNA was incubated in DNase I (0.2 U/μL, Invitrogen) for 30 min, extracted by phenol-chloroform, precipitated in isopropyl alcohol, and dissolved in diethylpyrocarbonate (DEPC)-treated water subsequently. The integrity of each sample was checked on a denaturing agarose gel. The concentration of total RNA was determined spectrophotometrically as pure if a ratio of optical density (OD) 260:280 > 1.6. Samples were stored at −80°C until used for specific oligonucleotide primer pairs used for amplification of Ang II, ERK1, and TGF-β_1 genes were designed according to the sequences obtained from GenBank. GenBank accession numbers were 50,689 (ERK1), 116,590 (ERK2), 497,229 (Ang II), 59,086 (TGF-β_1), and 012,580.2 (β-actin). The primers specific for each protein were: 5′ TAT CAA CAC CAC CTG CGA CCT 3′/5′ TTG GTG TAG CCC TTG GAG TTA 3′ (ERK1), 5′ TTC CCA AAC GCT GAC TCC AAA 3′/5′ GGC TCA TCA CTT GGG TCA TAA 3′ (ERK2), 5′ TGTGGTGCGAATGGAAGCC/5′-CAGGCAAGCCA TTCTCACA (Ang II), 5′ CCTGCTGAGGCTCAAGTTAAA 3′/5′ ACATCAAAGGACAGCCATTCT 3′ (TGF-β_1), and 5′ GCAGCGGGATTATGTCTTCTA 3′/5′ TTTCTTCATG GCCTTGTCAAT 3′ (β-actin). mRNA was transcribed into cDNA by TaqMan Reverse Transcription Reagents Kit. Relative mRNA expression values analysis were validated by real-time RT-PCR with SYBR Green (TAKARA Biotechnology Co.). Efficiency of the amplification reaction or potential variation in RNA loading was corrected by the expression of β-actin as endogenous control. The corresponding PCR products were 98 and 110 bp, respectively. The real-time RT-PCR consisted of 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 32 s. These procedures were carried out in instrument (ZhuHai Heima, Hema9700). Relative expression values were calculated as previously described.[14] The expression level of β-actin was used as an internal control.

Protein extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting

The left ventricular tissue (0.1 g/sample) was lysed in a homogenization buffer for 15 min at 35°C using the delipidation method. Protein extracts from the left atrium were

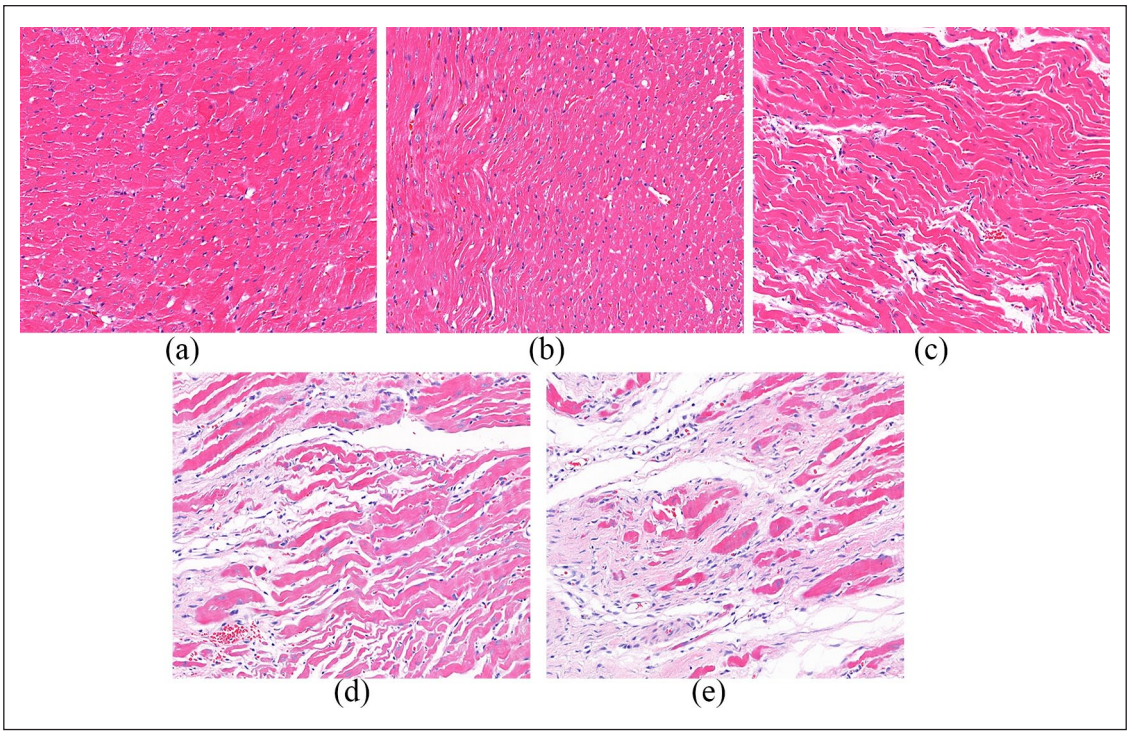


Figure 1. Panels 1(a–e) show the representative histologic sections of the left ventricle by HE staining from shame group (C group), NS control group (J group), low dose group (D group, 2.5 mg/kg), medium dose group (Z group, 5 mg/kg), and high dose group (G group, 10 mg/kg) respectively.

prepared with M-PER Mammalian Protein Extraction Reagent (Pierce) and quantified with BCA Protein Assay Kit (Pierce).

In brief, protein electrophoresis was performed on 10% Tri-HCl polyacrylamide ready gel and electroblotted onto Hybond-C extra nitrocellulose membranes. Membranes were incubated with antibody against Ang II (SAB, #48274), TGF-β_1 (proteintech, 21898-I-AP), ERK1/2 (proteintech, 67170-11g), and β-actin (SAB, #21612). ERK1/2 antibody wouldn't recognize the phosphorylated form of ERK. Then, membranes were incubated with the secondary antibody. Proteins were visualized by chemiluminescence using the ECLTM Western blotting system and the signal were detected and recorded by autoradiography (Beijing SaiZhi. Champchemi 610plus). The concentration of target proteins was determined by signal intensity (integral volume) of the appropriate bands on autoradiogram analyzed by a Scanner and the Quantity One software.

Statistical analysis

All data are expressed as mean ± SD and statistical significance is achieved for $p < 0.05$. Statistical comparisons among groups were performed with analyses of variance (ANOVAs). If significant effects were indicated by ANOVA, a *t*-test with Bonferroni's correction or Dunnett's test was used to evaluate the significance of differences between individual mean values. All data was analyzed in SPSS 25.

Results

Pathological examination

Representative histological sections from each group were shown by HE staining in Figure 1(a–e). Interstitial fibrosis was shown in blue by Masson trichrome staining in Figure 2(a–e). Left ventricular tissue from the sham rat appeared normal. In contrast, left ventricular tissue from the rat in the PM2.5 administrated groups showed a large amount of interstitial fibrosis distributed throughout the tissue. In addition, heterogeneity in the size and arrangement of cardiomyocytes was found in these tissues. These pathological abnormal findings were aggravated by higher dose of PM2.5 administrated. A quantitative analysis of fibrosis was shown in Figure 3. The percentage of fibrosis in the left ventricles in the control groups was markedly lower than that in the other PM2.5 administrated groups. Morever, it was elevated by higher concentration in PM2.5 administrated groups.

Expression of Ang II, ERK1/2, or TGF-β_1 mRNA

Figure 4(a–c) showed the relative mRNA expression values of Ang II, ERK1/2, and TGF-β_1 mRNA in six hearts (one independent determination per heart) from each

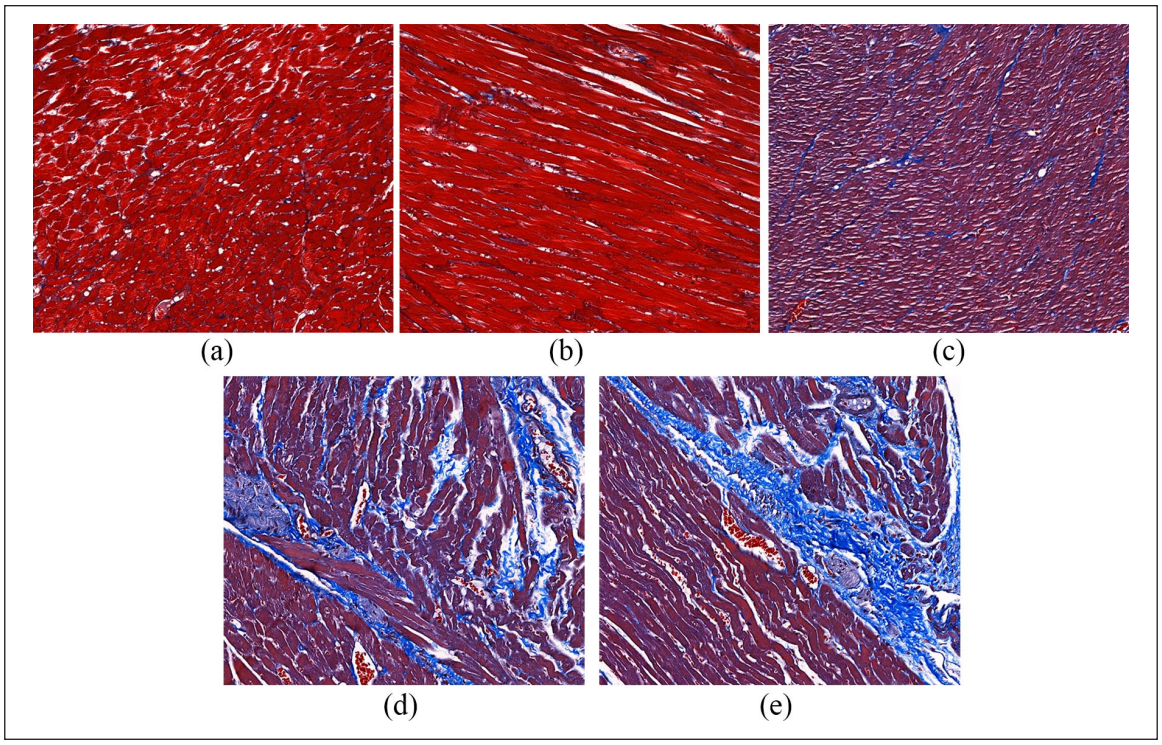


Figure 2. Interstitial fibrosis was shown in blue by Masson trichrome staining in Panels 2(a–e). Left ventricular tissue from the sham rat appeared normal. In contrast, left ventricular tissue from the rat in the PM2.5 administrated groups showed a large amount of interstitial fibrosis distributed throughout the tissue. In addition, heterogeneity in the size and arrangement of cardiomyocytes was found in these tissues. These pathological abnormal findings were aggravated by higher dose of PM2.5 administrated.

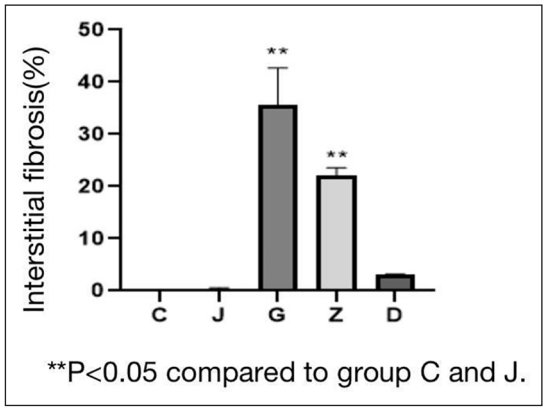


Figure 3. A quantitative analysis of fibrotic area in Masson staining, was shown in Figure 3. The percentage of fibrosis in the left ventricles in the control groups was markedly lower than that in the other PM2.5 administrated groups. Moreover, it was elevated by higher concentration in PM2.5 administrated groups (**$p < 0.05$).

group of rat. In the PM2.5 administrated groups, Ang II, ERK1/2, or TGF-β_1 mRNA expression was increased significantly compared to C and J groups. Higher dose of PM2.5 was dripped, higher the expression of above genes was increased.

Ang II, ERK1/2, or TGF-β_1 changes in protein level

Figure 5 showed the protein expression values of Ang II, ERK1/2, or TGF-β_1 in the C group, J group, G group, Z group, or D group, by the method of western blotting respectively. Ang II, ERK1/2, and TGF-β_1 expression was increased in the PM2.5 administrated groups significantly. The changes of Ang II, ERK1/2, and TGF-β_1 protein expression was aggravated by PM2.5 treating ($p < 0.05$). The above tendency of the proteins expression is accordance with their genes expressing in PCR experiments.

Discussion

The influence of PM2.5 on cardiovascular system

At present, the oxidative stress induced by PM2.5 exposure is considered as an important factor for cardiovascular system injury. Amount of reactive oxygen free radicals released can induce dysfunction of vascular endothelial cell function to promote blood pressure elevation, atherosclerotic plaque formation, and thrombosis. PM2.5 can also stimulate sympathetic and parasympathetic nerve centers directly in the body to affect blood pressure. At the same time, the above changes

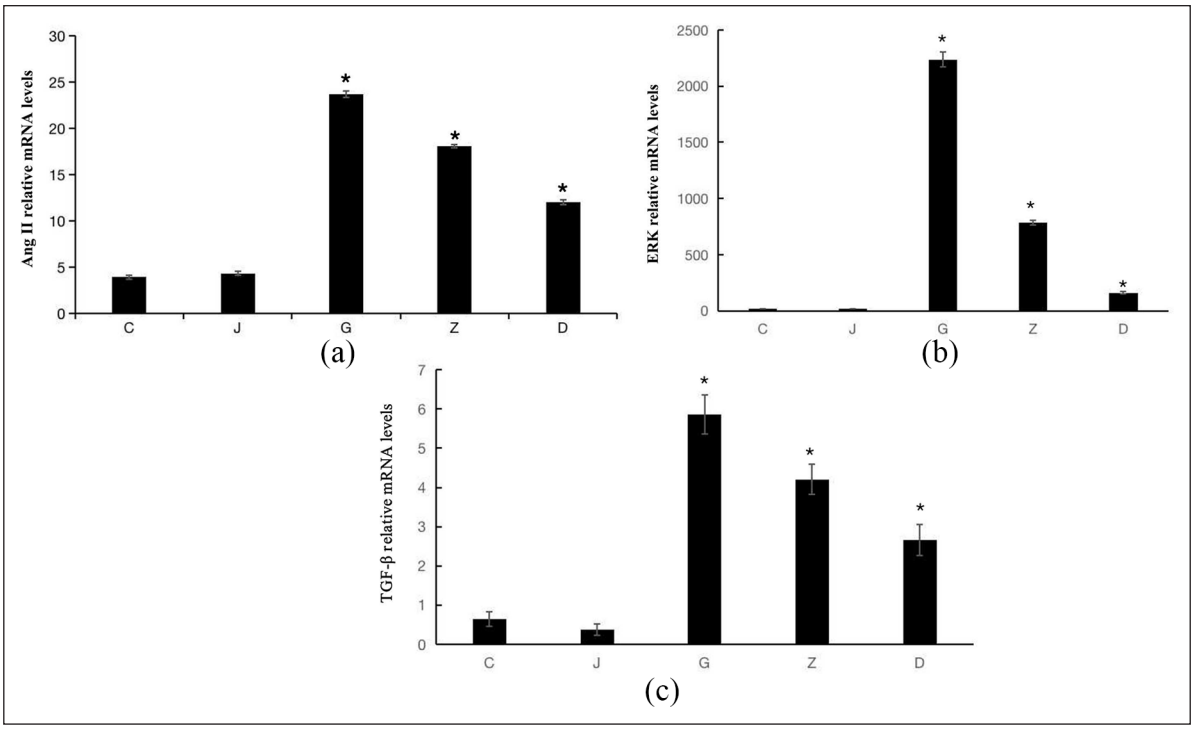
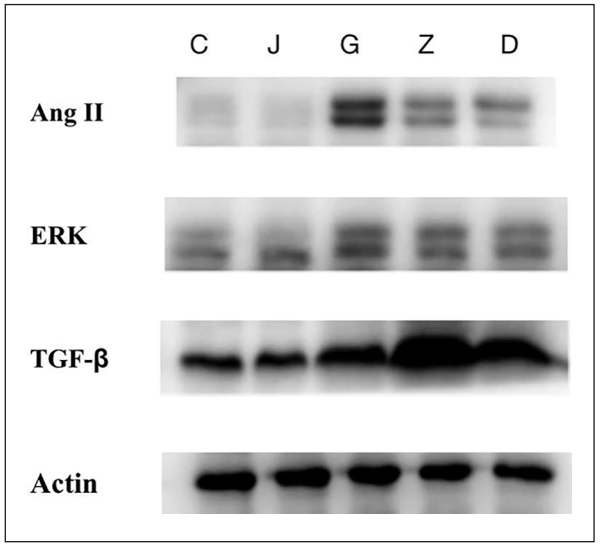


Figure 4. Figure 4(a–c) showed the relative mRNA expression values of Ang II, ERK1/2, and TGF-β_1 mRNA in six hearts (one independent determination per heart) from each group of rat. In the PM2.5 administrated groups, Ang II, ERK1/2, or TGF-β_1 mRNA expression was increased significantly compared to S and N groups. Higher dose of PM2.5 was dripped, higher the expression of above genes was increased.



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induced by PM2.5 were suggested to be relative with some tachyarrhythmia and bradyarrhythmia.[15] Several studies have shown that long-term exposure to PM2.5 increases the risk of cardiovascular disease. A Danish cohort study of 49,564 people from 1993 to 2015 showed that exposure to PM2.5 increased cardiovascular mortality at 18 μg/m^3.[16] The long-term adverse effects of air pollutants on cardiovascular mortality were also supported by the follow-up results of a comprehensive cohort analysis over 25 years in the UK.[17] A study from Asia also supported these results. The report from Korea involving 436,933 subjects clarified that the long-term exposure to PM2.5 was correlated with all-cause cardiovascular mortality linearly and significantly.[18] In addition, a number of experiments showed that short-term exposure to PM2.5 might induce cardiovascular disease. According to a latest study of Japan, short-term exposure to PM2.5 was indicated to increase the risk of cardiac arrest out of hospital.[19] Thus, amount of clinical researches have revealed that short-term and long-term exposure to PM2.5 would increase incidence of cardiovascular diseases.

The influence of PM2.5 on pulmonary and myocardial fibrosis

Pulmonary fibrosis is characterized by the deposition of collagen and other extracellular matrix molecules.[20] In a

recent study, Chen et al.[21] observed that PM2.5 increased the expression of TGF-β and collagen III deposition, which was responsible for right ventricle and pulmonary fibrosis. As was known to us, some particles in air such as PM2.5 were related to a lot of chronic respiratory diseases caused by pulmonary inflammation and fibrosis. In another report, it was found that the levels of IL-1β and TGF-β_1 was increased in bronchoalveolar lavage fluid of mice given PM2.5 via oropharynx for 21 days and the collagen deposition around small airway was obvious.[22] Cho et al.[23] demonstrated that oxidative stress caused by excessive ROS may be involved in the pathogenesis of human pulmonary fibrosis in their earlier study. A number of studies have shown that PM2.5 exposure can increase ROS in human alveolar epithelial cells and patients with idiopathic pulmonary fibrosis.[24–26]

As was known to us, cardiac fibrosis was closely related to many kinds of heart diseases, such as hypertensive heart disease, diabetic cardiomyopathy, dilated cardiomyopathy, hypertrophic cardiomyopathy, and viral myocarditis, which were manifested as cardiac interstitial fibrosis, excessive deposition of collagen, and abnormal distribution. Pressure overload caused by hypertension or aortic stenosis led to extensive myocardial fibrosis, initially associated with increased stiffness and diastolic dysfunction.[27] In addition, a variety of toxic insults (such as alcohol or anthracycline drugs)[28] induced progressive fibrosis in human patients and experimental models. Reactive fibrosis was considered as an adaptive response, which aimed to normalize the increased wall stress and maintain cardiac output. However, excessive interstitial fibrosis may lead to mechanical stiffness, even cardiac dysfunction and damage of electrical conduction.[29] Therefore, reactive myocardial fibrosis was closely related to physiological and pathological heart disease. Konstam et al.[30] suggested that the continuous activation of fibrosis led to the deposition of extracellular matrix protein in the heart. At meanwhile, cardiomyocytes became hypertrophic and the hardness of the ventricular wall increased. The decreasing of systolic and diastolic function of myocardium led to heart failure. Amount of studies have confirmed that the severity of myocardial fibrosis was closely related to the prognosis and death of patients with heart diseases, especially heart failure.[31] At present, the mechanism of myocardial fibrosis was not clarified very clearly. Some regulatory cytokines were involved, such as transforming growth factor-β_1 (TGF-β_1), connective tissue growth factor (CTGF), and interleukin (IL) family played an important role in the process of cardiac fibrosis. There have been a lot of researches on the effect of PM2.5 related to pulmonary fibrosis, but less on the effect of cardiac fibrosis. According to a report, PM2.5 can promote the deposition of myocardial fibrosis, but has no significant effect on infarcted myocardia. PM2.5 was indicated to regulate the immune response and oxidative stress in normal hearts, but not in infarcted hearts. In a latest report, male Wistar rats model with local exposure to PM2.5 was built successfully.[21] In this study, PM2.5 particles in the real atmosphere were collected by PM2.5 detection equipment of Binhai New Area Environmental Monitoring Station in Tianjin. Wistar rats were dripped saline and different concentration of PM2.5 suspension solution and killed after 6 weeks. By HE staining and Masson staining, it was showed that cardiomyocytes disordered arrangement, uneven cell size, and myocardial fibrosis significantly in PM2.5 groups, compared to control groups. Furthermore, the degree of cardiac fibrosis was aggravated in the higher concentration groups, compared to that of the lower concentration groups. PM2.5 was suggested to induce myocardial fibrosis.

Then, the mechanism related with cardiac fibrosis was discussed in present study. As was known to us, the activation of RAS was related with cardiac fibrosis closely. Previous studies have confirmed that Angiotensin (Ang) can increase the activation and proliferation of fibroblasts, promote collagen production and cardiomyocytes' hypertrophy and apoptosis.[32] Injection of a hypobaric dose of Ang II into mice can lead to cardiac hypertrophy and fibrosis.[33] Mitogen activated protein kinase (MAPK) was known as important pathway related to cardiac fibrosis in earlier researches. The activation of RAS was proved to increase the expressing of ERK to start up the MAPK pathway.[34] In latest studies, TGF-β_1 was clarified as an important effector of MAPK and other pathways related to cardiac fibrosis. According to Dr. Schultz et al.[35], Ang II treated did not result in cardiac hypertrophy or fibrosis in mice lacking of TGF-β_1. In our previous research, arial fibrosis complained with increasing of TGF-β_1 was observed in canine model of atrial fibrillation. Extra Ang-(1–7) administrated was indicated to decrease the atrial fibrosis and the TGF-β_1 expressing.[36]

Thus, in the present study, the expressing of ERK1/2 and TGF-β_1 was determined to discuss the mechanism of PM2.5 induced cardiac fibrosis. Compared to control groups, the expressing of ACE1/2 and TGF-β_1 was increased significantly. And the increasing of ERK1/2 and TGF-β_1 was promoted by higher concentration of PM2.5 administrated. Therefore, Ang II/ERK1/2/transforming factor-β signaling pathway was indicated to participate in the cardiac fibrosis induced by PM2.5 administrated significantly.

Study limitations

PM2.5 was clarified to induce the cardiac fibrosis primarily in our present study. Moreover, the activation of RAS was one of the mechanism possibly. The pathological abnormality and the changes of some protein expression was observed in our study. However, some protein, such as collagen I, should be determined by immunohistochemical method to reflect the cardiac fibrosis in further study.

Besides that, other important mechanism related to the fibrosis should be discussed in further study, such as TGFβ_1-Smad pathway, inflammation, oxidative stress, and mitochondrion dysfunction.

Conclusion

In mice model with PM2.5 dripped into trachea, PM2.5 induced ventricular fibrosis. The degree of cardiac fibrosis was aggravated by higher concentration of PM2.5 administrated. In accordant, increasing of ACE1/2 and TGF-β_1 was promoted by higher concentration of PM2.5. Thus, Ang II/ERK1/2/transforming factor-β signaling pathway was suggested as an important mechanism on PM2.5 induced cardiac fibrosis.

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Declaration of conflicting interests

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