Protective Effect and Mechanism of Shengmai Formula in Ultrafine Particle Matter-Mediated Atherosclerosis and Myocardial Ischemic Injury

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Abstract

Background: Long-term exposure to ultrafine particulate matter (UFPM) has been shown to enhance the development of atherosclerosis and impair myocardial function, leading to myocardial infarction (MI). The Shengmai (SM) formula is a kind of traditional Chinese medicine for cardiovascular diseases, which has been used in clinic for thousands of years. It is composed of Red ginseng, dwarf lilyturf tuber (Ophiopogon japonicus), and Chinese magnolia vine fruit and has been reported to improve myocardial ischemia and combat oxidative stress (OS) injury. The aim of this study was to explore the protective role of SM and the mechanism by which it acts against UFPM-induced cardiovascular injury. This study is expected to provide a basis for the development of potential adaptive signature of SM in the prevention of atherosclerotic cardiovascular disease (ASCVD) and premature death from global air pollution exposure.

Results: Pretreatment with SM reduced plaque vulnerability, improved cardiac systolic function, decreased the OS response, and attenuated UFPM-induced reactive oxygen species production and mitochondrial damage. In addition, mice pretreated with SM showed a smaller MI area. In vitro data further demonstrated that compared with the UFPM group, pretreatment with SM significantly increased the expression of nuclear factor erythroid 2-related factor2 (Nrf2) and HO-1, and to some extent protected the antioxidant defense system.

Conclusion: These results indicate that SM can inhibit the OS reaction in myocardial infarction mice, avoid cardiovascular injury induced by UFPM in atherosclerosis (AS)+MI mice, and improve cardiac function. Furthermore, the Keap1-Nrf2/HO-1 antioxidant signaling pathways might be involved during these processes. It is suggested that SM has a potential protective effect on the ASCVD population with prolonged exposure to excess particulate matter concentration.

Keywords: Shengmai, Atherosclerosis, Myocardial infarction, Ultra fine particulate matter, Oxidative stress, Keap1-Nrf2/HO-1 pathways.
1. Background

Cardiovascular disease (CVD) is the leading cause of death worldwide, responsible for 17.6 million deaths in 2016. Notably, three million of those deaths were attributable to air pollution [1]. Ultrafine particulate matter (UFPM) consists of airborne particles with an aerodynamic diameter of ≤2.5 μm. In China, for every 10 μg/m^3 increase in the annual exposure concentration of particulate matter (PM_{2.5}), the risk of cardiovascular disease and death increases by 25% and 16%, respectively [2]. Repeated and nearly continuous exposure to UFPM accelerates the process of AS [3, 4], leading to acute cardiovascular events, such as acute coronary syndrome and MI. At present, it is considered that the risk mechanism of UFPM-mediated systemic cardiovascular disease can be capsulated into several effective pathways, including endothelial barrier dysfunction, OS, inflammation, coagulation pathway, autonomic dysfunction, and epigenomic change [5]. However, most of the existing research is limited to the theoretical level, and there are still no effective prevention and treatment measures for clinical application. In recent years, a growing number of clinical trials have favored the acceptance of certain traditional Chinese medicine [6] for the treatment of CVD [7, 8]. The Shengmai (SM) formula, one of the most ancient prescriptions in TCM, is composed of Radix Ginseng, Radix Ophiopogonis, and Fructus Schisandrae Chinensis [9]. SM has the effects of invigorating qi, tonifying pulse, nourishing yin, and promoting fluid production [10]. Modern pharmacological research shows that SM has a wide range of pharmacological benefits, including anti-inflammation, anti-oxidation, anti-infection, and lipid-lowering effects. Clinically, SM can be used to treat shock, coronary heart disease, angina pectoris, myocardial infarction (MI), viral myocarditis, pulmonary heart disease, and heart failure. It is worth noting that SM is specially designated to treat coronary heart disease because of its efficacy in improving myocardial blood supply, heart failure, and myocardial fibrosis, thus alleviating the symptoms of CVDs [11].

Mechanically, SM causes an increase in calcium release from the sarcoplasmic reticulum (SR) through L-type Ca^{2+} current-activated ryanodine receptors (RyRs), thereby improving myocardial contractility. [12]. On the other hand, SM improves post-ischemic myocardial dysfunction by opening
mitochondrial $K_{ATP}$ channels [11]. SM can also inhibit myocardial fibrosis and regulate myocardial remodeling by regulating matrix metalloproteinases (MMPs) and collagen (COL-IV), thereby protecting the myocardium from damage.

It is worth noting that there is a common mechanism between UFPM exposure and SM protection—oxidation balance. Therefore, we hypothesized that SM not only has a pharmacodynamic effect on the treatment of cardiovascular diseases, but also has a potential protective effect on UFPM-mediated cardiovascular injury.

AS is the main cause and core pathological mechanism of MI. Clinically, most patients with MI suffer from AS as a basic disease. Therefore, to study the effect of PM$_{2.5}$ on MI, it is inevitable to build an AS-MI compound model in the same organism, so as to more appropriately simulate the occurrence and development of CVDs under air pollution conditions. We exposed ApoE/-/- mice with hyperlipidemia to PM$_{2.5}$ by repeated endotracheal instillation, and then ligated the left anterior descending (LAD) coronary artery to simulate the long-term exposure of patients with potential coronary artery disease.

Nuclear factor erythroid 2-related factor2 (Nrf2) is a transcription factor that regulates the induction and/or constitutive expression of antioxidant and phase II enzymes. Studies have shown that exposure to reactive oxygen species (ROS) and electrophiles induced a battery of genes encoding antioxidant and detoxifying enzymes/proteins via the Nrf2/Keap1 pathway [13, 14]. Genes encoding glutathione-S-transferase (GST), NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase (HO-1), $\gamma$-glutamylcysteine synthetase (GCS), and thioredoxin have been shown to be regulated through the antioxidant response element (ARE) or the electrophile response element (EpRE) within the regulatory region of target genes [15, 16].

Our new findings suggest SM as a potential therapy to prevent some of the premature deaths attributable to air pollution exposure and provide a new basis for its clinical application, which will also promote the development and application of TCM.

2. Results
2.1 General conditions of mice after UFPM and SM intervention.

In the first set of experiments, we documented general conditions in mice after UFPM and SM intervention. As shown in Figure 1A, after 12 weeks of a high-fat diet (HFD), ApoE−/− mice were exposed to UFPM by repeated endotracheal instillation with simultaneous drug administration. LAD coronary artery ligation was performed 24 hours after the last exposure. The body weight curves of mice in each group after UFPM treatment are shown in Figure 1B. The survival curve after UFPM exposure and MI is shown in Figure 1C. Compared with MI mice, the SM-treated mice showed an improved survival rate. Although the HFD led to significantly higher levels of plasma total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C), UFPM exposure did not affect the plasma lipid profile (Figure 1D–G).

2.2 SM reverses UFPM-induced AS development

We observed the longitudinal section of the aortic arch and the transverse section of the aortic root in ApoE−/− mice using ultrasound technology and pathological staining to explore the effect of SM on the progression of UFPM-mediated AS. Wall thickness of the aortic arch was measured by an ultrahigh resolution small animal ultrasound imaging system, as shown in Figure 2A–D. Compared with the NC, the wall thickness of the greater curvature (GC), lesser curvature (LC), and the origin of the brachiocephalic artery (BC) were significantly increased in the Model group after UFPM exposure. However, the SM group showed significantly reduced GC, LC, and BC wall thicknesses compared with the Model group. We quantified the longitudinal section of the aortic arch using hematoxylin and eosin (H&E) and Masson staining (Figure 2E). H&E, Oil Red O, and Masson trichromatic staining were used to quantify the aortic root cross-section, as shown in Figure 2F–I. H&E staining showed that the plaque area of each group with UFPM was significantly larger than that of the group treated with phosphate buffered saline (PBS). With or without UFPM exposure, the plaque area decreased after drug intervention compared to that in the Model group. Oil Red O staining also showed an increase in lipid content after UFPM intervention. Similarly, after the drug intervention, lipid levels in the plaques were lower than in the Model group, regardless of exposure to UFPM. Masson staining
showed that compared with the PBS group, the collagen content of APOE-/- mice exposed to UFPM was significantly reduced. After drug intervention, the plaque collagen content in the group without UFPM increased in contrast to the Model group with PBS. While in the presence of UFPM, the plaque collagen content did not decrease compared with the Model group. Overall, imaging and histological analysis of the aorta showed that SM alleviated the development of UFPM-mediated AS.

2.3 SM reduces fine particle matter-induced cardiac dysfunction.

To investigate the effect of SM on cardiac function, we examined the long-axis view of the heart and coronary flow by echocardiography. There were no significant differences in any of the indicators in the Sham group with or without UFPM exposure. With UFPM exposure intervention, the values of left ventricular ejection fraction percentage (LVEF %), left ventricular fractional shortening percentage (LVFS %), cardiac output (CO), stroke volume (SV), and interventricular septum dimension (IVS) in the group of MI plus SM were significantly lower than those in the group without UFPM exposure, while the values of LV internal dimensions at diastole (LVIDd) and LV internal diameter at systole (LVIDs) were significantly increased. No significant change was observed for LV posterior wall (LVPW), heart rate (HR), and LV mass among each group (Figure 3). However, there was a marked increase in LVEF % (Figure 3B), LVFS % (Figure 3C), CO (Figure 3D), and SV (Figure 3E), and LVIDs (Figure 3F), LVIDd (Figure 3G), LV posterior wall systole (LVPWs) (Figure 3J) were significantly reversed by SM treatment.

In addition, the effects of SM on the peak velocities of aortic flow and coronary artery flow were obtained in color Doppler mode at 24 h after MI with UFPM exposure (Figure 4A and 4D). As the picture shows, the aortic valve (AV) peak velocity (Figure 4B) and the AV peak pressure (Figure 4C) of the groups with UFPM showed no significant difference compared with that of the groups without UFPM. The decline in values of coronary peak velocity in the MI plus SM group were outstanding compared with that of the group without UFPM exposure. The decline was significantly reversed in the SM group compared to the MI group with or without UFPM intervention (Figure 4E). Taken together, these data indicate that the therapeutic effect of SM is significant in improving cardiac function in
UFPM-induced MI mice.

2.4 SM Reduces the area of MI and preserves the myocardial ultrastructure

The toxicological effect of UFPM in myocardial injury and the protective effects of SM in MI mice were investigated via quantifying infarct volumes in triphenyl-2H-tetrazolium chloride (TTC, T8170) stained heart slices (Figure 5A and 5B) and by observing the myocardial ultrastructure using transmission electron microscope (Figure 5C). TTC staining showed that the MI size of each group with UFPM was significantly larger than that of the group with PBS. SM treatment significantly reduced the MI size with or without UFPM exposure. In the Sham group, there was no significant difference in the myocardial ultrastructure of MI mice, with or without UFPM. Cardiomyocytes in MI mice after UFPM exposure exhibited myofibrillar disorganization, segmental loss of myofibrils, mitochondrion rupture, and mitochondria with swelling, partially disappeared cristae (Red arrow), and increased autophagosomes (Yellow arrow). SM treatment of MI after 24 hr showed cardiomyocytes with normal ultrastructure with or without UFPM. These results suggest that SM can ameliorate myocardial ultrastructural damage induced by PM-mediated MI in mice. An electrocardiogram (ECG) was recorded during the LAD ligation. The sinus rhythm and HR of mice in Sham group were normal. On the ECG, the ST segment of mice in the MI group was significantly abnormal, and the ST segment elevation was more significant after UFPM treatment, suggesting an acute inferior MI. The ST segment recovered after SM pretreatment compared with that in the MI group with UFPM exposure (Figure 5D).

2.5 SM attenuates UFPM-induced OS in MI mice

OS is an important factor for MI injury; its damages include mitochondrial dysfunction and apoptosis. We observed that UFPM induces and aggravates OS damage in aortic (Figure 6A and B) and cardiac tissues (Figure 6C and D), manifested as significant increases in ROS homeostasis levels, while SM treatment significantly reduces their levels. As shown in Fig. 6, the fluorescence density of ROS in each group with UFPM was significantly higher than that of the group with PBS. SM treatment
significantly reduced the fluorescence density of ROS.

2.6 SM plays an antioxidant role in vitro via the Nrf2 / HO-1 signal pathway

Nrf2, as a transcription factor, regulates the expression of several antioxidants and phase II detoxification enzymes, reduces ROS production, and thus plays an anti-oxidative stress role. We tested whether SM plays its protective effect by regulating the expression of Nrf2, HO-1, and NQO1, the key antioxidant agents in UFPM exposure. As shown in Figure 7, H9C2 cells were treated with siRNA for transfection for 6 hours, preincubated either with medium or SM (1.12 mg/ml) for 24 h, and then stimulated with UFPM (50 mg/ml) for 6 h. The expression of oxidation-stress-related enzymes, Nrf2, HO-1, and NQO1, were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

The expression level of Nrf2 in the group of UFPM was lower than that in the control group, but the difference was not statistically significant. After adding SM, the expression of Nrf2 was significantly higher than that of UFPM, indicating that SM could play the role of antioxidant by acting on NRF2. After siRNA intervention, the expression of Nrf2 decreased (Figure 7A). In the control group, the expression level of HO-1 was significantly reduced after exposure to UFPM and was effectively increased after adding SM. In the siRNA treatment group, there was no significant difference between PM and PBS, and SM increased the expression level of the HO-1 gene (Figure 7B). The expression level of NQO1 was decreased after exposure to UFPM in the control group. And the level HO-1 could be increased by adding SM. However, there was no statistical difference (Figure 7C).

3. Discussion

Extensive epidemiological investigations have revealed that short- and long-term exposure to air PM is associated with cardiovascular effects in humans, including MI, ischemic heart disease (IHD), AS, arrhythmia, and thrombosis [17]. Among them, the main mechanism of PM-increasing heart risk is the initiation and promotion of AS, which is a major cause of most CVDs [18]. Previous studies have established animal models of AS and MI, respectively, aiming to explain the possible mechanism of PM in atherosclerotic susceptible populations [19]. However, patients with MI often have a long-term
pathological basis of AS, so the establishment of a single animal model cannot well simulate the complex pathological state in humans. In this study, the formation and activation of AS plaques were induced by UFPM-infusion in the trachea of ApoE-/--mice, and the pathological and physiological changes during sudden myocardial ischemia injury were simulated by MI to provide clues for the population with ASVCD and prolonged exposure to UFPM.

PM$_{2.5}$ is now considered as a new risk factor for CVDs [20]. Serum lipid is a well-established mediator of CVD pathogenesis and progression. LDL-C lowering therapies are major targets of CVD risk reduction strategies [21]. One epidemiological study found that serum levels of LDL-C, TC, and TG increased with PM$_{2.5}$ exposure [22]. However, in our study, there were no significant changes in blood lipid levels after four weeks of exposure to UFPM. We hypothesized that insufficient exposure time and frequency contributed to this outcome. More recently, studies have shown that exposure of the particulate constituents of diesel exhaust increases both the size and complexity of atherosclerotic plaques in vivo and may therefore increase the susceptibility of plaques to rupture [23]. Our histological analysis showed that the lesion area of aortic root increased, lipid content increased, and collagen content decreased after four exposures to UFPM. However, after drug intervention, the lipid content in the aortic root decreased, although the collagen content did not change significantly, suggesting that the protective effect of SM is focused on inhibiting the accumulation of lipids mediated by UFPM in plaques.

Some studies suggest that the effects of PM may involve an imbalance in the autonomic control of the heart, leading to a decrease in HR and HR variability [24]. These changes are associated with poor prognosis in patients with CVD and may lead to adverse cardiovascular effects [25]. On the other hand, recent studies have found that exposure to PM$_{2.5}$ can lead to abnormal types of ECGs, such as ST segment depression in C57BL/6 mice and right bundle branch block in ApoE-/--mice. In our study, the mice in the combined model of AS and MI exhibited abnormal ECG types—including ST segment elevation after PM exposure. It can be considered that the abnormal ST segment on the ECG is related to myocardial ischemia and hypoxia induced by UFPM. SM intervention reversed the abnormal ST segment, suggesting that SM may play a protective role in UFPM-mediated ECG abnormalities.
Similar results have been found in epidemiological studies, in which PM predicted the increased risk of ST segment depression in patients with coronary artery disease [26]. In a nutshell, cardiac autonomic nerve control imbalance and myocardial ischemia-hypoxia effect may provide a new idea for the potential mechanism and drug prevention of CVD caused by PM$_{2.5}$ exposure.

PM and ischemia-induced OS act as key contributors to MI injury by inducing a cascade of pathological changes that include mitochondrial dysfunction, DNA damage, and protein aggregation, which in turn lead to apoptosis [27] MI-induced redox aberrance results not only from excessive ROS generation, but also from antioxidant system impairment [28]. SM dampened the OS by counterbalancing pro- and anti-oxidative machineries in the PM-induced ischemia myocardium. In the present study, we found that the ROS levels observed in the myocardial tissue of PM$_{2.5}$-exposed mice were higher than untreated PM$_{2.5}$-exposed mice. Moreover, similar restoration of PM$_{2.5}$-induced downregulation of ROS levels was observed in SM-treated and untreated mice. The ROS level after UFPM exposure increased, indicating that the imbalance of antioxidant capacity of the circulatory system increased. After SM intervention, ROS expression in the cardiovascular tissue was significantly reduced. Similar results were also found in other studies, wherein OS also occurred in lung and heart tissues of mice, and most studies have confirmed that OS is caused by ROS [29]. The elemental analysis of UFPM shows that the concentration of some metal elements is significantly higher than that of others, which has been proven to be related to the production of ROS and oxidative damage [30]. Transition metals’ catalyzed Fenton reaction can produce ROS on the particle surface and induce oxidative damage [31]. Wan found that prolonged inhalation exposure to PM results in upregulation of the potential inflammatory mediator, visfatin, which led to OS in ApoE-/- mice after a PM$_{2.5}$ challenge [32].

Some research has found that in bronchial cells exposed to diesel exhaust particles (DEP), the Nrf2 system positively regulates autophagy to maintain cellular homeostasis. Acute exposure to high PM$_{2.5}$ concentrations causes autophagy-related cell necrosis. The decrease in cytochrome C release and apoptosis by upregulation of HO-1 may assist PM$_{2.5}$-induced autophagy-related cell necrosis (Overexpression of HO-1 assisted PM$_{2.5}$-induced apoptosis failure and autophagy-related cell
In this study, by observing the myocardial ultrastructure, we found that UFPM increased the number of autophagosomes and damaged the myocardial mitochondria, while SM reduced the number of autophagosomes. As a key regulator of redox homeostasis in inactive cells, Nrf2 exerts cytoprotective functions encompassing anti-oxidative and anti-inflammatory responses under physiological conditions [33]. The absence of Nrf2 has been shown to aggravate myocardial damage and increase sensitivity [34]. Our previous research found that SM protective role against myocardial injury in UFPM-exposed myocardial ischemia rats is mediated via the PI3K/AKT/p38 MAPK/Nrf2 pathway. HO-1 and NQO1 are the two main members of the Nrf2-ARE system involved in the regulation of anti-oxidative responses [35]. The system exhibits antioxidant effects through upregulation of cytoprotective proteins, including HO-1 and NQO1, which are considered as the most powerful anti-oxidative enzymes due to their relatively long half-lives and low levels of degradation [36]. Most studies have shown that when OS occurs, defense mechanisms can be activated by upregulating Nrf2 and its target genes.

To further clarify the role of the Nrf2 related pathway during SM protection, we transfected H9c2 cells with silencing RNA (siRNA) specific to Nrf2 and exposed them to UFPM. The relative levels of mRNA for Nrf2, NQO1, and HO-1 were determined using RT-PCR, and we found that the downstream gene HO-1 expression decreased. Similar results have been seen in studies of paraquat induced OS in human nerve precursors, acquiring cells [37]. We consider this because exposure to extremely toxic conditions completely destroys the redox equilibrium. SM pretreatment significantly increased the expression of Nrf2 and HO-1. We suggest that the Nrf2-HO-I signaling pathway may be a defense mechanism for cardiovascular injuries mediated by UFPM and to some extent protected the antioxidant defense system.

4. Conclusion

In conclusion, this study found that repeated exposure to UFPM was associated with an increased vulnerability to atherosclerotic plaque, decreased cardiac systolic function, abnormal electrocardiogram, increased OS level, and myocardial ischemia and hypoxia in the compound mice.
model. Under the condition of UFPM exposure, the heart function of AS/MI mice was more vulnerable to damage than that of the pure AS mice model. SM intervention had a selective protective effect on the development of AS and myocardial insufficiency mediated by UFPM. In addition, the toxicological effect of UFPM on the human body may be mediated by the mechanism of OS, while SM can play a protective role by regulating oxidative balance.

In addition, the current results suggest that UFM-mediated OS injury may be related to maladjustment and the Nrf2 / HO-1 axis. SM pretreatment can induce the activation of Nrf2 / HO-1 and raise the expression level. These findings are important for understanding the pathogenesis of UFPM in cardiovascular disease and the protective effect of SM antioxidants by regulating defense systems such as Nrf2 / HO-1.

Our data provide important experimental data for further pharmacological research, and a rationale for future clinical trials and applications.

5. Materials and Methods

5.1 Drugs and reagents

We bought 2, 2, 2-Tribromoalcohol from Sigma (T48402, St. Louis, MO, United States). High-efficiency radioimmunoprecipitation assay tissue/cell lysate (R0010), 2, 3, 5-Triphenyl-2H-tetrazolium chloride (TTC, T8170), and bicinchoninic acid (PC0020) protein concentration assay kit was purchased from Solarbio (Beijing, China).

5.2 Preparation of PM$_{2.5}$

The PM$_{2.5}$ sample was provided by the US National Institute of Standards and Technology (NIST). PM$_{2.5}$ was diluted with Dulbecco's PBS after sonication in double distilled water and freeze-drying in vacuum. The final concentration of PM$_{2.5}$ was 1 mg/ml.

5.3 Animals

Male 6-week-old ApoE-/mice (22 ± 2 g) were purchased from Peking University Health Science Center (Beijing, China, Certificate no.: SYXK (Jing) 2016-0010). This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Care and Use of
Laboratory Animals issued by the Ministry of Science and Technology of China. The protocol was approved by the Laboratory Animal Ethics Committee of China Academy of Chinese Medical Sciences.

5.4 Myocardial ischemia injury model and drug administration

Mice were fed high-cholesterol diets for 12 weeks and then randomly assigned to the following groups: AS+Sham, AS+MI, SM (3.12 mg·kg\(^{-1}·d\^{-1}\), equivalent to twice the clinical dosage). Then, the mice were divided into 6 groups with given PBS or UFPM. Mice were treated with a standardized UFPM from the US NIST of 50μg/animal, once a week for four weeks, intratracheally. The SM group was administrated by gavage once a day for 2 weeks before UFPM exposure. 24 hours after the last exposure of UFPM, the mouse model of myocardial (MI) was performed. Briefly, except for the AS+Sham mice, other mice were anesthetized by intraperitoneal injection of tribromoethanol; after which they underwent tracheal intubation and were connected to a ventilator. Next, the chest of each mouse was opened at the intercostal space between the third and fourth sternal rib via a left thoracotomy. Once the heart was exposed, the proximal LAD coronary artery under the left auricle was transiently ligated by a slipknot utilizing a 7-0 silk suture. Following the thorax closure and as soon as spontaneous respiration was sufficient, the mice were released and placed on an electric blanket. ST segment elevation on an ECG monitor represented a success in MI model surgery. In AS+Sham mice, the surgical procedures were identical, except the left anterior descending coronary artery was not tied.

5.5 The measurement of lipid index

After the treatment, blood samples were collected and centrifugated at 4°C, and plasma TG, TC, HDL-C, and LDL-C levels were determined using an automatic biochemical analysis system (chemical-180, Sysmex, Japan).

5.6 Echocardiographic measurement

Mice were anesthetized by inhalation of 3% isoflurane driven by 100% O2 in an induction chamber and then maintained with 1.5% isoflurane using an inhalation mask in the supine position. The noninvasive assessment of left ventricular function and coronary blood flow in the heart were made using an ultrahigh resolution small animal ultrasound imaging system (Vevo 2100, Visual Sonics,
Toronto, ON, Canada) equipped with a 30-MHz transducer. Mice were anesthetized by inhaling 1.5–2.0% isoflurane and transferred to the dorsal position and placed on a heated imaging platform. Parameters indicating cardiac functions were measured by M-mode and color Doppler mode as follows: SV, LVEF, LVFS, CO, LVIDd, LVIDs, LVPWd, LVPWs, interventricular septum dimension systole (IVSs), interventricular septum dimension diastole (IVSd), HR, and LV mass, along with AV peak velocity and coronary peak velocity.

5.7 Quantitative measurement of myocardial infarct size

At 24 h after ligation, the myocardial tissues of the euthanized mice were removed. The tissue was then crosscut into four pieces of 2-millimeter-thick slices in a heart matrix device, which were added to the 2% solution of triphenyltetrazolium chloride (TTC), incubated at 37°C in the dark for 15 min. After staining by TTC, the sections were photographed to measure the MI size using Image J analysis software (National Institutes of Health, Bethesda, MD, United States) and calculated as the proportion of MI size (percentage) = infarct size (while pale area)/total area of transverse slice 100%.

5.8 Procedure of ROS immunofluorescence assay for frozen sections

A chemical pen was used to draw a circle around the Cardiac and vascular tissue. A spontaneous fluorescence quenching agent was added to the circle for 5 min, and then it was water rinsed for 10 min. A ROS dyeing solution was then added, diluted with PBS at a dilution ratio of 1:200. After incubating in a light-proof incubator at 37° for 30 min, the slides were placed in PBS (PH 7.4) and washed by shaking on the decolorizing shaker three times, for 5 min each time. After the sections were dried, diamidino-2-phenylindole (DAPI) dye was added in the circle and incubated at room temperature in a dark condition for 10 min. The slides were again placed in PBS (PH 7.4) and washed by shaking on the decolorizing shaker three times, for 5 min each. The slices were slightly dried and sealed with an anti-fluorescence quenching sealing tablet. The sections were observed under a fluorescence microscope and the images were collected. DAPI ultraviolet excitation wavelength 330–380 nm, emission wavelength 420 nm, emitting blue light; fluorescein isothiocyanate (FITC) excitation wavelength 465–495 nm, emission wavelength 515–555 nm, emitting green light; the excitation wavelength of CY3 is 510–560, and the emission wavelength is 590 nm, emitting red light.
5.9 Histological analysis

All collected tissues were fixed in 4% paraformaldehyde and embedded with optimal cutting temperature compound (OCT) or paraffin for histological assessment. H&E, Oil Red O, Masson’s trichrome, and EVG staining was performed for lesion area, lipid and collagen quantification, and for measurement of fibrous cap thickness and broken elastic fibers. The lipid and collagen contents were measured using red and blue-stained area in Oil Red O and Masson’s trichrome staining. Representative images were analyzed by the Image Pro Plus software.

5.10 Cell culture and treatment

Rat myoblastic cells (H9C2) were purchased from the American Type Culture Collection and cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Madison, USA) under sterile conditions. The cells were incubated with 5% CO₂ at 37ºC. Cells were incubated with siRNA for 6 h followed by a medium change into fresh DMEM and maintenance for 24 h. For the mechanism analysis of SM, cells that were preincubated either with medium or SM (1.12 mg/ml) for 24 h were stimulated with UFPM (50 mg/ml) for 6 h.

For mRNA analysis, total RNA was isolated from H9c2 cell cultures in six well plates; RNA was converted to cDNA using the Reverse Transcription System from Promega following the manufacturer’s protocol. Relative cDNA levels were assayed using the LightCycler 480 system by Roche (Basel, Switzerland). Primer sequences of Nrf2, HO-1, NQO1, and β-actin are shown in Supplementary Table S1.

5.11 RT-qPCR analysis

RT-qPCR assays for selected genes, including Nrf2, HO-1, and NQO1, as well as β-actin (housekeeping gene), were performed for further validation. Specifically, RNA was isolated, and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Invitrogen Life Technologies). The gene expression levels were determined by RT-qPCR using the SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) and normalized to the housing gene GAPDH. The expression levels of the genes in the SM-treated group were expressed as the fold-change when compared to the
levels in the UFPM-exposed group using the 2−∆∆ct method; likewise, the expression levels in the UFPM-exposed group were determined by calculating the fold-change when compared with the control group.

5.12 Statistical analysis

Experimental data are presented as the mean ± standard error of measurement (SEM) or mean ± standard deviation (SD). GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, United States) was utilized for statistical analysis, which was performed with Student’s two-tailed t-test for comparison between two groups or one-way analysis of variance for multiple groups. A value of P < 0.05 was defined as statistically significant.

List of Abbreviations:
ultrafine particulate matter (UFPM); myocardial infarction (MI); Shengmai Formula (SM); atherosclerotic cardiovascular disease (ASCVD); oxidative stress (OS); Cardiovascular disease (CVD); traditional Chinese medicine (TCM); sarcoplasmic reticulum (SR); ryanodine receptors (RyRs); matrix metalloproteinases (MMPs); collagen (COL-IV); atherosclerosis (AS); reactive oxygen species (ROS); left anterior descending (LAD); Nuclear factor erythroid 2-related factor2 (Nrf2); glutathione-S-transferase (GST); antioxidant response element (ARE); electrophile response element (EpRE); high-fat diet (HFD); total cholesterol (TC); triglyceride (TG); low-density lipoprotein-cholesterol (LDL-C); high-density lipoprotein-cholesterol (HDL-C); hematoxylin and eosin (H&E); phosphate buffered saline (PBS); standard deviation (SD); standard error of measurement (SEM); left ventricular ejection fraction percentage (LVEF %); left ventricular fractional shortening percentage (LVFS %); cardiac output (CO); optimal cutting temperature compound (OCT); triphenyltetrazolium chloride (TTC); stroke volume (SV); interventricular septum dimension (IVS); LV internal dimensions at diastole (LVIDd); LV internal diameter at systole (LVIDs); LV posterior wall (LVPW); LV posterior wall systole (LVPWs); aortic valve (AV); triphenyl-2H-tetrazolium chloride (TTC, T8170); electrocardiogram (ECG); heart rate (HR); reverse transcription- quantitative polymerase chain reaction (RT-qPCR); ischemic heart disease (IHD); diesel exhaust particles (DEP); National Institute of Standards and Technology (NIST); LV
posterior wall diastole (LVPWd); interventricular septum dimension systole (IVSs); interventricular septum dimension diastole (IVSd); diamidino-2-phenylindole (DAPI); fluorescein isothiocyanate (FITC); Dulbecco’s Modified Eagle Medium (DMEM); DHE: dihydroethidium

**Declarations**

**Ethics Approval and Consent to participate**

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of China. The protocol was approved by the Laboratory Animal Ethics Committee of China Academy of Chinese Medical Sciences.

**Consent for publication**

The Author confirms: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, review, or thesis); that it is not under consideration for publication elsewhere; that its publication has been approved by all co-authors, if any; that its publication has been approved (tacitly or explicitly) by the responsible authorities at the institution where the work is carried out.

**Availability of data and materials**

All data generated or analyzed in this study are included in this paper (and its supplementary information documents). Other datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions
Conceptualization, T.Y. and Y.-J.L.; methodology, T.Y. and S.-Q.Q.; software, T.Y. and S.-Q.Q.; validation, L.-N.C. and S.-Q.Q.; formal analysis, Y.-M.Y. and H.L.; investigation, S.-Q.D., Z.-Y.Z., and Y.Z.; resources, T.Y. and S.-Q.Q.; data curation, T.Y. and L.-N.C.; writing—original draft preparation, T.-Y.; writing-review and editing, Y.-J.L. and L.-N.C.; visualization, T.-Y.; supervision, Y.-J.L. and X.-X.Z.; funding acquisition, Y.-J.L. and L.-N.C. All authors have read and agreed to the published version of the manuscript.

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FIGURE 1 General condition of mice after UFPM and SM intervention.

(A) After 12-week of HFD feeding, mice were administered SM (3.12 mg/kg/day) and exposed to particulate matter via inhalation once a week for four consecutive weekdays. At the end of the day, a LAD ligation operation was induced in the coronary artery.

(B) Body weight before and after UFPM exposure and LAD ligation was recorded in indicated groups (n= 15, *P < 0.05, **P < 0.01)

(C) Survival curves of mice after UFPM exposure and LAD ligation. (n= 15, *P < 0.05, **P < 0.01)

(D-G) Effect of UFPM and SM on the serum levels of TC (C), TG (D), LDL (E) and HDL (F) levels in mice. (n = 6, *P < 0.05, **P < 0.01)

UFPM: ultrafine particulate matter; SM: Shengmai Formula; HFD: high-fat diet; LAD: left anterior descending; TC: total cholesterol; TG: triglyceride; LDL: low-density lipoprotein-cholesterol; HDL: high-density lipoprotein-cholesterol

FIGURE 2 SM reverses fine particle matter -induced AS development

(A-D) ultrasound images of the aortic arch in ApoE−/− mice. Wall thickness of the aortic arch was observed at the greater curvature (GC), the lesser curvature (LC) and the origin of the brachiocephalic artery (BC). Values are expressed as mean ± SD (n = 6, *P < 0.05, **P < 0.01) Thickened wall of the aortic arch sections were observed by ultrasound and indicated with white arrows in representative pictures. (n = 6 per group, p* < 0.05, p** < 0.01).

(E) Representative images of H&E and Masson staining of longitudinal section of aortic arch; (n = 6 per group, p* < 0.05, p** < 0.01).

(F) Representative images of H&E, Masson, and Oil Red O staining of the transverse section of the aortic root; bar = 500μm. (n = 6 per group, p* < 0.05, p** < 0.01).

(G-I) Statistical analyses of plaque area, lipid content, and collagen area in aortic roots. Values are expressed as mean ± SD (n = 6 per group, p* < 0.05, p** < 0.01).

H&E: hematoxylin and eosin; SM: Shengmai Formula; SD: standard deviation
FIGURE 3 Changes of echocardiographic characterization of cardiac function in myocardial ischemia (MI) mice.

After 24 h of ischemia, the effect of SM (SM, 3.12 mg/kg/day) on cardiac function was quantitatively evaluated.

(A) Representative echocardiography images of different groups. Bar graph quantitation of echocardiographic changes in cardiac function in different groups detected in M-mode:

(B) LVEF %, (C) LVFS %, (D) CO, (E) stroke volume, (F) IVSs, (G) IVSd, (H) LVIDs, (I) LVIDd, (J) LVPWd, (K) LVPWs, (L) Heart Rate, and (M) LV mass. Values were expressed as mean SD (n = 6, *P < 0.05, **P < 0.01)

LV: left ventricular; EF: ejection fraction; FS: LV fractional shortening; CO: cardiac output; IVSd: interventricular septum dimension diastole; IVSs: interventricular septum dimension systole; LVIDd: LV internal dimensions at diastole; LVIDs: LV internal diameter systole; LVPWd: LV posterior wall diastole; LVPWs: LV posterior wall systole.

FIGURE 4 SM improves the coronary blood flow and left ventricular function after UFPM exposure in MI mice. After 30 min

At 24 h after ischemia, the effect of SM (3.12 mg/kg/day) on cardiac function was quantitatively evaluated.

(A) Representative echocardiography images of aortic blood flow were determined with different groups.

(B) AV peak velocity and (C) AV peak pressure was detected in color Doppler mode. Values are expressed as mean ± SD (n = 6, *P < 0.05, **P < 0.01)

(D) Representative echocardiography images of coronary artery flow were determined with different groups.

(E) Coronary peak pressure was detected in color Doppler mode. Values are expressed as mean ± SD (n = 6, *P < 0.05, **P < 0.01)

AV: aortic valve; SM: Shengmai Formula
FIGURE 5 SM reduces the area of myocardial infarction and preserves the myocardial ultrastructure

After 24 h of ischemia, myocardial tissue was stained by 2, 3, 5-Triphenyl-2H-tetrazolium chloride (TTC). Normal myocardial tissues were represented in red, whereas the white marked areas of infarct. (A) Representative images of TTC staining in different groups, including Sham, MI model, and SM groups. TTC staining was performed at 24 h after MI. Quantitation of TTC stain of myocardial tissue slices as the percentage of infarct volumes of each group (n = 6, *P < 0.05, **P < 0.01) (B) The ultrastructure of mice myocardia was observed under an electron microscopy (n = 6, Scale bars 80 um). (C) Electrocardiogram types before and after UFPM instillation in ApoE-/ mice of each group. (n = 6, The black arrow represents the abnormal S-T segment)

SM: Shengmai Formula; UFPM: ultrafine particulate matter

FIGURE 6 SM attenuates UFPM-induced OS in MI mice

ROS fluorescent probe-DHE. ROS were stained with DHE (red) and nucleus with DAPI (blue). (A and D) UFPM-induced cellular accumulation of reactive oxygen species (ROS) and rescue by SM was detected using ROS fluorescent dye (red color). ROS was induced by UFPM exposure in both the myocardial and vascular tissue of MI mice, and this was ameliorated by treatment with SM. (n = 6, *P < 0.05, **P < 0.01, Scale bars 200 um and 80 um). (B and C) ROS accumulation in myocardial and vascular tissue was quantified (n = 6, *P < 0.05, **P < 0.01).

SM: Shengmai Formula; UFPM: ultrafine particulate matter; ROS: reactive oxygen species; DAPI: diamidino-2-phenylindole; DHE: dihydroethidium
FIGURE 7 SM plays an antioxidant role in vitro via the Keap1-Nrf2 / HO-1 signal pathway

Expression of mRNA levels of Nrf2, HO-1, and NQO1 after UFPM exposure in Control and siNrf2-H9c2 cells.

(A) The fold change of Nrf2 in Control and siNrf2-H9c2 cells. (n = 6, *P < 0.05, **P < 0.01).

(B) The fold change of HO-1 in Control and siNrf2-H9c2 cells. (n = 6, *P < 0.05, **P < 0.01).

(C) The fold change of NQO1 in Control and siNrf2-H9c2 cells. (n = 6, *P < 0.05, **P < 0.01).

SM: Shengmai Formula; UFPM: ultrafine particulate matter