The Effects of $PM_{2.5}$ Exposure and Freeze-dried Strawberry Supplementation on Atherosclerosis and Inflammation in a Mice Model

DISSERTATION

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Abstract

Both epidemiological and empirical data have demonstrated the association between long-term exposure to fine particulate matters ($PM_{2.5}$) and the burden of atherosclerosis. Strawberry, a rich source of micronutrients and several bioactive phytochemicals, has been implicated in the prevention of cardiovascular diseases and metabolic syndromes. This study was designed to explore the effects of freeze-dried strawberry supplementation on $PM_{2.5}$-potentiated atherosclerosis in $apoE^{-/-}$ mice, which is a spontaneous atherosclerotic model. Mice, fed with high-fat chow or high-fat chow supplemented with 10% strawberry powder, were exposed to either filtered air or concentrated ambient particles using "Ohio Air Pollution Exposure Systems for Interrogation of Systemic Effects" for 6 months. After $PM_{2.5}$ exposure, plaque area and lipid area of aorta were evaluated using hematoxylin & eosin staining and oil-red O staining, respectively. In addition, total cholesterol, high-density lipoprotein and fasting glucose were measured using diagnostic kits. Furthermore, the levels of inflammatory cytokines in plasma were determined via ELISA kits, and the mRNA expressions of inflammatory cytokines within lung were quantitated using real-time PCR. Our data showed that long-term $PM_{2.5}$ exposure potentiated atherosclerosis and inflammation in these mice; strawberries reduced inflammation and improved lipid profile, but did not attenuate atherosclerosis after $PM_{2.5}$ exposure. These findings indicate that other

mechanistic pathways, e.g. systemic oxidative stress, autonomic nervous system imbalance, and the direct toxic effects of particles, may diminish the effects of strawberries on $PM_{2.5}$-potentiated atherosclerosis.

Dedication

This document is dedicated to my family.

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Fields of Study

Major Field: Molecular, Cellular and Developmental Biology

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Chapter 1: Introduction

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous plaque in the large arteries. Although the etiology of atherosclerosis is very complicated, numerous risk factors have been identified and can be grouped into factors with an important genetic component and those that are largely related to environment. Particulate matters (PM) air pollution poses the great risks to the development of atherosclerosis in human; experimental studies, particularly using genetic mouse model, reveal that systemic inflammation may play a central role in PM-mediated atherosclerosis. This study aimed to identify key inflammatory mediators that regulate the enhancement of atherosclerosis after PM exposure. In addition, this study was designed to evaluate the effects of strawberry, a natural anti-inflammatory fruit, on PM-potentiated atherosclerosis.

Central Hypothesis: Strawberry supplementation can attenuate the development of atherosclerotic plaque and reduce inflammation after $PM_{2.5}$ exposure in a mouse model.

To test this hypothesis, we proposed three specific aims.

Aim 1: To determine the concentration and compositions of $PM_{2.5}$ used in our study.

$PM_{2.5}$ sample was collected using Teflon filters via the whole body exposure system "Ohio Air Pollution Exposure Systems for Interrogation of Systemic Effects". The

concentration of $PM_{2.5}$ was measured using a microbalance; $PM_{2.5}$ was extracted from the filters and the extracts were then analyzed for trace elements using high-resolution inductively coupled plasma-mass spectrometry.

Aim 2: To identify inflammatory mediators regulating the enhancement of atherosclerosis after $PM_{2.5}$ exposure. $ApoE^{-/-}$ mice, fed with high-fat chow, were exposed to either filtered air or concentrated $PM_{2.5}$ for 6 months. Plaque area and lipid area were evaluated by morphological staining. Total cholesterol, high-density lipoprotein (HDL) and glucose were measured using diagnostic kits. The levels of plasma cytokines were determined via ELISA kits, and the mRNA expressions of cytokines within lungs were quantitated via real-time PCR.

Aim 3: To evaluate the effects of strawberry supplementation on $PM_{2.5}$-potentiated atherosclerosis. $ApoE^{-/-}$ mice, fed with high-fat chow with or without strawberry powder, were exposed to either filtered air or concentrated $PM_{2.5}$ for 6 months. After exposure, atherosclerotic plaque, lipid profile, and inflammatory cytokines were evaluated as described in aim 2.

Taken together, our study can provide compelling evidence on the effects of strawberry on $PM_{2.5}$-mediated atherosclerosis. In addition, the exposure data collected from this study are useful for government to compare local $PM_{2.5}$ levels and may provide a new target for monitoring air pollution.

Chapter 2: Particulate Matter Air Pollution and Atherosclerosis: Mechanistic Insights

Background

Atherosclerosis, the primary cause of heart disease and stroke, is a progressive disease characterized by the accumulation of lipids and fibrous plaque in the large arteries. Although the etiology of atherosclerosis is very complicated, numerous risk factors have been identified and can be grouped into factors with an important genetic component and those that are largely related to environment. Genome-wide association studies in human have revealed dozens of discrete genetic loci that are associated with myocardial infarction, coronary artery disease and other circulating biomarkers related to atherosclerosis [1]. The availability of novel animal models accelerates the process of identifying the causal gene at each loci and elucidating the molecular and physiological mechanisms. Mice deficient in apolipoprotein E (apoE) or the low-density lipoprotein (LDL) receptor develop advanced atherosclerotic lesions and are the models most used in genetic and physiological studies [2]. Previous studies indicate that diet appear to be the most significant environmental factor for atherosclerosis [3]. High-fat or high-cholesterol diet is mostly required for development of atherosclerotic lesion in experimental animals [4, 5]. In addition, other environmental factors associated with atherosclerosis include smoking [6], lack of exercise [7], and infectious agents [8].

Particulate matter (PM) air pollution is a mixture of microscopic solid and liquids droplets suspended in air, consisting of numerous components, e.g. acids, organic chemicals, metals, solids or dust particles, and allergen. According to its aerodynamic diameter, PM is classified into coarse (10 to 2.5 μm; PM_{10}), fine (< 2.5 μm; $PM_{2.5}$), and ultrafine (<0.1 μm; $PM_{0.1}$) particles. It has been suggested that PM fraction poses the great risks to the development of atherosclerosis in human (Table 1). Although PM_{10} and $PM_{0.1}$ may have the capacity to induce atherosclerosis, few studies have focused on these size fractions. Most data to date have provided the consistent association of increased atherosclerotic risk with exposure to $PM_{2.5}$. The present review integrates the current experimental evidence to discuss the mechanistic pathways whereby PM inhalation can promote atherosclerosis.

Development of Atherosclerotic Plaque

A normal artery consists of three distinct layers (Fig.1A). The intima, the innermost layer, is bounded by a monolayer of endothelial cells (ECs) and is supported by an internal elastic lamina. The ECs are in direct contact with the blood flow and serve as a selectively permeable barrier between blood and tissues. Massive extracellular connective tissue matrix, primarily proteoglycans and collagen, exists in the intima. The media, the middle layer, consists of smooth muscle cells (SMCs). The adventitia, the outer layer, consists of connective tissues with interspersed fibroblasts. Atherosclerotic lesion occurs in the intima and involves multiple cell types, e.g. monocytes, T cells, ECs and SMCs.

The key events of atherosclerotic plaque development consist of lesion initiation, foam-cell formation and fibrous plaque formation, which have been well clarified by animal

studies [9]. Lesion initiation is characterized by the disruption of vascular endothelial integrity and the accumulation of LDL in the extracellular matrix (Fig.1 B). The risk factors, including aging, genetic and environmental factors, may cause EC injury and change the lipid profile in circulation. For example, overexpression of human apoB in transgenic rabbits results in increased levels of LDL and decreased levels of high-density lipoprotein (HDL) [10]. After passing through EC junction, LDL tends to bind to the subendothelial extracellular matrix, a process known as LDL retention. The interactions between apoB, the ligand of LDL receptor, and matrix proteoglycans play an important role in LDL retention in the artery wall [11]. LDL in the intima undergoes the first oxidation to form minimally oxidized LDL. Injured ECs and 12/15-lipoxygenase may provide a source of reactive oxygen species (ROS) and seed the extracellular LDL through inserting molecular oxygen, respectively [12].

HDL is strongly protective against atherosclerosis, although the beneficial effects remain elusive in particular patients, e.g. the patients with established coronary artery disease [13]. Several mechanisms may claim its protective effects. First, HDL can directly bind unesterified cholesterol and remove excess cholesterol from cells and extracellular tissues [14]. Second, HDL may reverse endothelial dysfunction and improve endothelial barrier integrity. Several *in vitro* studies have shown that treatment of ECs with HDL attenuate apoptosis in response to pro-atherogenic signals such as oxidized LDL [15], tumor necrosis factor [16], and growth factor deprivation [16]. Third, HDL carries an esterase known as serum paraoxonase that can degrade certain biologically active oxidized

phospholipids [17]. The antioxidant properties of HDL inhibit LDL oxidation, thereby reducing the inflammation and attenuating the uptakes by macrophages.

As shown in Fig.1 C, LDL must be further modified before it can be taken up by macrophages to form foam cells. Minimally oxidized LDL has pro-inflammatory properties and stimulates ECs, resulting in the release of adhesion molecules and chemotactic proteins. These molecules facilitate leukocytes, e.g. monocytes and T cells, to roll on along the endothelial surface and direct them to the lesion sites [18]. LDL can be highly oxidized via ROS produced by ECs and macrophages and several enzymes such as myeloperoxidase, sphigomyelinase and a secretory phospholipase [9, 19, 20]. Macrophages recognize the modified LDL via two major scavenger receptors, SR-A [21] and CD 36 [22], and rapidly uptake these particles, leading to foam-cell formation.

Fibrous plaques are characterized by a growing necrotic core, accumulation of SMCs and SMC-derived extracellular matrix (Fig. 1D). The death of foam cells generates a growing mass of lipid debris, forming a necrotic core. The interaction between CD40 ligand and CD40 expressed on T cells and macrophages contribute to the release of pro-inflammatory cytokines and growth factors [23]. ECs also express CD40 and the binding with its ligand causes the release of adhesion molecules [24]. These mediators recruit more leukocytes from circulation into the lesion site and stimulate SMC proliferation. Elevated homocysteine levels and hypertension also contribute to the stimulation of SMC proliferation [25, 26]. Excess SMCs migrate into intima and secrete extracellular matrix, forming a fibrous cap around necrotic core.

Potential Mechanisms Involved in PM-mediated Atherosclerosis

Previous studies have now corroborated the pro-atherosclerotic effects of PM in animals such as rabbits and mice [27-29]. Using more physiological relevant whole body exposure to concentrated ambient PM, the researchers demonstrated that long-term exposure to PM can potentiate plaque development and vascular inflammation in apoE deficient mice [28, 30]. Although some important aspects remain elusive, subsequent studies have revealed the possible pathways of PM-mediated atherosclerotic lesions, which may involve multiple organs, cell types and molecular mediators (Fig.2). PM inhalation can provoke an inflammatory response and oxidative stress within lungs with mediators (e.g. cytokines, ROS and constituents of PM) "spilling over" into circulation [31]. These mediators can cause EC injury and disrupt endothelial barrier integrity, thereby enhancing the barrier permeability. An *in vitro* study demonstrated that PM increased ROS level of ECs and disrupted endothelial barrier integrity through p38 mitogen-activated protein kinase- and heat-shock protein 27-dependent pathways [32]. LDL diffuses passively through EC junctions and is oxidized by elevated ROS caused by PM or EC injury.

PM not only promotes the initiation of atherosclerotic lesion via oxidative stress, it also accelerates foam-cell formation by modulating inflammatory pathways. Pro-inflammatory cytokines released from alveolar macrophages, e.g. TNF-α, IL-6 and IFN-γ [33, 34], can induce systemic inflammation and activate adipose tissues, secreting pro-inflammatory adipokines. In addition to contribution to systemic inflammation, some adipokines, including adiponectin, resistin, omentin-1 and chemerin, directly modulate the atherogenic environment of arterial wall by regulating the function of ECs, SMCs and

macrophages [35]. PM can be recognized by intimal macrophages via toll-like receptors (TLRs) such as TLR2 and TLR4 [36, 37]. Upon recognition, cytokines and chemotactic proteins are released from macrophages possibly through the activation of NF-κB pathway [38, 39]. MCP-1 is a key molecule that recruits monocytes from circulation via binding to CCR-2 on the cell surface [40]. Recruited monocytes by oxidized LDL and PM differentiate into intimal macrophages that uptake the highly oxidize LDL through scavenger receptors, forming the foam cells.

PM inhalation is known to trigger hypertension through abnormal activation of sympathetic nervous system [41]. Epidemiological studies have shown elevated blood pressure is strongly associated with atherosclerosis [9, 42]. Studies using spontaneously hypertensive rats indicate that raised blood pressure stimulates the expression of platelet-derived growth factor that is a potent mitogen for SMCs [26]. PM-primed macrophages also secrete growth factors that may be important for SMC migration and proliferation [43]. In addition, macrophages may uptake and remove PM by apoptosis, generating cell debris. This debris, together with extracellular lipids from foam cells, facilitates the formation of necrotic core.

Conclusions

Both *in vivo* and *in vitro* studies confirm the pro-atherosclerotic properties of PM exposure and reveal the underlying mechanistic pathways. PM-mediated enhancement of atherosclerosis is likely due to its pro-oxidant and pro-inflammatory effects, involving multiple organs, different cell types, and various molecular mediators. There are many issues, however, that have yet to be addressed. The responsible components and the

combined effects of other pollutants (e.g. ozone) remain unclear. It is urgent to evaluate the toxicity of $PM_{0.1}$ in human because it may be highly toxic to the cardiovascular system. The mechanistic pathways whereby PM exposure mediates dysfunctional HDL need to be investigated in details since they may lead to the identification of a therapeutic target for atherosclerosis. Future studies on these issues may yield a better understanding of PM-mediated atherosclerosis and provide useful information for policy makers to determine acceptable levels of PM air pollution.

Table 1. Human Studies Linking PM Exposure with Atherosclerosis.

Authors	Study Design	Key Findings	Pollutants	Subjects	Year	Location	Reference
Künzli et al	Cross-sectional study	Exposure associated with an increase in CIMT; with a cross-sectional exposure contrast of 10 μg/m^3 $PM_{2.5}$, CIMT increased by 5.9%; larger significant effects seen in women ≥ 60 years old and subjects taking lipid-lowering medications	$PM_{2.5}$	798 subjects ≥ 40 years old without diabetes or CVD	1998-2003	Los Angeles basin, U.S.	[44]
Pope et al	Case-crossover study	Exposure $PM_{2.5}$ to strongly associated with AIC, with $PM_{2.5}$ level elevated by 10 μg/m^3 $PM_{2.5}$, AIC increased by 4.5%;	$PM_{2.5}$ and PM_{10}	12865 subjects	1994-2004	Utah, U.S.	[45]
Hoffmann et al	Cross-sectional study	Trends for an increase in CAC of 17.2% per 3.91 μg/m^3 of $PM_{2.5}$ after controlling for other risk factors	$PM_{2.5}$	4494 subjects 45-74 years old	2002	Three Germany cities*	[46]
Diez Roux et al	Cross-sectional study	CIMT associated with exposures to $PM_{2.5}$ and PM_{10} after controlling for other risk factors	$PM_{2.5}$ and PM_{10}	5172 subjects 44-84 years old without CVD	1982-2002	Six U.S. regions$	[47]

Continued

Table 1 continued.

Bauer et al	Population-based prospective cohort study	$PM_{2.5}$ exposure associated with CIMT; higher effects seen in subjects working full time within the past five years	$PM_{2.5}$ and PM_{10}	3380 subjects 45-75 years old	2000	Three Germany cities*	[48]
Kälsch et al	Cross-sectional study	$PM_{2.5}$ exposure associated with an increase in TAC; with a cross-sectional exposure contrast of 2.4 μg/m^3 $PM_{2.5}$, TAC increased by 18.1%	$PM_{2.5}$ and PM_{10}	4814 subjects 45-75 years old	2000-2003	Three Germany cities*	[49]

CIMT: carotid intima-media thickness; PM: particulate matters; CVD: cardiovascular disease; AIC: acute ischemia coronary; CAC: coronary artery calcium; TAC: thoracic aortic calcification. * Essen, Mülheim, and Bochum. $ Baltimore, Maryland; Chicago, Illinois; Forsyth County, North Carolina; Los Angeles, California; New York, New York; and St. Paul, Minnesota.

Figure 1. Development of Atherosclerotic Plaque. (A) Normal structure of artery. (B) Initiation of atherosclerosis. (C) Foam-cell formation. (D) Fibrous plaque formation. LDL: low-density lipoprotein; HDL: high-density lipoprotein; EC: endothelial cell; ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; PCAM: platelet cell adhesion molecule; VLA: very late antigen; ROS: reactive oxygen species; NOS: nitric oxide synthases; CD: cell differentiation; IFN: interferon; TNF: tumor necrosis factor; SMC: smooth muscle cell.

Figure 1

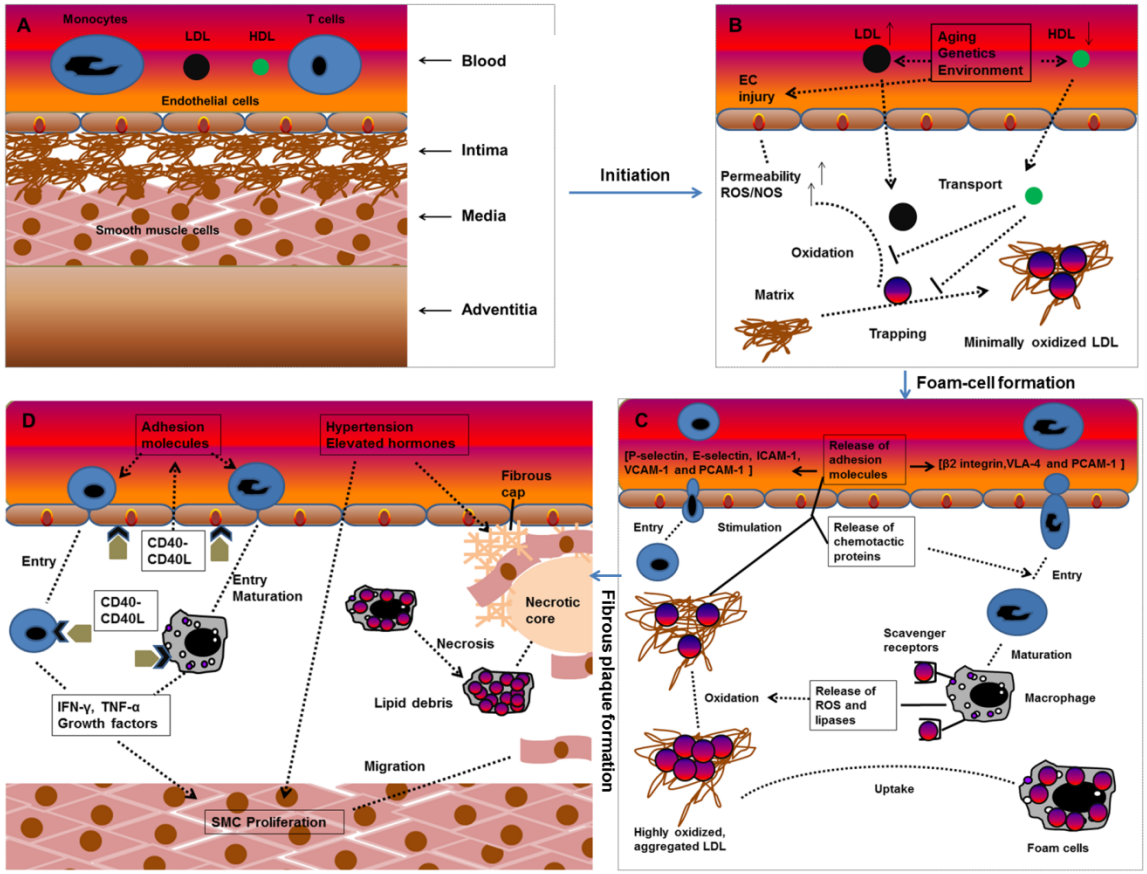
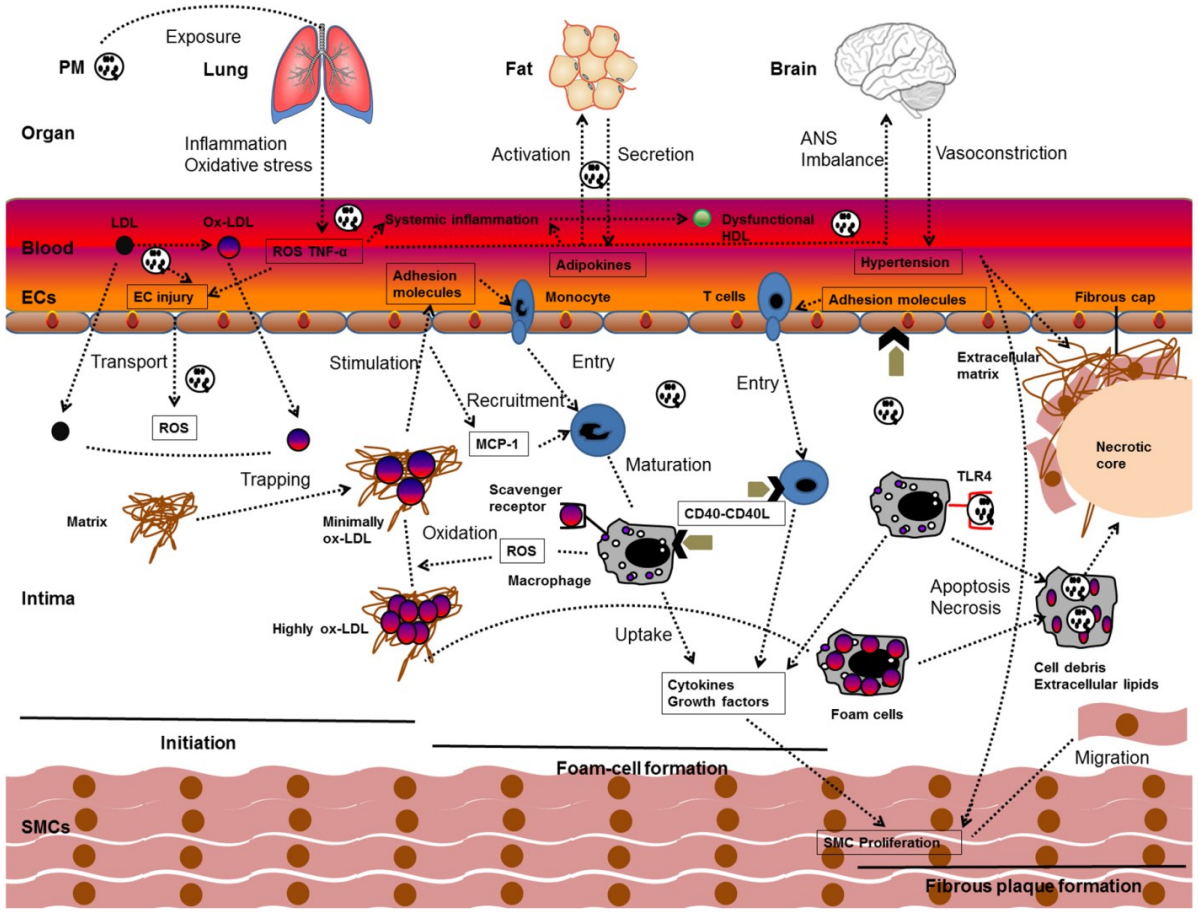

A
Monocytes
T cells
LDL
HDL
Endothelial cells
Blood
Intima
Media
Smooth muscle cells
Adventitia
Initiation
B
LDL
Aging
Genetics
Environment
HDL
EC injury
Permeability ROS/NOS
Transport
Oxidation
Matrix
Trapping
Minimally oxidized LDL
Foam-cell formation
C
[P-selectin, E-selectin, ICAM-1, VCAM-1 and PCAM-1]
Release of adhesion molecules
[β2 integrin,VLA-4 and PCAM-1]
Entry
Stimulation
Release of chemotactic proteins
Entry
Scavenger receptors
Maturation
Oxidation
Release of ROS and lipases
Macrophage
Uptake
Highly oxidized, aggregated LDL
Foam cells
Fibrous plaque formation
D
Adhesion molecules
Hypertension
Elevated hormones
Fibrous cap
Entry
CD40-CD40L
Entry
Maturation
CD40-CD40L
Necrotic core
Necrosis
Lipid debris
IFN-γ, TNF-α
Growth factors
Migration
SMC Proliferation

Figure 2 Mechanistic Pathways of PM-mediated Atherosclerosis. PM: particulate matters; LDL: low-density lipoprotein; HDL: high-density lipoprotein; ANS: autonomic nervous system; ROS: reactive oxygen species; TNF: tumor necrosis factor; EC: endothelial cell; MCP: monocyte chemotactic protein; TLR: toll-like receptor; SMC: smooth muscle cell

Figure 2


Exposure
PM
Lung
Organ
Inflammation
Oxidative stress
Fat
Activation
Secretion
Brain
ANS
Imbalance
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LDL
Ox-LDL
ROS TNF-α
Systemic inflammation
Dysfunctional HDL
Adipokines
Hypertension
EC injury
Adhesion molecules
Monocyte
T cells
Adhesion molecules
Fibrous cap
Transport
ROS
Stimulation
Recruitment
MCP-1
Entry
Entry
Extracellular matrix
Necrotic core
Trapping
Matrix
Minimally ox-LDL
Maturation
Scavenger receptor
CD40-CD40L
TLR4
Oxidation
ROS
Macrophage
Intima
Highly ox-LDL
Uptake
Apoptosis
Necrosis
Cytokines
Growth factors
Foam cells
Cell debris
Extracellular lipids
Initiation
Foam-cell formation
Migration
SMCs
SMC Proliferation
Fibrous plaque formation

Chapter 3: Chronic Inflammation in Adipose Tissues

Background

Obesity is characterized as a chronic state of low-grade inflammation with progressive immune cell infiltration into adipose tissue (AT). Obesity and ectopic lipid deposition are major risk factors for diseases ranging from insulin resistance (IR) to type 2 diabetes mellitus (T2DM) and atherosclerosis. The incidences of these diseases are increased with obesity and are thought to arise from the chronic inflammation [44]. It is now accepted that AT is the primary source of many pro-inflammatory cytokines. The novel discovery that pro-inflammatory macrophages are recruited to obese AT prompted an increased interest in the interplay between immune cells and metabolism [45, 46].

The findings from animal studies and *in vitro* experiments have suggested that adipose tissue macrophages (ATMs) play critical roles in the establishment of the chronic inflammatory state and metabolic dysfunctions such as T2DM and IR [47, 48]. Either genetic or diet-induced adipocyte expansion promotes the accumulation of macrophages in AT in the mice and the majority of humans [45, 46, 49]. Upon activation, immature bone-marrow derived peripheral monocytes migrate into the site of inflammation and differentiate into tissue macrophages [50]. Macrophage numbers and/or pro-

inflammatory gene expression in AT are positively associated with adipocyte size in obese mice and are negatively associated with weight loss in obese humans [45, 51].

Although recruitment of macrophages into AT involves interactions of innate and adaptive immunity in multiple organs, at its core lays a unique crosstalk between adipocytes and macrophages. In the chapter, we discuss the obesity-mediated adipose tissue remodeling, and particularly, the role of adipokines/chemokines in macrophage recruitment to obese adipose tissue.

Adipose Tissue and Adipose Tissue Macrophages

In addition to the storage of energy in the form of lipids, AT has been recognized as the largest endocrine organ secreting several hormones (leptin and resistin) [52], adiponectin [53], growth factors (vascular endothelial growth factor) [54], pro- and anti-inflammatory mediators (α4 integrin, interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α) [46, 55], and complement proteins [56, 57]. These factors that are released by AT are collectively referred to as adipokines [58]. AT is mainly composed of adipocytes and a heterogenous constellation of endothelial cells, adipocyte precursors, nerve terminals, fibroblasts, blood vessels, and leukocytes. Each of these cells and structural components contribute to the synthesis and turnover of extracellular matrix components that collectively create unique microenvironments within AT [59].

Macrophages and their monocyte precursors are highly heterogeneous cell populations. Upon the cytokine polarization, macrophages are divided into classically activated macrophages (M1) and alternatively activated macrophages (M2). M1 can be induced by interferon-γ alone or in concert with microbial stimuli or cytokines, while M2 can be

induced by IL-4, IL-10, IL13, and IL-33 [60]; in general, M1 are characterized by an IL-12^{high}, IL-23^{high}, and IL-10^{low} phenotype, in contrast, the various forms of M2 generally express an IL-10^{high}, IL-12^{low}, and IL-23^{low} phenotype [60]. In 2007, Lumeng *et al*. extended this M1/M2 macrophage paradigm into ATMs [61]. Their data indicated that ATM phenotype changed from an anti-inflammatory M2 polarized state to a pro-inflammatory M1 state when obesity occurred, which contributed to the chronic low-grade inflammation of obesity.

Obesity-mediated Adipose Tissue Remodeling

With excessive weight gain, extreme increases in adipocyte size are accompanied by an elevated frequency of adipocyte death and macrophage recruitment [62, 63]. Obesity-associated AT remodeling has been first described by Cinti in 2005 as the existence of significant numbers of so-called "crown-like structures (CLS)", consisting of macrophages surrounding dead adipocytes in both obese mice and humans [62] (Fig.3). The high prevalence of CLS is highly correlated to AT inflammation and metabolic disorder and considered to be pathological lesions in AT of obese subjects [64].

During AT remodeling, adipocyte death may be sufficient to initiate macrophage infiltration and induce AT inflammation [59]. This hypothesis has been further substantiated by the study demonstrating that the preponderance of ATMs in lean and obese mice and humans is selectively localized to individual dead adipocytes and that the frequency of adipocyte death is increased over 30-fold in an obese mouse model as well as in obese humans [62]. A nearly complete remodeling of the epididymal fat depot has

been observed in a murine diet-induced obesity model, characterized as frequency of adipocyte death, an increase of depot weight and ATM accumulation [65].

Crosstalk between Adipocytes and Macrophages

The interaction between adipocytes and macrophages aggravates the chronic inflammation in obese AT [66]. Pro-inflammatory adipokines such as monocyte chemotactic protein [67]-1 and TNF-α, and saturated FAs released by adipocytes interact with TLR4 complex, inducing nuclear factor-κB activation in resident macrophages [68, 69]. Conversely, activated macrophages also release pro-inflammatory chemokines including MCP-1, recruiting the monocytes from circulation into the site of AT inflammation [70]. Once infiltrated into the AT, monocytes become matured and interact with adipocytes in a paracrine manner through TNF-α production, increasing the production of pro-inflammatory adipokines and reducing the production of anti-inflammatory adiponectin [68]. This crosstalk between adipocytes and macrophages establishes and maintains the chronic inflammation state in obese AT through persistently recruiting new macrophages/monocytes from circulation (Fig.4).

Adipokines and chemokines are key mediator linking the adipocytes and ATMs and regulating AT inflammation. As described above, AT has been recognized as the largest endocrine organ secreting a variety of adipokines. Following the onset of obesity, the secretory status of adipocytes can be modified by the changes in the cellular composition of the tissue, including alterations in the number, phenotype and localization of immune, vascular and structural cells. Recent evidence suggests that obesity-induced changes in adipokine secretion can influence the AT function through recruiting the immune cells

and promoting inflammatory responses [44]. The current section discusses the key adipokines and chemokines that have roles in ATM accumulation.

TNF-α

TNF-α is a pro-inflammatory cytokine that has been noted as one of active participants in the development of obesity-related diseases [71]. TNF-α may contribute to adipokine dysregulation in adipocytes and an increased level of TNF-α was found in ATs of obese murine models [72]. In humans, TNF-α expression correlated with body mass index, percentage of body fat, and hyperinsulinemia, whereas weight loss decreased TNF-α level [73]. TNF-α levels were also found to positively associated with other markers of IR [74]; while short-term treatment with TNF-α inhibitor in obese patients reduced systemic inflammatory markers without improving insulin sensitivity [75].

TNF-α converting enzyme (TACE) is the major factor that induces soluble TNF-α and has been implicated as a central regulator in obesity and AT inflammation [76]. Previous studies have shown that TACE activity is significantly higher in diet-induced obese mice compared with control group [77]; and that TACE knock-out mice are protected against obesity-induced IR and diabetes [78]. In addition, a recent study showed that serum macrophage-related chemokine levels and the number of CLS were significantly elevated in ATs of TACE-transgenic mice fed with HFD [79]. These findings collectively suggested that TACE could be a possible therapeutic target of obesity-related diseases.

Leptin

Leptin is one of the most important AT-derived hormones, involving in both innate and adaptive immunity [80]. Obesity is associated with an increased level of leptin prevailing in the expanding AT [81], suggesting the potential role of leptin in obesity-mediated inflammation. Leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice have been well generated and shown less macrophage infiltration and inflammatory gene expression in AT, in spite of increased weight gain and adiposity [46]. Furthermore, Dib *et al.* report that C57BLJ mice reconstituted with db/db bone marrow, when placed on a high-fat diet, have significant lower body weight and adiposity, attenuated macrophage infiltration, and subsequently diminished AT inflammation [82].

Leptin may affect macrophage infiltration to AT through the upregulation of adhesion molecules in the endothelial cells of the stromal vascular compartment [83]. However, despite the convincing nature of these findings from the different groups, there are equally convincing data showing that leptin does not influence weight gain and macrophage infiltration in AT [84, 85]. The contrasting results generated from these studies may be caused by different background strain, potential effects of gut microbiota, different baseline of body weight, and different percent fat in the diet [86]. Taken together, these key elements must be considered to further evaluate the role of leptin in the macrophage recruitment in the future studies.

Adiponectin

Adiponectin is another novel AT-derived hormone that modulates a number of metabolic processes, such as glucose regulation and fatty acid oxidation [87]. Adiponectin is almost

exclusively synthesized by adipocytes and is present at high levels (3-30 μg/ml) in the blood relative to other adipokines [88]. Previous studies have shown that adiponectin may suppress lipid accumulation and have anti-inflammatory effects on macrophages [89, 90]; and that adiponetin promotes macrophage polarization to an anti-inflammatory phenotype [91]. Ohashi K *et al.* report that ATMs isolated from adiponectin knock-out mice displayed increased M1 markers and decreased M2 markers; while *in vitro*, the treatment of macrophages with recombinant adiponectin led to an increase in the levels of M2 markers [91]. Thus, adiponectin-mediated modulation of macrophage function and phenotype may contribute to its role in reducing inflammation within AT.

Fatty Acids

AT regularly releases FAs through the process known as lipolysis, in which involves the breakdown of lipids and hydrolysis of triglycerides into glycerol and free FAs. Macrophages express a variety of free FA receptors including TLRs, CD36, and GPCRs [92-94]. TLR4 binds to free FAs such as palmitate and stearate indirectly via an endogenous ligand like Fetuin-A [95], causing the activation of nuclear factor-κB and subsequent formation of pro-inflammatory cytokines and prostaglandins via increased expression of cyclooxygenase-2 [69, 96]. CD36, the class B scavenger receptor, facilitates uptake of long chain FAs and contributes to inflammatory responses by promoting macrophage infiltration into AT and expression of pro-inflammatory cytokines [97]. CD36 knockout mice show improved insulin sensitivity and reduced inflammation within AT [98]. GPCR120 selectively binds omega-3 FAs and leads to blockage of nuclear factor-κB resulting in reduced inflammatory responses [94].

In vitro, saturated FAs such as palmitate induce extracellular release of histone H3 in part through reactive oxygen species and c-Jun N-terminal kinases signaling. Extracellular H3 activates endothelial cells to express adhesion molecules such as ICAM-1 and VCAM-1 that recognize and interact with integrins, thereby facilitaing the firm adhesion of leukocytes to endothelial cells and contributing to leukocyte trafficking [99].

MCP-1

To date, MCP-1-CCR2 system is the most well studied chemokine-chemokine receptor system in ATM recruitment. Human study indicates that although obesity is associated with the increased expression of several chemokine genes in AT, only MCP-1 is secreted into the extracellular space, where it influences the function of adipocytes and acts as recruitment factor of macrophages [100]. MCP1 directs recruitment of pro-inflammatory macrophages to sites of inflammation [101]. In addition to blood monocyte recruitment, local proliferation of macrophages (only in visceral adipose tissue) driven by MCP-1 contributes to obesity-associated adipose tissue inflammation [102]. CCR2 plays a role in the maintenance of ATMs and IR once obesity is established [103]. Both in vivo and in vitro evidence supports that CCR2 in BM cells plays a role in the recruitment of macrophages into obese AT [104]. CCR2 antagonist (CCX417) can reduce inflammation in AT in models of T2DM [105].

However, controversy exists regarding the exact role of this system. In contrast to these findings, Inouye et al. report that MCP-1 may not be crucial for AT macrophage recruitment [106]. In that study, they used male 6-week-old MCP-1 deficient mice to evaluate the macrophage infiltration after 28 weeks of HFD feeding. Surprisingly, MCP-

1 deficient mice on HFD showed alterations of metabolic function, but no reductions in ATMs compared with wild-type mice, indicating that MCP-1 has effects on metabolism that are independent of its macrophage-recruiting capabilities [106]. Their results were confirmed by another group in the following year [107]. Taken together, all reported studies show that over-expression of MCP-1 in AT elevates macrophage recruitment [70, 108], and that CCR2 deficiency or inhibition reduces macrophage recruitment [103-105, 109, 110]. However, whether deficiency of MCP-1 can reduce macrophage recruitment and improve insulin sensitivity remains conflicting [106, 107, 111]. The complexity and redundancy of chemokine signaling may account for these conflicting results. In fact, CCR2 is a functional receptor shared by several other chemokines including MCP-2, MCP-3, CCL7 and CCL8, which are all expressed in obese AT and may affect macrophage recruitment [112, 113].

Conclusion

Significant progress has been conducted to identify the molecules that regulate ATM recruitment and the roles of these molecules in obesity-related diseases. Adipokines have both pro-inflammatory and anti-inflammatory effects and the balance between these effects in normal state of nutrition determines the local and systemic inflammatory status. Imbalanced adipokine production during over-nutrition contributes to AT inflammation and obesity-related diseases such as IR and atherosclerosis. Chemokines have roles in recruitment of monocytes from circulation and *in situ* proliferation of ATMs. There are many questions, however, that have yet to be addressed particularly due to the complexity and redundancy of chemokine signaling. Thus, further investigation of the

functions and mechanisms of key adipokines/chemokines will lead to a better understanding of the pathogenesis of obesity and related disorders. Moreover, therapeutic strategies that counteract the dysregulation of adipokine production could be a potential and promising means for treating obesity-related diseases, e.g. IR and atherosclerosis.

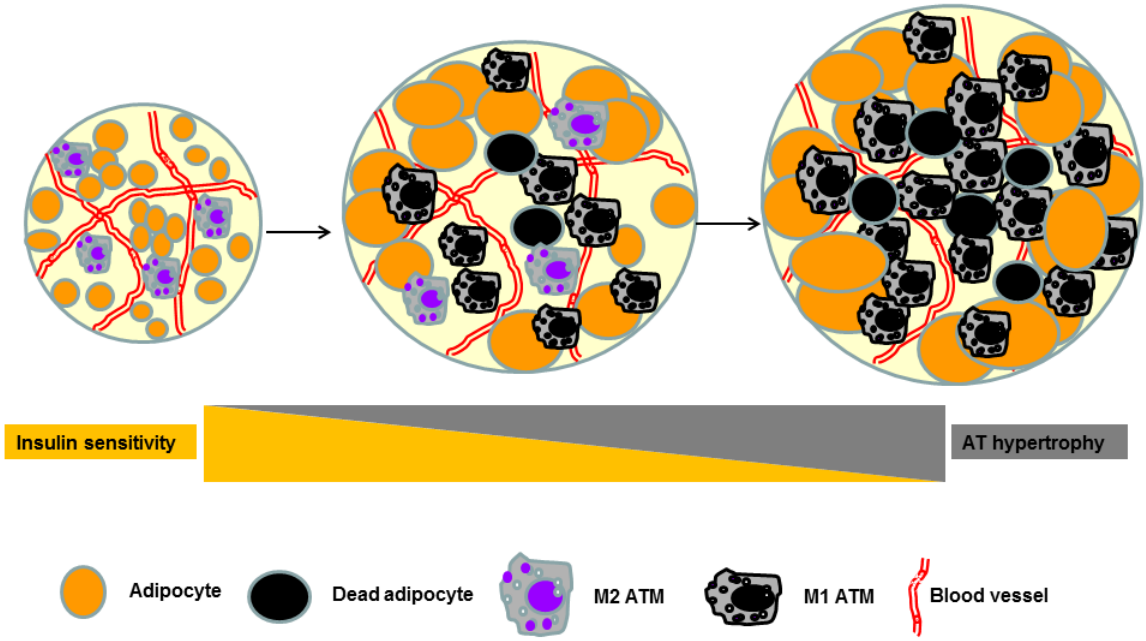

Figure 3. Obesity-mediated Adipose Tissue Remodeling. AT: adipose tissue. ATM: adipose tissue macrophages.

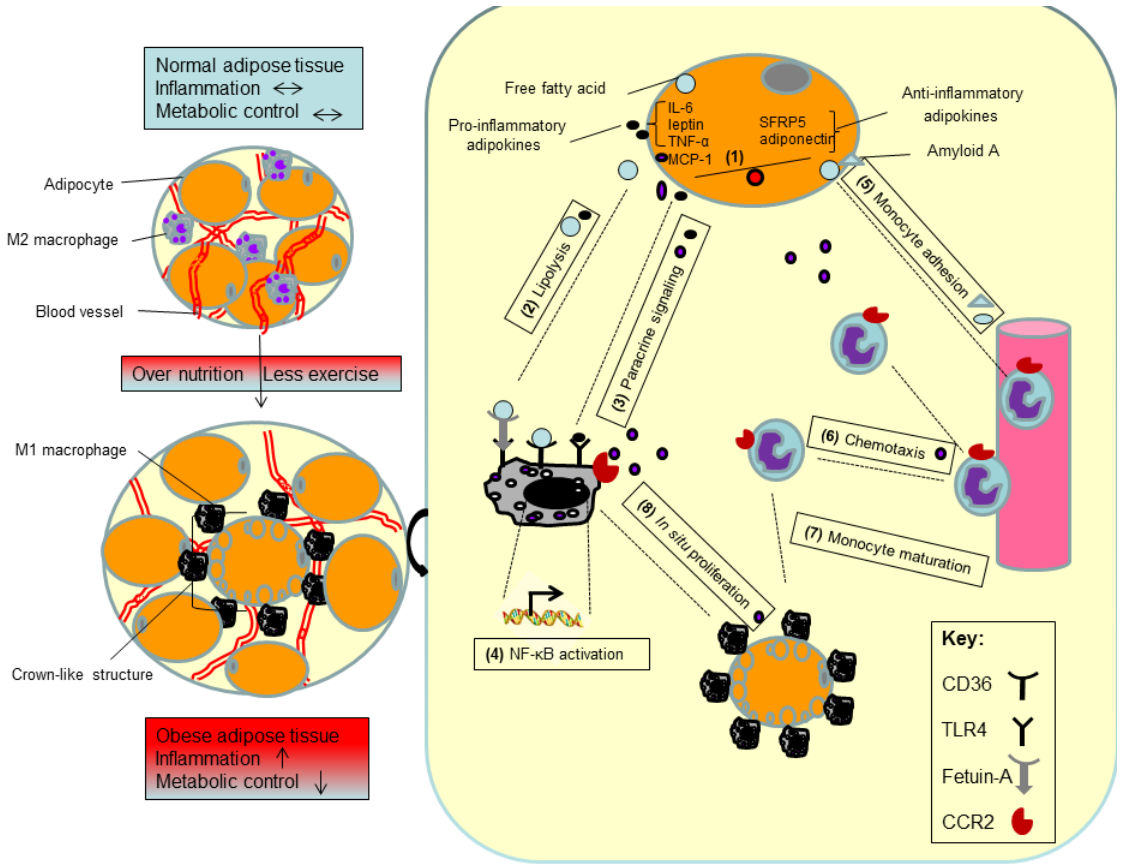

Figure 4. Chronic Inflammation in Obese Adipose Tissue. TLR: toll-like receptor; CD36: cluster of differentiation 36; MCP-1: monocyte chemotactic protein-1; CCR2: C-C chemokine receptor type 2; NF-κB: nuclear factor-κB.

Chapter 4: Potential Impacts of Strawberries on Cardiovascular Diseases

Background

Strawberries are a rich source of polyphenols, ellgtigannins, vitamin C, folic acids, postassium, and fiber. Approximately 40 phenolic compounds, including vitamin C, ellagitannins and anthocyanins, have been identified in strawberries. The antioxidant activity of these compounds has been well documented and accounts for the benefits of strawberries. Flavonoids are potent antioxidants as they are effective scavengers of hydroxyl and peroxyl radicals. *In vitro* studies indicate that many flavonoids are more evident antioxidants than vitamin C and E [114]. Phenolic acids are also promising antioxidants and work synergistically with other antioxidants. The most prevalent phenolic acids in strawberries include ellagic acid, tannic acid, and capsaicin [115]. The cellular antioxidant activity assay revealed that apples and strawberries are the largest contributors to dietary antioxidant activity [116]

Strawberries are now considered a functional food with multiple health benefits, e.g. lowering lipids, anti-oxidation, and anti-inflammatory responses. It has been shown that fresh strawberries can attenuate postprandial hyperglycemia in health subjects when compared to those receiving a matched glucose [117]. A similar study conducted among

hyperlipidemic adults showed that freeze-dried strawberry supplementation can significantly attenuate postprandial lipemia and lipid oxidation in response to a high-fat diet challenge [118]. The prevailing evidence indicates that strawberries may have promising implications in the management of metabolic syndrome and cardiovascular disease (CVD). Thus, the main objective of this chapter is to discuss the nutrients of strawberry related to human health and summarize the key findings from both epidemiological and experimental studies.

Strawberry Nutrients and Phenolic Compounds

Strawberries are a rich source of several nutrients, phytochemicals and fiber (Table 2); these elements, particularly phytochemicals, contribute to their biological effects. Strawberries contain significant amounts of vitamins, potassium, folic acid, carotenoids, and specific flavonoids, e.g. pelargonidin, quercetin, and catechin. Strawberries also have significant amounts of ellagic acid, tannins, and phytosterols [119]. Phenolic compounds are the best-studied phytochemicals in strawberries and are very bioactive in animals and humans who consume them. These compounds are composed of one or more aromatic rings between one or more hydroxyl groups. There are hundreds of different phenolic compounds in plant, but flavonoids and phenolic acids are the most common phenolic compounds found in strawberries. This section discusses these compounds and their bioactivities.

Flavonoids

The main flavonoids found in strawberries are anthocyanin, catechin, quercetin, and kaempferol. These compounds share similar chemical structure and are present in plants as aglycones or flavonoid glycosides. Anthocyanin is a large subclass of flavonoid plant pigments and its structures depend on environmental pH. In strawberries, anthocyanins are the most abundant flavonoids. Catechin is a monomeric flavanol compound present in strawberries as part of a complex mixture of phenolic substances. Quercetin and kaempferol belong to the subclass of flavonoids known as flavonols, which represent about 11% of the phenolic compounds in strawberries [120]. It has been suggested that all of these flavonoids are potent antioxidants and have protective effects on CVDs and cancer [114].

Both *in vivo* and *in vitro* studies have shown that cyanidin, an isoform of anthocyanins, acts as a potent scavenger against hydroxyl radicals and superoxide [121, 122]. In a mouse model of skin cancer, high dose of anthocyanin extracts can reduce the promotion of tumors via inhibition of protein kinase C [123]. Catechin has also been claimed as a potential anticancer compound. In a spontaneous tumor model, HTLV-1 *tax* transgenic mice, dietary catechin (4 mmol/kg) can postpone the onset of tumor development by 45% [124]. Quercetin and kaempferol have been shown to block the activation of certain carcinogens via inhibition of DNA adducts formation [125]. In rats treated with several carcinogens, quercetin at 1% of the diet can significantly inhibit adenomas and carcinomas in small intestines instead of other organs [126].

Ellagic Acid

Ellagic acid is widely distributed phenolic acids in food, e.g. berries, grapes and walnuts. It presents both in the free form and esterified to glucose in water soluble hydrolysable ellagitannins. It has been reported that ellagic acid comprises 51% of the phenolic acids in strawberries [127]. In comparison to other fruits such as raspberries, pinapples, and pomegranates, strawberries appear to contain higher ellagic acid.

The antioxidant properties of ellagic acid have been reported in numerous *in vitro* and animal studies [128, 129]. Its four hydroxyl and two lactone functional groups act as hydrogen bond acceptors and donors, respectively. This enables ellagic acid to work as a potent scavenger against a variety of ROS [130]. In addition, ellagic acid exerts indirect effects against oxidative stress via upregulation of Nrf2, which is a redox-sensitive transcription factor regulating the antioxidant response in the cell [130]. Furthermore, ellagic acid can increase glutathione (GSH) levels through upregulation of GSH synthetase [131]. These biological effects of ellagic acid collectively attribute to its antioxidant properties.

Previous studies have also claimed that ellagic acid has anti-proliferative properties and may prevent cancer in animals and humans. The treatment of ellagic acid can significantly reduce the incidence of lung tumors in newborn mice receiving carcinogen injection [132]. In rats, ellagic acid supplementation inhibits the development of esophageal cancer induced by N-nitrosomethylbenzylamine by 25-50% [133]. An *in vitro* study using human breast cancer cell line MCF-7 showed that 30 μM of ellagic acid inhibited carcinogenesis by 45% [134]. Anticancer effects of ellagic acid may be due to

multiple mechanisms. First, ellagic acid can inhibit the activation of microsomal cytochrome P450 enzymes, which are primary enzymes known to activate certain carcinogens, e.g. nitrosamine, aniline and benzene [135]. Second, ellagic acid may induce hepatic phase II detoxifying enzymes including glutathione S-transferase, NADPH, and UDP glucuronosyl-transferse [114]. At last, ellagic acid may inhibit cancer initiation by removing oxygen free radicals and the reactive metabolites of carcinogens [114].

Strawberries and CVDs

As previously discussed, oxidation of LDL is the key step in the development of atherosclerosis. The antioxidant properties of phenolic compounds have been elucidated as primary reasons for the health benefit of strawberries. These compounds may reduce the oxidation of LDL and thus prevent the progression of atherosclerosis.

Epidemiological Studies

Previous epidemiological studies have shown that consumption of fruits and vegetables rich in flavonoids is associated with lower risk for CVDs [136, 137]. The data collected from the INTER-HEART study, investigating three dietary patterns from 52 countries, indicate that the consumption of fruits and vegetables is inversely associated with the risk for myocardial infarction [138]. The researchers concluded that an unhealthy dietary intake increased the risk of myocardial infarction globally and accounted for approximate 30% of the population-attributable risk.

The findings of a large population study reveal that flavonoid intake, e.g. anthocyanins, may contribute to the prevention of hypertension [139]. In this study, 46,672 women

from the Nurses' Health Study I, 87,242 women from the NHS II, and 23,043 men from the Health Professionals Follow-Up Study were followed up for 14 years to monitor the hypertension and flavonoid intakes. The pooled multivariate-adjusted analyses found that subjects with the highest anthocyanin intake had an 8% reduction in risk for hypertension compared to those with the lowest intake [139]. Another prospective study including 34,489 postmenopausal women in the Iowa Women's Health Study showed that significant inverse associations were observed between anthocyanin intake and CVD, coronary heart disease (CHD) and total mortality [RR (95% CI)] after multivariate adjustment [140]. These observational data suggest that consumption of fruits rich in flavonoids may contribute to the reduction of the risk for CVD.

Clinical Studies

Human intervention studies, using fresh, frozen, or freeze-dried strawberries, have shown the therapeutic potentials of strawberries in counteracting oxidative challenges caused by high-fat diet, hyperlipidemia or metabolic syndromes. In 27 subjects with metabolic syndrome, freeze-dried strawberries (4 cups a day for 8 weeks) can significantly reduce the levels of total cholesterol, LDL, and circulating adhesion molecules, e.g. VCAM-1 [141]. A similar study, conducted by the same group, showed that even short-term strawberry supplementation (2 cups a day for 4 weeks) decreased the levels of total cholesterol and LDL by 5% and 6%, respectively ($p < 0.05$) [142]. The researchers found that plasma ellagic acid was elevated after four-week strawberries treatment [142], indicating that this compound may claim the effects on lowering the risk factors for CVDs.

Postprandial hyperglycemia and hyperlipidemia can cause endothelial dysfunction and contribute to the development of atherosclerosis [143]. The effects of strawberries on postprandial hyperglycemia, hyperlipidemia, oxidative stress and inflammatory responses have been studied in clinical trials with a high-fat or high-glucose challenge. In obese subjects (BMI: 29.2 ± 2.3 kg/m^2), six-week strawberry intervention significantly lowered postprandial platelet activation factor (PAF)-1 and IL-1β [144]. In healthy subjects or the patients with hyperlipidemia, strawberries were suggested to increase postprandial plasma antioxidant ability [145], reduce the postprandial glucose response [146], and attenuate postprandial hyperlipidemia or lipid oxidation [118], after a high-fat diet challenge. The postprandial benefits of strawberries on lipid profile, oxidation and inflammation suggest their protective potentials in CVDs.

Experimental Studies

Mechanistic studies in animal and cell culture models provide compelling evidence on the roles of strawberry fruits, extracts, or purified anthocyanins in the ameliorating inflammation, oxidative stress, hypertension, and hyperlipidemia, which are the risk factors for CVDs. It has been shown that quercetin and kaempferol reduce the release of mast cells from guinea pigs and rats, respectively [147, 148]. Emerging evidence has suggested the contribution of mast cells to CVDs, in particular, by the effects on atherosclerotic plaque progression and destabilization [149]. In mouse primary peritoneal macrophages treated with lipopolysaccharide (LPS), strawberry treatment for 48 hours significantly reduced pro-inflammatory cytokines, e.g. IL-1β and IL-6, while markedly increased the levels of IL-10 [150]. The anti-inflammatory properties of strawberries are

also found in preclinical mouse model, showing the decreased levels of TNF-α, IL-1β and IL-6 after strawberry intervention [151]. Since the roles of inflammation in atherosclerosis have been well documented, these anti-inflammatory effects of strawberries may help reduce the risks for CVDs.

Antioxidants are crucial in the prevention of CVDs since they can inhibit LDL oxidation. Antioxidants may promote plaque stability, improve vascular endothelial functions and reduce tendency of thrombosis. An *in vitro* study found that most of phenolic compounds, e.g. catechin, quercetin, cyaniding and caffeic acid, have antioxidant effects [152], although no synergistic effects were seen among these compounds. Strawberry extracts such as gallic acid equivalents and acetone were shown to inhibit oxidation of human LDL and hydroperoxide formation in a dose-dependent manner [153]. Ellagic aicd treatment was also shown to inhibit oxidized LDL-induced proliferation of rat aortic smooth muscle cells [154]. These antioxidants from strawberries prevent CVDs through inhibition of LDL oxidation, the key step for initiation and development of atherosclerosis.

A recent study using *in vitro* models reported that phytochemicals from strawberries inhibited the activities of α-glucosidase, α-amylase and angiotensin I converting enzyme (ACE) [155]. The inhibitory effects on these enzymes are relevant for the management of hyperglycemia and hypertension. Further details were released from a cultivar-specific study: among all strawberry cultivars, Ovation exhibited the greatest α-glucosidase inhibitory activity; Honeoye, Idea, and Jewel exhibited moderate α-amylase inhibition; Jewel and Ovation showed moderate ACE inhibition [156]. In addition, it has been

reported that these polyphenols from strawberries inhibit the protease and lipase activities, suggesting a novel therapeutic potential of this fruit as macronutrient enzyme inhibitors in obesity, dyslipidemia and hyperglycemia [157, 158].

Conclusion

As one of the most popular fruits in the United States, strawberries rich in phytochemicals (e.g. flavonoids and ellagic acid), micronutrients (e.g. vitamins and carotenoids) and fibers are termed as a functional food. Studies on the individual compounds have demonstrated anticancer, antioxidant, and anti-inflammatory activities. Because of these properties of the phenolic compounds, consumption of strawberries may help reduce the risk for cancer and heart diseases. Indeed, inhibitory effects of strawberries on LDL oxidation and inflammation have been well understood on a basis of animal and *in vitro* studies. These findings need further investigation in larger clinical trials. Future studies are needed in defining the optimal dose, form and duration of strawberry intervention in the management of CVDs. Such studies will provide evidence in the mechanistic pathways of strawberry-mediated protective effects on heart disease.

Table 2. Nutritional Content of Strawberries.

Value/100 g	**Strawberries, raw**	**Strawberries, frozen, unsweetened**	**Strawberries, frozen, sweetened, whole**	**Strawberries, frozen, sweetened, sliced**
Water (g)	90.95	89.97	78.05	73.18
Energy (kcal)	32	35	78	96
Protein (g)	0.67	0.43	0.52	0.53
Carbohydrate (g)	7.68	9.13	21	25.92
Fiber (g)	2.0	2.1	1.9	1.9
Calcium (mg)	16	16	11	11
Iron (mg)	0.41	0.75	0.47	0.59
Magnesium (mg)	13	11	6	7
Phosphorus (mg)	24	13	12	13

Continued

Table 2 continued.

Potassium (mg)	153	148	98	98
Sodium (mg)	1	2	1	3
Zinc (mg)	0.14	0.13	0.05	0.06
Vitamin C (mg)	58.8	41.2	39.5	41.4
Thiamin (mg)	0.024	0.022	0.015	0.016
Riboflavin (mg)	0.022	0.037	0.077	0.051
Niacin (mg)	0.386	0.462	0.293	0.401
Vitamin B-6 (μg)	0.047	0.028	0.028	0.03
Vitamin A, IU	12	45	27	24
Fatty acids, total saturated (g)	0.015	0.006	0.007	0.007
Fatty acids, total polyunsaturated (g)	0.155	0.054	0.068	0.064

Source: National Nutrient Database for Standard Reference Release 27, United States Department of Agriculture.

Chapter 5: The Characteristics of $PM_{2.5}$ Air Pollution Collected via Ohio Air Pollution Exposure System for Interrogation of Systemic Effects

Abstract

Previous evidence has provided a link between fine particulate matter ($PM_{2.5}$) air pollution and human health. To explore the local level of $PM_{2.5}$ and its characteristics, $PM_{2.5}$ was collected using "Ohio Air Pollution Exposure Systems for Interrogation of Systemic Effects" at Columbus, Ohio. Mass concentration and major elements were determined by gravimetric analysis and high-resolution inductively coupled plasma-mass spectrometry, respectively. In addition, black carbon was collected using Aethalometer; correlation between the two pollutants was calculated. Our data showed that $PM_{2.5}$ level in our sampling site was below Environmental Protection Agency standards, and peaked on Jul-Aug and Nov-Dec; chemical composition of $PM_{2.5}$ was dominated by sulfates. Our data also revealed that black carbon and $PM_{2.5}$ were modest related, indicating that black carbon could serve as another marker for air pollution.

Background

Fine particulate matter ($PM_{2.5}$) air pollution has undoubtedly raised a global concern to the governments and public. Although epidemiological studies have provided compelling evidence linking $PM_{2.5}$ and human health effects [159-161], *in vivo* animal studies are indispensable to elucidate the underlying molecular and cellular mechanisms. One of

major challenges in animal studies is how to deliver $PM_{2.5}$ to experimental subjects. Intranasal instillation is a feasible method and has been used in recent particle exposure studies [162-164]. However, this method has significant flaws. First, it requires anesthesia to successfully deliver $PM_{2.5}$, which may change the animal physiology and affect the outcomes. Second, most of air pollution exposure in "real world" is chronic process; the concentration and composition of $PM_{2.5}$ are usually dynamic during the exposure. Intranasal instillation may not mimic this process. Finally, this method requests well-trained lab technicians to perform consistently, which may not be suitable for large number of animal exposure.

Our $PM_{2.5}$ sampling and exposure system, the "Ohio Air Pollution Exposure System for Interrogation of Systemic Effects (OASIS)", is a versatile aerosol concentration and enrichment system. This system was designed and constructed by Chen Lab at New York University to perform air pollution health effect studies [165]. This system consists of two $PM_{2.5}$ concentrator and four reinforced stainless steel Hinner-type whole-body inhalation chambers. The $PM_{2.5}$ concentrators are three-stage aerosol concentrator that utilizes the technology of virtual impactors to concentrate ambient $PM_{2.5}$. Whole-body inhalation chambers are used to hold mice and the maximum capacity of each are 32 mice. Two chambers are exposed to concentrated ambient particles (CAPs), while the others are exposed to filtered air (FA) as control with the same air flow rate. This system allows us to collect local $PM_{2.5}$ samples and perform systemic analysis of $PM_{2.5}$-associated physiology and pathology in mouse models.

The current study was designed to determine the local $PM_{2.5}$ level and its characteristics, and to explore another potential marker for air pollution. In addition, we aimed to compare the composition distribution of CAPs with that of ambient air (AA).

Material and Methods

$PM_{2.5}$ Sampling Sites

$PM_{2.5}$ samples were collected via "OASIS" that was located in 2001 Polaris Parkway, Columbus, OH 43240. Fig.5 is a map of Columbus showing the location of our sampling site as well as three Environmental Protection Agency (EPA) sampling sites (site 1: 5750 Maple Canyon, Columbus, OH 43229; site 2: State Fairgrounds, Columbus, OH 43211; site 3: 1700 Ann Street, Columbus, OH 43207). Our study site experiences heavy motor vehicle traffic due to its proximity to interstate 71 (I-71) and the entrance to Polaris Fashion Place.

$PM_{2.5}$ Sampling Protocol

$PM_{2.5}$ was collected using "OASIS", 6 hour a day, 5 days a week for 12 months. The particles in FA, AA and CAPs were deposited on Teflon filters (37 mm, 0.2 μm pore, Pall Corporation, Ann Arbor, MI) for gravimetric and trace element analyses.

Gravimetric Analysis

Gravimetric analysis was conducted using a microbalance (MT-5 Mettler Toledo, Columbus, OH) in a temperature/humidity-controlled room as described in Federal Reference Method (Core of Federal Regulations, EPA 1999b). $PM_{2.5}$ mass concentration was calculated using equation: mass concentration ($\mu g/m^3$) = [net weight (mg) x 1000] ÷

[air flow (liter per minute) x exposure time (minute) ÷ 1000]. After gravimetric analysis, the filters were delivered to University of Michigan for trace element analysis.

Trace Element Analysis

Teflon filters were placed in 15 mL centrifuge tubes and were wetted with 150 µl of ethanol before extraction in 10 mL of 10% HNO3. The extraction solution was sonicated for 48 hours and then given two-week acid-digest. The extracts were analyzed for trace elements using high-resolution inductively coupled plasma-mass spectrometry (Element2, Thermo Finnigan, San Jose, CA).

Black Carbon Measurement

Ambient black carbon was monitored by Aethalometer (Anderson Instruments Incorporated) between Jan 2012 and May 2013. Air sample was collected as a spot on a roll of filter tape. When the density of the spot reached a pre-set limit, the tape advanced to a new spot and the collection continued. The average concentration of absorbing black carbon was calculated based on the measurement of sample flow rate and the instrument's optical and mechanical characteristics.

Data Analyses

Pearson product-moment correlation coefficient between ambient $PM_{2.5}$ and black carbon was calculated using EXCEL. $PM_{2.5}$ concentrations were presented as mean ± S.D., and were analyzed by unpaired student *t*-test (two-sided) using Prism 4.0 (GraphPad Software, Inc, San Diego, CA).

Results

Ambient $PM_{2.5}$ Level in Our Study Site

The overall trend of ambient $PM_{2.5}$ collected by our system was consistent with that reported by EPA (Fig.6A). An average of ambient $PM_{2.5}$ concentration in our study site was 9.5 ($\mu g/m^3$), ranging between 2.1 and 18.5 ($\mu g/m^3$); while that reported by EPA was 10.2 ($\mu g/m^3$), ranging between 2.0 and 16.7 ($\mu g/m^3$). Our data indicate that ambient $PM_{2.5}$ levels in Columbus area are below either EPA 24-hour $PM_{2.5}$ standard (35 $\mu g/m^3$) or annual $PM_{2.5}$ standard (15 $\mu g/m^3$) (Revised by EPA on Sep 21, 2006).

In our study site, ambient $PM_{2.5}$ concentration in the first half year was slightly lower than that in the second half year (Fig.6A). Ambient $PM_{2.5}$ levels peaked on Jul-Aug and Nov-Dec during exposure time, which was coincident with the traffic peak during summer and winter holidays. This indicates that motor vehicle emission may be the primary source of ambient $PM_{2.5}$ at our study site. Indeed, our sampling site is located near the I-71 (< 0.5 mile) and the entrance to Polaris Fashion Place (< 1 mile), experiencing the heavy traffic particularly during the holiday seasons.

Ambient Black Carbon Level in Our Study Site

In addition to monitoring ambient $PM_{2.5}$ level, we also detected black carbon in AA using Aethalometer. As shown in Fig.6B, the relative higher level of black carbon clustered in the second half year, with a peak on Oct during the sampling period. To explore the linear correlation between ambient $PM_{2.5}$ and black carbon, we calculated Pearson product-moment correlation coefficient. Our result showed that ambient $PM_{2.5}$ and black carbon were positively related ($R^2 = 0.3$) (Fig.7), indicating a modest linear relationship between two air pollutants.

Characteristics of CAPs

During the sampling periods, the average mass concentrations of AA and CAPs were 9.5 $\mu g/m^3$ and 86.3 $\mu g/m^3$, respectively. $PM_{2.5}$ mass concentration in CAPs was significantly higher than that in FA (Fig.8A). The major chemical composition in our study site was dominated by sulfates; other significant elements included calcium, sodium and iron in AA and CAPs (Fig.8B). Our data showed that $PM_{2.5}$ composition distributions of AA and CAPs were identical, indicating that "OASIS" only concentrated AA without changing the chemical composition. Table 3 shows the details on elemental mass concentrations of FA, AA and CAPs in 2012. As expected, elemental levels in FA were quite low and can be ignored compared with those in CAPs.

Discussion

In the present study, we identified the seasonal trend of ambient $PM_{2.5}$ and explored the association between ambient $PM_{2.5}$ and black carbon at Columbus, Ohio. In addition, we characterized the compositions of CAPs collected by our exposure system, providing the details on the chemical species that may contribute to the adverse effects of ambient $PM_{2.5}$ on experimental animals.

The overall trend of ambient $PM_{2.5}$ collected by our system was consistent with the average reported by three EPA sites, although the level of $PM_{2.5}$ collected in our study site appeared to be slightly lower than those reported by EPA. This indicates that our exposure system "OASIS" is feasible for monitoring the local $PM_{2.5}$ and is, therefore, suitable for systemic studies in the physiological effects of $PM_{2.5}$ on animal models. Indeed, this system has been extensively used by several laboratories investigating the adverse effects of $PM_{2.5}$ on murine models, e.g. spontaneously hypertensive rats, diet-

induce obese mice and atherosclerotic mice [166-168]. This system has a great capacity to expose up to 128 mice, allowing us to study multiple projects or treatments simultaneously.

Our data, combined with the data released by EPA, showed that ambient $PM_{2.5}$ levels in Columbus area were below EPA standard. In our study site, ambient $PM_{2.5}$ concentration in the first half year was slightly lower than that in the second half year; ambient $PM_{2.5}$ levels tended to peak on Jul-Aug and Nov-Dec during sampling time. This indicates that motor vehicle emission may be the primary source of ambient $PM_{2.5}$ at our study site. As shown in the map, our sampling site is located near the I-71 (< 0.5 mile) and the entrance to Polaris Fashion Place (< 1 mile). Heavy traffic during school opening and holidays may contribute to the increases in ambient $PM_{2.5}$ levels. Modified land use regression models have provided a clear link between traffic and PM air pollution [169]. Future study, focusing on the association of traffic pattern with ambient $PM_{2.5}$ at our study site, will be critical to monitor and control this pollutant.

Our result showed that ambient $PM_{2.5}$ and black carbon were positively related ($R^2 = 0.3$), indicating a modest linear relationship between two air pollutants. Black carbon is the sooty part of PM, emitted by incomplete combustion of biofuel, fossil fuel and biomass [170]. In contrast to $PM_{2.5}$ and other particulates, black carbon absorbs sunlight efficiently. This absorption clearly leads to local heating of the atmosphere and global climate change [171, 172]. Furthermore, black carbon may trigger airway inflammation and lung cancer, particularly in children and occupational exposure workers [173, 174]. As the effects of black carbon have become understood in recent year, it is urgent that

this pollutant is monitored more comprehensively and treated as an important target of environmental control, which is currently patchy.

It has become evident that $PM_{2.5}$ is associated with airway inflammation, cardiovascular diseases, and metabolic disorders [31, 175, 176]. In addition to $PM_{2.5}$ mass, different chemical compositions of this pollutant contribute to divergent health effects. A study conducted in Detroit and Grand Rapids revealed that despite similar mass concentrations in CAPs at both sites, exposed animals exhibited divergent airway allergic responses which may be dependent on some specific chemical characteristics of $PM_{2.5}$ [177]. However, chemical-specific effects of $PM_{2.5}$ are poorly understood. The major chemical composition in our study site was dominated by sulfates, followed by other significant elements including calcium, sodium and iron. Previous *in vitro* studies claim that transition metals, particularly iron, are major factors mediating oxidative stress in response to PM exposure [178].

Our data showed that $PM_{2.5}$ composition distributions of AA and CAPs were identical, indicating that "OASIS" only concentrated AA without changing the chemical composition. As previously discussed, chemical compositions also play crucial roles in $PM_{2.5}$-mediated health effects. This property of our exposure system allows us to mimic the effects of $PM_{2.5}$ air pollution in "the real world".

Taken together, the studies described here enable us to reveal the annual level and seasonal trend of $PM_{2.5}$ in Columbus, Ohio. Black carbon could be used as another marker to monitor local air pollution. Future studies of tracking emission sources and

determining chemical-specific toxicological responses will help clarify the relationship between the compositions of $PM_{2.5}$ and human health effects.

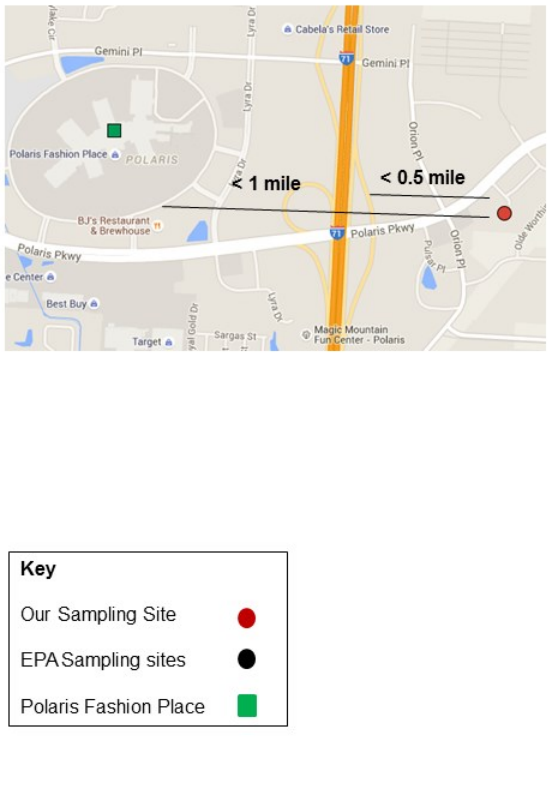

Figure 5. Map of Our Study Site. Bottom: the map showing our study site as well as EPA sampling sites. Top: the enlarged map showing the distances from our study site to highway (I-71) or shopping mall (Polaris Fashion Place). The distance was measured by google map. EPA: Environmental Protection Agency.

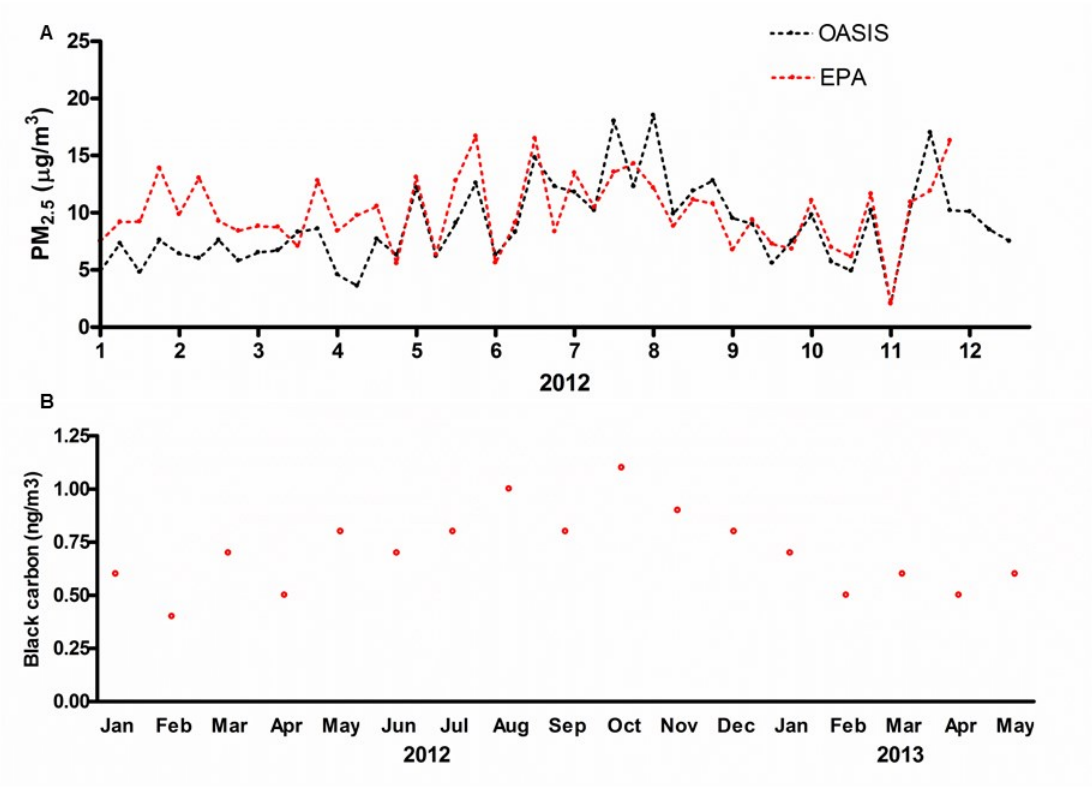

Figure 6. Ambient $PM_{2.5}$ and Black Carbon. (A) The concentrations of ambient $PM_{2.5}$ collected from our study site (OASIS) and by EPA (averaged values) in 2012. (B) Ambient black carbon monitored in our study site from Jan, 2012 to May, 2013. $PM_{2.5}$: fine particulate matters; OASIS: "Ohio Air Pollution Exposure Systems for Interrogation of Systemic Effects"; EPA: Environmental Protection Agency.

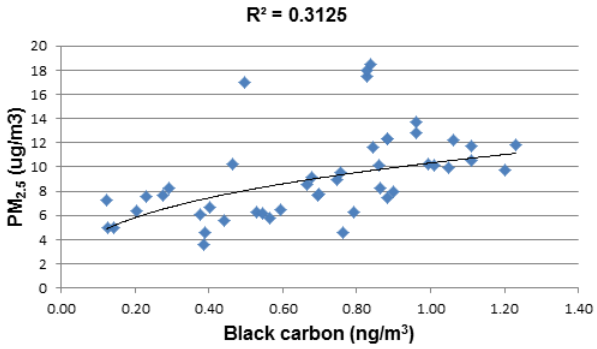

Figure 7. Correlation between Ambient $PM_{2.5}$ and Black Carbon. Pearson product-moment correlation coefficient was calculated using EXCEL.

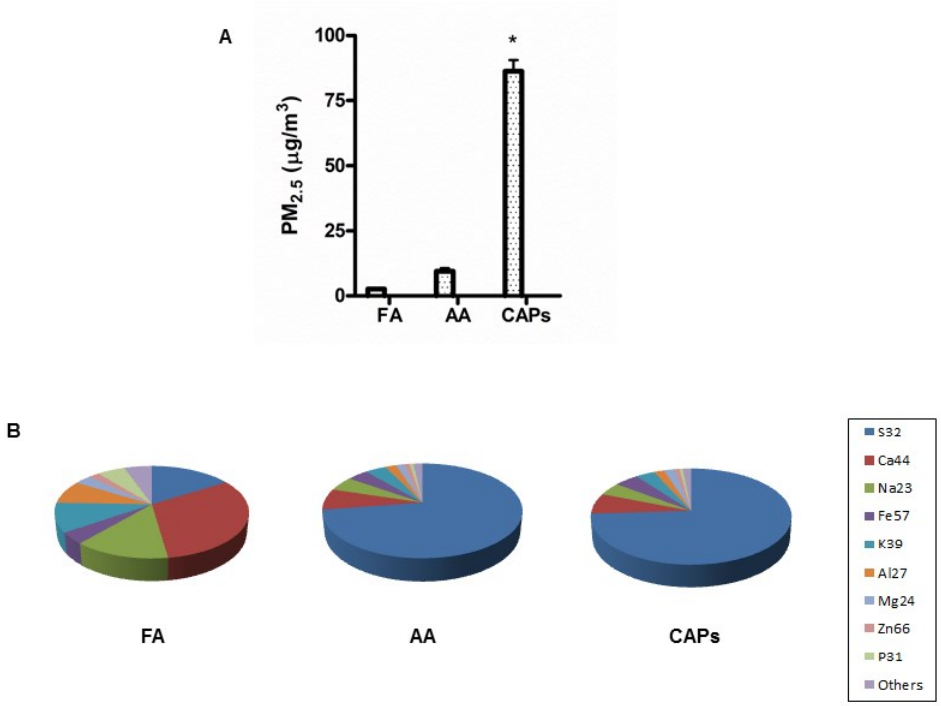

Figure 8. Characteristics of $PM_{2.5}$ Collected via OASIS. (A) Mass concentrations of FA, AA and CAPs. (B) Major composition distributions of FA, AA and CAPs. PM2.5: fine particulate matters; OASIS: "Ohio Air Pollution Exposure Systems for Interrogation of Systemic Effects"; FA: filtered air; AA: ambient air; CAPs: concentrated air particles. * $p < 0.05$ vs. FA group.

Table 3. Trace Elements of $PM_{2.5}$ Collected via OASIS in 2012.

Trace Element (ng/m^3)	**OASIS**		
	FA	AA	CAPs
S32	39.8	810.4	7335.8
Ca44	79.0	80.9	686.6
Na23	33.8	55.1	422.5
Fe57	10.1	45.1	442.7
K39	25.4	45.0	342.5
Al27	20.3	23.1	187.4
Mg24	8.0	17.6	183.6
Zn66	5.4	11.9	92.3
P31	3.6	8.4	60.7
Cr52	7.0	3.3	14.1
Ba137	0.6	3.0	28.5
Pb208	2.5	3.0	22.4
Cu63	1.7	2.8	24.9
Mn55	0.2	1.9	18.4
Ti47	0.1	0.9	9.0
Se77	0.0	0.8	7.9
Sb123	0.1	0.7	6.2
As75	0.0	0.6	5.1
Ni60	0.4	0.5	2.4
Sr88	0.1	0.5	4.2
Mo95	0.2	0.3	2.8
Cd111	0.5	0.3	1.7
V51	0.0	0.2	2.0
Co59	0.1	0.2	0.7
Rb85	0.0	0.1	0.6
Ce140	0.0	0.0	0.4
La139	0.0	0.0	0.3

$PM_{2.5}$: fine particulate matters; OASIS: "Ohio Air Pollution Exposure Systems for Interrogation of Systemic Effects"; FA: filtered air; AA: ambient air; CAPs: concentrated air particles.

Chapter 6: Long-term $PM_{2.5}$ Air Pollution Exposure Potentiated Atherosclerosis and Inflammation in a Mouse Model

Abstract

Both epidemiological and empirical data have demonstrated the association between long-term exposure to fine particulate matters ($PM_{2.5}$) and the burden of atherosclerosis. This study was designed to explore the underlying mechanisms and identify potential targets for prevention of atherosclerosis. $ApoE^{-/-}$ mice, a spontaneous atherosclerotic model, were exposed to filtered air or concentrated ambient particles using "Ohio Air Pollution Exposure Systems for Interrogation of Systemic Effects" for 6 months. After $PM_{2.5}$ exposure, plaque area and lipid area of aorta were evaluated using hematoxylin – eosin staining and oil-red O staining, respectively. In addition, total cholesterol, high-density lipoprotein and fasting glucose were quantitated using diagnostic kits. Furthermore, the levels of plasma cytokines were determined via ELISA kits and the mRNA levels of cytokines in the lung tissues were measured using real-time PCR. Our data showed that long-term $PM_{2.5}$ exposure potentiated atherosclerosis and modified the lipid profile in circulation; that interleukin-6 was the key inflammatory cytokine that may play a role in this process. These findings lead us to explore the protective effects of anti-

inflammation using natural nutrients such as strawberry on the development of atherosclerosis after long-term $PM_{2.5}$ exposure.

Background

Atherosclerosis is a specific form of arteriosclerosis in which an artery wall thickens and eventually reduces elasticity due to a chronic vascular inflammation. Cholesterol and inflammation are major components of atherosclerosis and considered to act as partners in all developmental stages of the disease [179, 180]. Lipoproteins retained by matrix proteoglycans in the intimal layers of the arterial wall are susceptible to undergo oxidative modifications, and this event is followed and potentiated by an immediate innate immune response [181, 182]. This process is promoted by low-density lipoprotein (LDL) that can transport fat molecules into artery walls, recruit macrophages, and thus drive atherosclerosis; in contrast, high-density lipoprotein (HDL) prevents or even reverses this process through removing fat molecules from macrophages in the artery walls [183].

The etiology of atherosclerosis is very complicated. Epidemiological studies over the past 60 years have revealed that numerous risk factors for atherosclerosis. These factors can be clarified into factors with an important genetic component, and those that are largely related to environment. Men below 60 years old develop cardiovascular diseases including atherosclerosis at more than twice the rate of women under 60 years old [184]. When all known risk factors are controlled for, family history remains a crucial independent factor for atherosclerosis [185]. In addition, other health conditions, e.g.

hypertension, metabolic syndrome, systemic inflammation and depression, may contribute to the development of atherosclerosis [9].

The importance of environment in human atherosclerosis has been examined in twin studies. Population migration studies clearly show that the environmental factors explain much of the variation in atherosclerosis incidence between populations [186]. High-fat and high-cholesterol diets appear to be the most significant factor and are usually required for development of atherosclerosis in experimental animals [42]. Numerous epidemiological studies have strongly supported the association of smoking with the development of atherosclerosis; clinical trials have demonstrated the beneficial effects of stopping smoking [42]. Other environmental factors, e.g. lack of exercise, infectious agents and low antioxidant levels, may contribute to the development of atherosclerosis as well [9].

Substantial epidemiological studies have demonstrated long-term $PM_{2.5}$ exposure as another risk factor for atherosclerosis in humans [187-189]. Subsequent animal studies have now corroborated these findings using "whole body $PM_{2.5}$ exposure system". Sun et al., reported that long-term $PM_{2.5}$ exposure (6 hour a day, 5 days a week for 6 months) in conjunction with high-fat chow accelerated plaque development and increased vascular inflammation in an apolipoprotein (apo) $E^{-/-}$ mice [28]. $PM_{2.5}$ exposure can induce inflammation and oxidative stress within lungs and then release inflammatory mediators into circulation; these mediators thereafter convey an inflammatory signal to the vasculature, causing vasomotor dysfunction and chronically promoting atherosclerosis [31, 190].

ApoE$^{-/-}$ mice and LDL receptor (LDLR)$^{-/-}$ mice are the most common animal models for atherosclerosis. ApoE$^{-/-}$ mice, first introduced in 1992, exhibit five times normal serum cholesterol and develop spontaneous atherosclerotic lesions [196-198], while LDLR$^{-/-}$ mice develop atherosclerosis with the assistance of dietary cholesterol. In addition, the atherosclerotic lesions in apoE$^{-/-}$ mice are primarily composed of cholesterol clefts, while those in LDLR$^{-/-}$ mice are primarily composed of foam cells [2]. Existing studies have confirmed that apoE$^{-/-}$ mice are suitable model to investigate molecular basis of $PM_{2.5}$-mediated atherosclerosis [28, 174, 199]. This study was designed to evaluate atherosclerosis and identify key inflammatory mediators in response to long-term $PM_{2.5}$ exposure in apoE$^{-/-}$ mice.

Material and Methods

Animal Model

Six-week-old apoE$^{-/-}$ male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained at 21 °C on a 12-h light/12-h dark cycle with free access to water and food. They were fed with 42% high-fat chow (n = 8; Adjusted Calories Diet, TD 88137, Harlan, Indianapolis, Ind); body weights and food consumption were monitored weekly. The Ohio State University Animal Care and Use Committee approved the protocols and the use of animals.

Exposure Protocol

Mice were exposed to either filtered air (FA) or concentrated ambient particles (CAPs) via our exposure system “Ohio Air Pollution Exposure System for Interrogation of Systemic Effects (OASIS)”, which was previously described. Mice were exposed for 6

hours per day, 5 days per week for a total of 6 months. Exposure began on March, 2012 and was stopped on September, 2012. $PM_{2.5}$ collection, gravimetric and elemental analyses were previously described in chapter 5.

Morphometric Analysis

The abdominal aorta was fixed in 10% zinc formalin and embedded in paraffin for hematoxylin-eosin (H & E) staining. Segments of thoracic aorta were frozen in liquid nitrogen and embedded in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek USA Inc, Torrance, CA) for oil red-O staining. Plaque area and lipid content were evaluated via H & E positive areas of aortic arch and oil-red O positive areas of thoracic aorta, respectively. At least 10 sections from 3 consecutive slides per area per mouse (abdominal and thoracic aortas) were detected. Each image was digitized under a microscope (Zeiss Axioskop with Spot I digital camera, Jena, Germany) and analyzed by National Institutes of Health Image J software. Plaque and lipid areas were normalized for the cross-sectional vessel cavity area and expressed as a percentage value.

Blood Lipid and Glucose

In the end of exposure, mouse was fasting overnight and the blood was obtained from the tip of the tail. Glucose levels were determined using a Contour Blood Glucose Meter (Bayer, Mishawaka, IN). After the exposure, mice were sacrificed after blood collection directly from left ventricle puncture. Blood samples were centrifuged at 3000 rpm for 5 minutes and serum was separated. Total cholesterol and HDL levels were assayed using diagnostic kits (Thermo Electron, Louisville, CO).

Plasma Levels of Inflammatory Cytokines

Whole blood was collected into EDTA-treated tubes. Cells were removed from plasma by centrifugation for 10 minutes at 2000 rpm using a refrigerated centrifuge. Following centrifugation, plasma (the liquid component) was transferred into a clean polypropylene tube using a Pasteur pipette. Plasma levels for tumor necrosis factor (TNF)-α, interferon (IFN)-γ, monocyte chemoattractant protein-1, interleukin (IL)-6 and -12, and adiponectin were detected using Mix-N-Match ELISArray Kit (Qiagen, Austin, TX) according to manufacturer instructions.

Quantitative RT-PCR

Lung was removed and homogenized by TRIzol Reagent (Invitrogen, Carlsbad, CA. Total RNA was then converted into cDNA with M-MLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA). Gene expression for TNF-α, MCP-1, IL-6 and -10 were determined using inventoried primer and probe assays (Applied Biosystems, Foster City, CA) on an ABI 7500 Fast Real Time PCR System using Taqman Universal PCR Master Mix. All reactions were performed under the following conditions: 50 °C for 2 minutes, 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. All data were standardized for β-actin and represented as relative mRNA expression. The primers are shown in table 4.

Statistical Analyses

Data were presented as mean ± S.D. unless otherwise stated. Unpaired student t-tests (two-sided) were performed using Prism 4.0 (GraphPad Software, San Diego, CA). In all cases, a *p* value less than 0.05 was considered to be statistically significant.

Results

$PM_{2.5}$ Characteristics during the Study Period

The mean daily $PM_{2.5}$ concentration at the study site in Columbus, OH, was 9.9 $\mu g/m^3$ during the exposure. The average $PM_{2.5}$ concentrations in FA and CAPs were 2.5 $\mu g/m^3$ and 109.6 $\mu g/m^3$, respectively. The average chemical composition of CAPs during the exposure period was summarized in table 5. As shown, the CAPs constituents were dominated by sulfate, which is typical for the study site. The results from the epidemiologic and toxicologic studies with PM sulfate are inconsistent and fail to support for a causal association of PM sulfate and health risk [195]. This indicates that sulfate in PM may affect health-related outcomes indirectly via interaction with certain metal species or secondary organic matter.

Body Weight and Food Consumption

Mice between FA and CAPs groups had similar body weights in the beginning of the study (Fig.9A), while after 6-month exposure, mice exposed to CAPs appeared to gain less body weights compared with those exposed to FA (Fig.9B). This was further confirmed by our data showing that mice exposed to CAPs consumed less food compared with those exposed to FA (Fig.9C). Our data indicate that long-term $PM_{2.5}$ exposure may affect animal appetite and metabolism.

$PM_{2.5}$ Induced Atherosclerosis

Fig.10 provided representative sections from morphometric analyses of the aorta and quantitated column bars. H & E staining showed that plaque area of the aorta was significantly increased in mice exposed to CAPs compared with those exposed to FA, while lipid area of the aorta detected by oil-red O staining was similar between two

groups. Our data indicate that long-term $PM_{2.5}$ exposure potentiates atherosclerosis in an $apoE^{-/-}$ mouse model, which verifies the previous findings [28, 168].

To further evaluate the $PM_{2.5}$-mediated vascular effects, we detected the serum levels of cholesterol and HDL in exposed mice. Our results showed that $PM_{2.5}$ exposure caused an increase in cholesterol level ($p < 0.05$), but a decrease in HDL level ($p < 0.01$) (Fig.11A-B). Our data, together with previous evidence [194], indicate that long-term $PM_{2.5}$ exposure may induce atherosclerosis via changing the lipid profile in blood.

Previous studies have shown that $PM_{2.5}$ exposure can exaggerate insulin resistance in both genetic and diet-induced obese mouse models [196, 197]. It has become evident that high glucose can increase the cardiovascular risk and serve as a marker for atherosclerosis [198-200]. In our study, the mice exposed to CAPs exhibited higher fasting glucose level relative to those exposed to FA (Fig.11C). Collectively, our data strongly show that long-term $PM_{2.5}$ exposure induces atherosclerosis and changes the levels of the lipids and glucose in blood.

$PM_{2.5}$ Induced Inflammation

Inflammation is involved in the development of atherosclerosis and anti-inflammatory mediator therapy can slow atherosclerosis progression in both animal and human studies [201-203]. To evaluate $PM_{2.5}$-mediated inflammation, plasma levels of inflammatory mediators, e.g. TNF-α, IFN-γ, MCP-1, IL-6, IL-12 and adiponectin, were measured via ELISA kits. The results showed that plasma levels of TNF-α and IL-6 were significantly elevated in mice exposed to CAPs, but not the others (Fig.12). Our data are consistent

with the previous studies showing that $PM_{2.5}$ exposure can increase the plasma levels of TNF-α and IL-6 [194, 204].

Pulmonary inflammation was also detected via measuring mRNA levels of TNF-α, MCP-1, IL-6 and IL-10. We found that relative mRNA level of IL-6 in the lung tissue was apparently elevated after exposure to CPAs, while others showed no significant changes (Fig.13). Our data indicate that IL-6 plays a crucial role in $PM_{2.5}$-mediated inflammation in both circulation and tissue.

Discussion

The present study demonstrated $PM_{2.5}$-potentiated atherosclerotic plaques in $apoE^{-/-}$ mice. In addition, long-term $PM_{2.5}$ exposure caused the increases in total cholesterol and glucose level, but the decrease in HDL level in mice. Furthermore, this study found that TNF-α and IL-6 in plasma were elevated after long-term $PM_{2.5}$ exposure; that mRNA expression of IL-6 within lung was upregulated in response to long-term $PM_{2.5}$ exposure.

Our data showed that long-term $PM_{2.5}$ exposure significantly reduced the food intake compared with FA group; body weight gain in mice exposed to CAPs appeared to be less than those exposed to FA. This indicates that long-term $PM_{2.5}$ exposure may change the appetite and metabolism. Our previous data showed that 5-week $PM_{2.5}$ exposure can significantly increase the level of circulating leptin [196]. Leptin is a hormone secreted by adipose tissue that regulates energy balance by inhibiting hunger. Elevated leptin may inhibit the appetite and then reduce the food consumption in mice exposed to CAPs. Although these mice gain less body weights, they exhibit more severe metabolic disorders. In our study, the data suggested that the blood glucose in mice exposed to

CAPs was almost twice as that in FA groups. It has become evident that high glucose can increase the cardiovascular risk and serve as a marker for atherosclerosis [198-200].

Morphological analyses suggested that long-term $PM_{2.5}$ exposure significantly increased the atherosclerotic plaque area in an $apoE^{-/-}$ mouse model, which verifies the previous findings [28, 168]. Our data also showed that $PM_{2.5}$ exposure caused an increase in cholesterol level, but a decrease in HDL level. Cholesterol is an essential structural component of cell membrane and serves as a precursor for the synthesis of steroid hormones [205]. Although cholesterol is essential for all animal life (not bacteria), high level of cholesterol in the circulation is strongly associated with the progression of atherosclerosis [206, 207]. HDL is one of lipoproteins and prevents atherosclerosis through transporting fat molecules, e.g. cholesterol and triglycerides, out of artery walls [208, 209]. Several mechanisms may claim its protective effects. First, HDL can directly bind unesterified cholesterol and remove excess cholesterol from cells and extracellular tissues [14]. Second, HDL may reverse endothelial dysfunction and improve endothelial barrier integrity. Third, HDL carries an esterase known as serum paraoxonase that can degrade certain biologically active oxidized phospholipids [17]. The antioxidant properties of HDL inhibit LDL oxidation, thereby reducing the inflammation and attenuating the uptakes by macrophages.

Inflammation has a prominent role in atherosclerosis and its complications. Circulating monocytes migrate into the intima in the presence of adhesion molecules and chemoattractant proteins. Once resident in the arterial intima, monocytes acquire the morphological characteristics of macrophages, undergoing a series of changes that lead

ultimately to foam cell formation [18]. Inflammatory mediators, e.g. TNF-α, IL-6 and IFN-γ, are crucial for activation of macrophages and foam cell formation. In the present study, our data suggested that long-term exposure to $PM_{2.5}$ can upregulate mRNA expression of IL-6 within lung, and cause increases in circulating TNF-α and IL-6.

TNF-α is a pro-inflammatory cytokine secreted by a variety of cells including adipocytes, macrophages, natural killer cells, T-cells and endothelial cells [210]. Elevated circulating TNF-α predicts vascular damage since it is associated with early atherosclerosis in middle-aged healthy men [211]. TNF-α induces vascular inflammation via activation of nuclear factor-κB, thereby releasing adhesion molecules (e.g. ICAM-1 and VCAM-1) and chemoattractant proteins (e.g. MCP-1) from endothelial cells and smooth muscle cells. Previous *in vitro* studies have suggested that TNF-α may promote platelet aggregation and ROS production primarily through activation of the arachidonic acid pathways [212, 213]. Thus, TNF-α is deeply involved in vascular inflammation and plaque formation, facilitating the development of atherosclerosis.

IL-6 is a multifunctional cytokine that has both pro-inflammatory and anti-inflammatory properties, depending on locations [214]. In healthy subjects, IL-6 expression is highly regulated by a complex hormonal network related to glucocorticoid and catecholamine secretion [215]. After $PM_{2.5}$ exposure, IL-6 level can be elevated and then trigger innate immune system via binding to toll-like receptors [31, 216]. Increased IL-6 is highly associated with metabolic and vascular diseases, such as obesity, type 2 diabetes, atherosclerosis [215]. IL-6 plays a crucial role in plaque development and destabilization via release of other cytokines, oxidation of LDL, stimulation of ROS production, and

activation of MCP-1 [217]. These effects explain clinical observation that elevated IL-6 levels in coronary and systemic circulation are a risk factor for atherosclerosis.

Taken together, this study provided compelling evidence supporting that long-term $PM_{2.5}$ exposure mediated atherosclerosis and changed lipid profile in $apoE^{-/-}$ mice. In addition, we identified TNF-α and IL-6 as the key inflammatory mediators in this process. These findings lead us to explore the protective effects of anti-inflammation using natural nutrients such as strawberry on the development of atherosclerosis after long-term $PM_{2.5}$ exposure.

Table 4. Primers Used for RT-PCR.

Primer	Forward oligonucleotides	Reverse oligonucleotides
TNF-α	5'-TCTCATGCACCACCATCAAGGACT-3'	5'-TGACCACTCTCCCTTTGCAGAACT-3'
MCP-1	5'-TGCTGTCTCAGCCCAGATGCAGTTA-3'	5'TACAGCTTCTTTGGGACACCTGCT-3'
IL-6	5'-TGGCTAAGGACCAAGACCATCCAA-3'	5'-AACGCACTAGGTTTGCCGAGTAGA-3'
IL-10	5'-TTGCTCTTGCACTACCAAAGCCAC-3'	5'-AGTAAGAGCAGGCAGCATAGCAGT-3'
β-actin	5'-TGTGATGGTGGGAATGGGTCAGAA-3'	5'-TGTGGTGCCAGATCTTCTCCATGT-3'

RT-PCR: real time-polymerase chain reaction, TNF-α: tumor necrosis factor-α, MCP-1: monocyte chemoattractant protein-1, IL-6: interleukin-6, IL-10: interleukin-10.

Table 5. PM2.5 Concentration and Compositions Collected via OASIS, March-September, 2012.

$PM_{2.5}$	**OASIS**		
	FA	AA	CAPs
Mass Concentration ($\mu g/m^3$)	2.5	9.9	109.6
Trace Element (ng/m^3)			
S32	39.8	842.7	7821.8
Ca44	76.6	73.9	669.6
Na23	34.8	57.1	450.5
Fe57	10.1	40.1	441.7
K39	24.5	40.8	336.6
Al27	20.4	24.9	197.4
Mg24	8.2	17.5	190.2
Zn66	5.6	9.9	84.8
P31	5.6	9.1	63.7
Cr52	6.7	3.1	13.6
Ba137	0.6	2.5	26.9
Pb208	2.5	2.8	22.5
Cu63	1.4	2.3	23.4
Mn55	0.2	1.7	18.3
Ti47	0.1	0.7	8.9
Se77	0.0	0.8	8.0
Sb123	0.0	0.6	6.1
As75	0.0	0.5	5.0
Ni60	0.3	0.5	2.4
Sr88	0.1	0.4	4.0
Mo95	0.1	0.3	2.7
Cd111	0.5	0.2	1.7
V51	0.0	0.2	2.1
Co59	0.1	0.2	0.7
Rb85	0.0	0.0	0.6
Ce140	0.0	0.0	0.4
La139	0.0	0.0	0.3

$PM_{2.5}$: fine particulate matters; OASIS: "Ohio Air Pollution Exposure Systems for Interrogation of Systemic Effects"; FA: filtered air; AA: ambient air; CAPs: concentrated air particles.

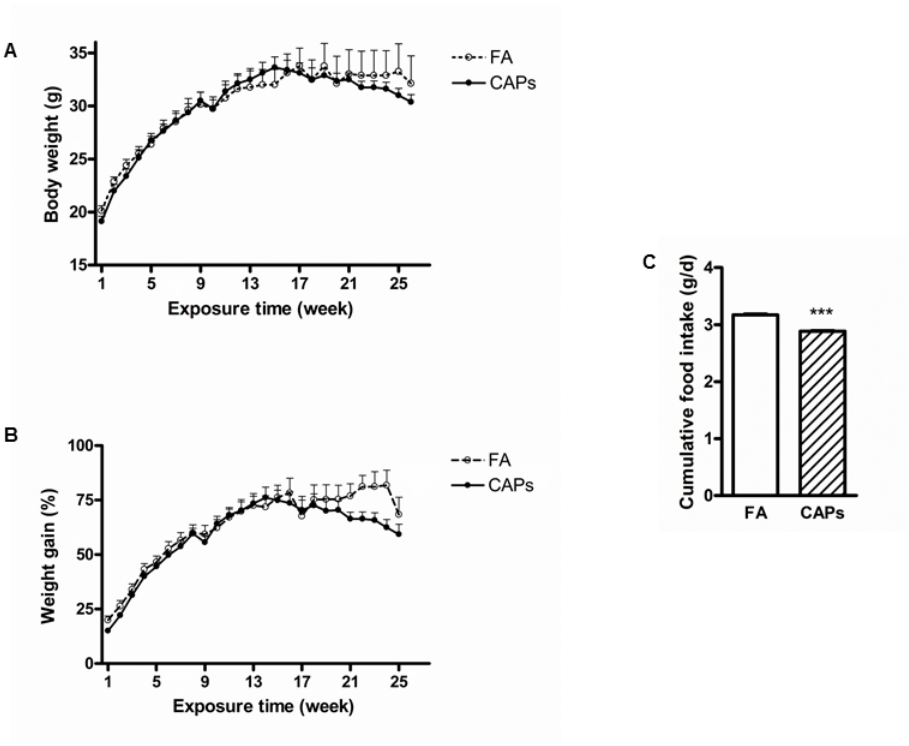

Figure 9. Body Weights and Food Consumption. (A) The curves of body weights during the exposure. (B) The percentages of body weights gained during the exposure. (C) Cumulative food intakes during the exposure. FA: filtered air; and CAPs: concentrated ambient particles. *** $p < 0.001$ vs. FA group.

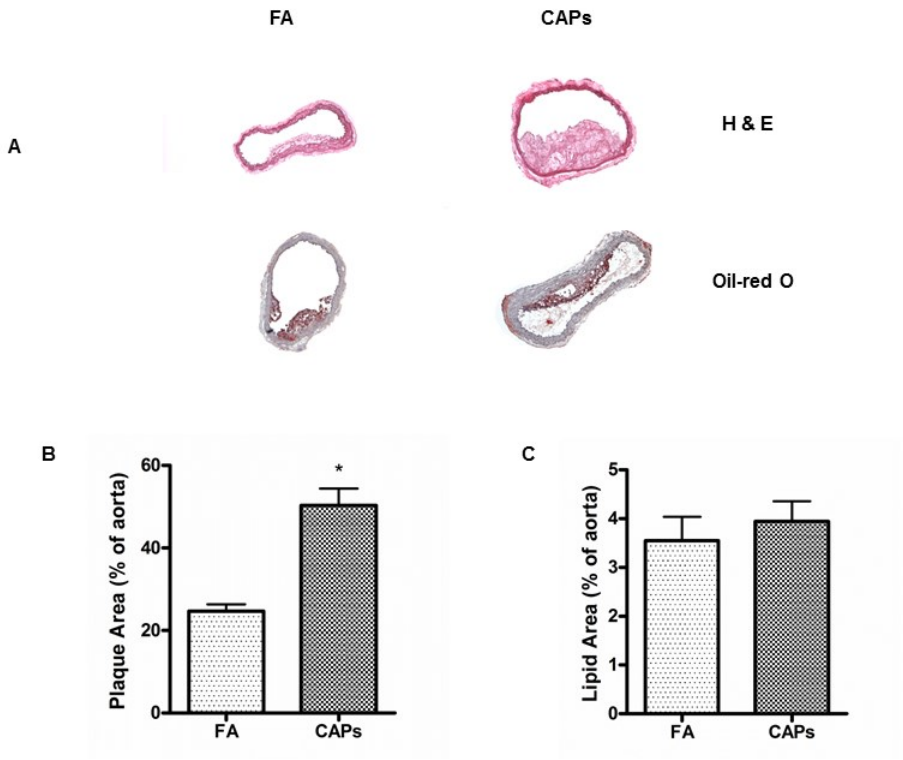

Figure 10. Development of Atherosclerotic Plaque after $PM_{2.5}$ Exposure. (A) Representative images of H & E staining of abdominal aortic sections and Oil-red O staining of aortic arch sections. (B) Plaque area was analyzed from H & E staining. (C) Lipid area was analyzed from Oil-red O staining. H & E staining: hematoxylin-eosin staining; FA: filtered air; and CAPs: concentrated ambient particles. * $p < 0.05$ vs. FA group.

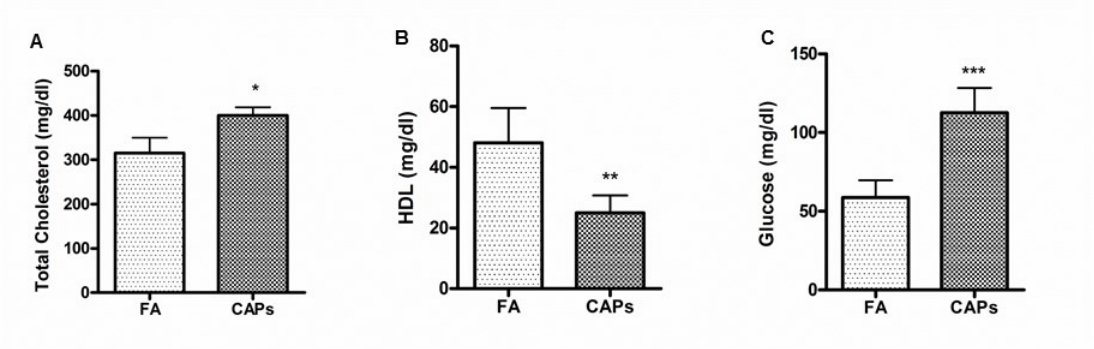

Figure 11. Serum Lipids and Glucose after $PM_{2.5}$ Exposure. (A) The concentration of serum cholesterol. (B) The concentration of serum HDL. (C) The concentration of fasting glucose. HDL: high-density lipoprotein; FA: filtered air; and CAPs: concentrated ambient particles. * $p < 0.05$ vs. FA group; ** $p < 0.01$ vs. FA group; *** $p < 0.001$ vs. FA group

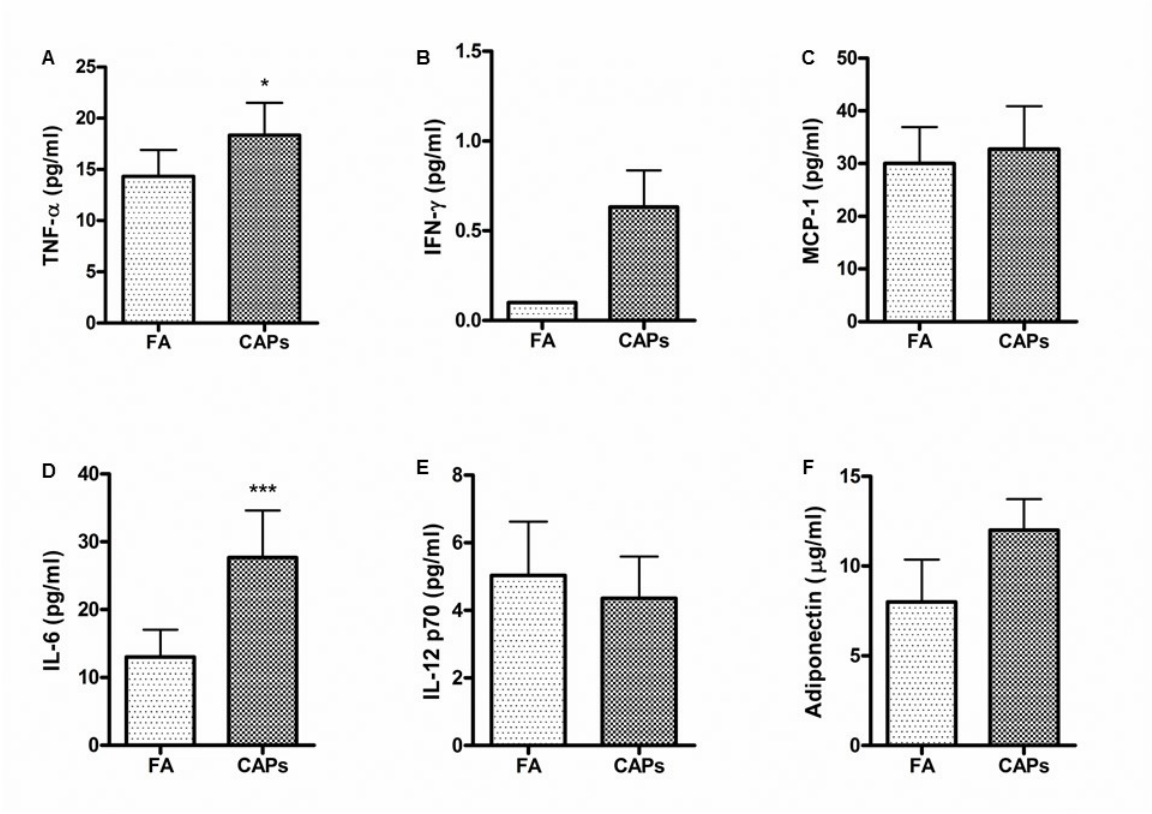

Figure 12. Plasma Levels of Inflammatory Cytokines after $PM_{2.5}$ Exposure. (A) The concentration of plasma TNF-α. (B) The concentration of plasma IFN-γ. (C) The concentration of plasma MCP-1. (D) The concentration of plasma IL-6. (E) The concentration of plasma IL-12. (F) The concentration of plasma adiponectin. TNF: tumor necrosis factor; IFN: interferon; MCP: monocyte chemoattractant protein; IL: interleukin; FA: filtered air; and CAPs: concentrated ambient particles. * $p < 0.05$ vs. FA group; *** $p < 0.001$ vs. FA group.

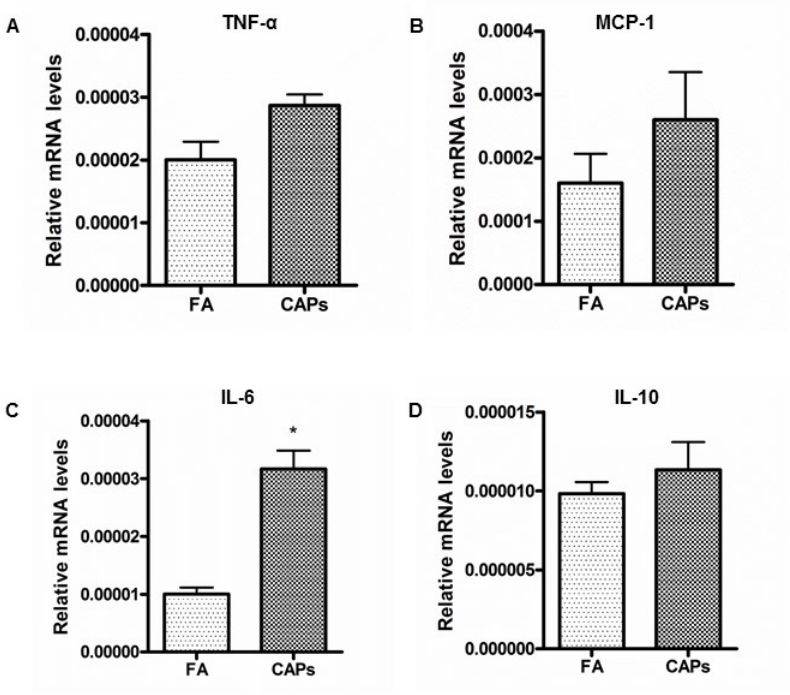

Figure 13. Relative mRNA Levels in Lung Tissues after $PM_{2.5}$ Exposure. (A) TNF-α mRNA level. (B) MCP-1 mRNA level. (C) IL-6 mRNA level. (D) IL-10 mRNA level. TNF: tumor necrosis factor; MCP: monocyte chemoattractant protein; IL: interleukin; FA: filtered air; and CAPs: concentrated ambient particles. * $p < 0.05$ vs. FA group.

Chapter 7: Effects of Freeze-dried Strawberry Supplementation on Atherosclerosis after Long-term $PM_{2.5}$ Exposure in a Mouse Model

Abstract

Strawberry, a rich source of micronutrients and several bioactive phytochemicals, has been implicated in the prevention of cardiovascular diseases and metabolic syndromes. This study aimed to evaluate the effects of strawberries on $PM_{2.5}$-mediated atherosclerosis and inflammation in $apoE^{-/-}$ mice. These mice were fed with high fat chow with or without 10% freeze-dried strawberry powder. In the meantime, they were exposed to either filtered air or concentrated ambient particles for 6 months. After exposure, plaque areas and lipid areas were evaluated using H & E staining and Oil-red O, respectively. Serum lipids and plasma cytokines were measured via ELISA kits. Relative mRNA expressions in lung were quantitated using RT-PCR. Our results showed that strawberries can significantly improve lipid profile and reduce the inflammation, but failed to improve atherosclerosis after $PM_{2.5}$ exposure. This indicates that other mechanistic pathways may play roles in $PM_{2.5}$-mediated atherosclerosis and diminish the effects of strawberries.

Background

Strawberries, a unique combination of several nutrients, phytochemicals and fiber, are a great source of B-vitamins, vitamin C, vitamin E, potassium, folic acid, carotenoids, and specific flavonoids, e.g. pelargonidin, quercetin, and catechin. Strawberries also contain significant amounts of ellagic acids, tannins, and phytosterols [119]. These polyphenols have diverse structure and functions, accounting for most of the health benefits of strawberries. Ellafic acids have shown to exert significant free radical scavenging and anti-proliferative effects in several experimental models [119]. Flavonoids present free radical scavenging activity as well as anti-inflammatory and vasodilatory functions [218]. Phytosterols are plant-derived sterols that have similar structure and function to cholesterol. Numerous clinical trials have reported that dietary phytosterols present cholesterol lowering effects [219].

Strawberry has been implicated in the prevention of cardiovascular diseases [118, 220, 221], and metabolic syndromes [117, 146]. In a randomized double-blind controlled trial, 6-week strawberry supplementation can substantially reduce lipid peroxidation and inflammation, and improve glycemic control and antioxidant status in subjects with type 2 diabetes [220]. Another study carried out in diabetic subjects showed that just two weeks on a diet rich in flavonols exhibited a protective effect against oxidative DNA damage in lymphocytes [222]. In an *in vitro* study, strawberry extract inhibited platelet aggregation and decreased the levels of inflammatory mediators, e.g. sP-selectin, sCD40L and IL-1β, in human platelet-rich plasma [221]. Treatment of rabbit aortic rings with aqueous extract of freeze-dried strawberry powder produced a dose-dependent endothelial relaxation [223].

Phytochemicals in strawberries, including anthocyanins, ellagic acids, catechin, quercetin and kaempferol, may account for the observed cardio-protective effects in both clinical and experimental studies. It has been shown that anthocyanin treatment can upregulate endothelial nitric oxide synthase (eNOS) in bovine artery endothelial cells [224]. This indicates that strawberry phytochemicals may reverse endothelial dysfunction and inhibit the initiation of atherosclerosis since eNOS has a crucial role in maintaining cardiovascular homeostasis. Subsequent animal studies demonstrate that purified anthocyanins from strawberries added to drinking water can prevent the development of dyslipidemia and obesity in mice fed with a high-fat diet for 3 months [225, 226]. Antioxidant effects and inhibition of platelet aggregation have also been reported in other studies investigating phytochemicals, e.g. catechin, ellagic acid, kaempferol and quercetin, suggesting their potential roles in the prevention of atherosclerosis [119].

Emerging evidence from animal and *in vitro* cell culture studies supports the roles of strawberries in ameliorating obesity, inflammation, hypertension, and oxidative stress, which are the risk factors for cardiovascular diseases [119]. Our previous studies have demonstrated that long-term exposure to $PM_{2.5}$ potentiates atherosclerosis through upregulating inflammatory mediators, e.g. TNF-α and IL-6, in $apoE^{-/-}$ mice. This study was designed to evaluate the effects of strawberries on $PM_{2.5}$-mediated atherosclerosis and inflammation in these mice.

Material and Methods

Animal Model and Feeding Study

Six-week-old apoE$^{-/-}$ male mice were purchased from the Jackson Laboratory [227]. All mice were maintained at 21 °C on a 12-h light/12-h dark cycle with free access to water and food. They were fed either with 42% high-fat chow (Adjusted Calories Diet, TD 88137, Harlan, Indianapolis, Ind), or with high-fat chow with 10% freeze-dried strawberry. This diet was ingredient-matched to the high-fat chow except that the 100g of freeze-dried strawberry power replaced 100g of sucrose in the custom formulation. The freeze-dried strawberry powder was provided by the California Strawberry Commission. Body weights and food consumption were monitored weekly. The Ohio State University Animal Care and Use Committee approved the protocols and the use of animals.

Exposure Protocol

Mice were exposed to either filtered air (FA) or concentrated ambient particles (CAPs) via our exposure system "Ohio Air Pollution Exposure System for Interrogation of Systemic Effects (OASIS)", which was previously described. Mice were exposed for 6 hours per day, 5 days per week for a total of 6 months. Exposure began on March, 2012 and was stopped on September, 2012. $PM_{2.5}$ collection, gravimetric and elemental analyses were previously described in chapter 5.

Morphometric Analysis

The abdominal aorta was fixed in 10% zinc formalin and embedded in paraffin for hematoxylin-eosin (H & E) staining. Segments of thoracic aorta were frozen in liquid nitrogen and embedded in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek USA Inc, Torrance, CA) for oil red-O staining. Plaque area and lipid content were evaluated via H & E positive areas of aortic arch and oil-red O positive areas of

thoracic aorta, respectively. At least 10 sections from 3 consecutive slides per area per mouse (abdominal and thoracic aortas) were detected. Each image was digitized under a microscope (Zeiss Axioskop with Spot I digital camera, Jena, Germany) and analyzed by National Institutes of Health Image J software. Plaque and lipid areas were normalized for the cross-sectional vessel cavity area and expressed as a percentage value.

Blood Lipid and Glucose

In the end of exposure, mouse was fasting overnight and the blood was obtained from the tip of the tail. Glucose levels were determined using a Contour Blood Glucose Meter (Bayer, Mishawaka, IN). After the exposure, mice were sacrificed after blood collection directly from left ventricle puncture. Blood samples were centrifuged at 3000 rpm for 5 minutes and serum was separated. Total cholesterol and HDL levels were assayed using diagnostic kits (Thermo Electron, Louisville, CO).

Plasma Levels of Inflammatory Cytokines

Whole blood was collected into EDTA-treated tubes. Cells were removed from plasma by centrifugation for 10 minutes at 2000 rpm using a refrigerated centrifuge. Following centrifugation, plasma (the liquid component) was transferred into a clean polypropylene tube using a Pasteur pipette. Plasma levels for tumor necrosis factor (TNF)-α, interferon (IFN)-γ, monocyte chemoattractant protein-1, interleukin (IL)-6 and -12, and adiponectin were detected using Mix-N-Match ELISArray Kit (Qiagen, Austin, TX) according to manufacturer instructions.

Quantitative RT-PCR

Lung was removed and homogenized by TRIzol Reagent (Invitrogen, Carlsbad, CA. Total RNA was then converted into cDNA with M-MLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA). Gene expression for TNF-α, MCP-1, IL-6 and -10 were determined using inventoried primer and probe assays (Applied Biosystems, Foster City, CA) on an ABI 7500 Fast Real Time PCR System using Taqman Universal PCR Master Mix. All reactions were performed under the following conditions: 50 °C for 2 minutes, 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. All data were standardized for β-actin and represented as relative mRNA expression.

Statistical Analyses

Data were presented as mean ± S.D. unless otherwise noted. One-way ANOVA followed by Bonferroni's *post*-hoc test was performed using Prism 4.0 (GraphPad Software, Inc, San Diego, CA). In all cases, a *p* value less than 0.05 was considered to be statistically significant.

Results

CAPs Characteristics, Body Weights and Food Consumption

$PM_{2.5}$ concentration and average chemical compositions of CAPs have previously summarized in table 5. The average $PM_{2.5}$ concentration in FA and CAPs were 2.5 $\mu g/m^3$ and 109.6 $\mu g/m^3$, respectively. The CAPs constituents were dominated by sulfate. After 6-month exposure, animals exposed to CAPs appeared to gain less body weights compared with those exposed to FA, in both high-fat chow and strawberry treatment groups (Fig.14A-B). $PM_{2.5}$ exposure significantly reduced food intakes in mice fed with high-fat chow or strawberry diet (Fig.14C).

Effects of Strawberry Supplementation on $PM_{2.5}$-mediated Atherosclerosis

As previously described, long-term $PM_{2.5}$ exposure potentiated atherosclerosis in $apoE^{-/-}$ mice. To test the effects of strawberry supplementation on $PM_{2.5}$-mediated atherosclerosis, mice were fed with high-fat diet with 10% freeze-dried strawberry during the exposure. Our date showed that mice with strawberry supplementation exhibited similar plaque areas and lipid areas compared with those without strawberry supplementation after long-term exposure to CAPs (Fig.15A-C). It was demonstrated that long-term $PM_{2.5}$ exposure can upregulate the levels of total cholesterol and glucose, and decrease the HDL level in blood (Fig.16A-C); strawberry supplementation, however, almost restored these molecules to normal levels after $PM_{2.5}$ exposure (Fig.16A-C).

Effects of Strawberry Supplementation on $PM_{2.5}$-mediated Inflammation

To detect the effects of strawberries on $PM_{2.5}$-mediated Inflammation, plasma levels of cytokines and mRNA expression of cytokines in lung were determined. Exposure to CAPs can significantly increase the levels of TNF-α and IL-6 in plasma relative to exposure to FA; while strawberry supplementation can decrease IL-6 level but not TNF-α level in plasma after $PM_{2.5}$ exposure (Fig.17A and D). In addition, strawberries can robustly increase the plasma levels of IFN-γ and adiponectin in response to long-term $PM_{2.5}$ exposure (Fig.17B and F). There are no differences detected for MCP-1 and IL-12 in plasma after $PM_{2.5}$ exposure and strawberry supplementation (Fig.17C and E).

In addition to the increase in plasma, mRNA level of IL-6 was also elevated in lung in response to $PM_{2.5}$ exposure; strawberries almost restored its level to normal (Fig.18C). Compared with the group without strawberries, the supplementation significantly

increased IL-10 mRNA expression in lung after $PM_{2.5}$ exposure (Fig.18D). But no differences were observed for TNF-α and MCP-1 mRNA expressions among these groups (Fig.18A and B). Our data verified the previous findings that strawberries had anti-inflammatory properties and changed cytokine profile [150, 228].

Discussion

The present study demonstrated that strawberry supplementation improved serum lipid profile and reduced inflammation after long-term exposure to $PM_{2.5}$. However, our data showed that strawberry supplementation appears to have no effects on $PM_{2.5}$-potentiated atherosclerosis in $apoE^{-/-}$ mice. These data must be fully considered when the protective effects of strawberries on atherosclerosis are mentioned in future studies.

Previous studies showing that strawberry supplementation can improve lipid profile and glucose level are consistent with our findings [229, 230]. As previously discussed, elevated cholesterol and glucose levels in circulation can increase the cardiovascular risk [198-200, 206, 207]; while serum HDL can prevent atherosclerosis through removing cholesterol and triglycerides from artery walls [208, 209]. This indicates that strawberry supplementation may prevent cardiovascular diseases in mice.

Inflammation has been implicated in the progression of atherosclerosis and inflammatory cytokines may be used as potential therapeutic targets for accelerated atherosclerosis. Anti-inflammatory properties of strawberry have been well documented and may play a role in the prevention of cardiovascular diseases [114, 119]. Indeed, our data support that strawberries can reduce $PM_{2.5}$-mediated inflammation via downregulating pro-inflammatory cytokines, e.g. IL-6, and upregulating anti-inflammatory mediators, e.g.

adiponectin and IL-10. Collectively, these data indicate that strawberries may prevent the development of atherosclerosis in experimental mice.

Surprisingly, strawberry supplementation appeared to have no effects on $PM_{2.5}$-potentiated atherosclerosis, although it improved serum lipid profile and modulated the inflammatory responses after $PM_{2.5}$ exposure. Morphological analyses showed that strawberry supplementation failed to reduce both plaque area and lipid area of atherosclerotic lesion. Several mechanisms may explain these results. First, multiple risk factors for atherosclerosis in our model may diminish the benefits of strawberries. Our study employed $apoE^{-/-}$ mice to investigate the development of atherosclerosis. This mouse exhibits five times normal serum cholesterol and develops spontaneous atherosclerotic lesions [191-193]. To accelerate the development of atherosclerosis in mice, we fed them with 40% high-fat chow. It has been suggested that high-fat diet is the most significant factor for atherosclerosis [42]. Furthermore, the animals were exposed to concentrated $PM_{2.5}$ for 6 months, which has been verified by our data as another environmental risk factor for atherosclerosis. Taken together, these risk factors may be too complicated and powerful to be recovered by strawberries. A modified model considering all these aspects will be needed to evaluate the benefits of strawberries on cardiovascular diseases.

Second, although our data showed the anti-inflammatory effects of strawberries (e.g. downregulating IL-6 and upregulating adiponectin and IL-10), the supplementation did not change the level of TNF-α. TNF-α is one of the upstream cytokines that regulate systemic inflammation and induce atherosclerosis. In patients with systemic

inflammatory disorders, the antibody against TNF-α can reverse endothelial dysfunction and reduce vascular event rates [231]. In addition, our data showed that IFN-γ in plasma was elevated by strawberries in response to $PM_{2.5}$ exposure. This mediator regulates both innate and adaptive immune responses and may have distinct roles at different stages of atherosclerosis [232]. During the early phases, it is generally thought that IFN-γ stimulates cell recruitment to the plaque and disrupts lipid development; while in the late phases, IFN-γ seems to be pro-apoptotic and therefore accelerates atherosclerosis progression [232]. These two key cytokines may hold central roles in $PM_{2.5}$-mediated atherosclerosis. The underlying pathways related to TNF-α and IFN-γ will be further investigated to explore why strawberries fail to improve atherosclerotic lesions.

Third, other mechanistic pathways may diminish the effects of strawberry supplementation on $PM_{2.5}$-mediated atherosclerosis. Indeed, the general mechanistic pathways whereby $PM_{2.5}$ exposure may promote atherosclerosis progression include systemic oxidative stress and inflammation, autonomic nervous system imbalance, and the toxic effects of soluble metals and organic compounds in $PM_{2.5}$ [31]. Although strawberries may attenuate inflammation after long-term exposure to $PM_{2.5}$, the disruption of autonomic nervous system and the direct toxicity of PM components may contribute to the development of atherosclerosis is our study.

Taken together, our data indicate that strawberry supplementation can improve serum lipid profile and reduce inflammation in response to long-term $PM_{2.5}$ exposure. However, the morphometric data show no effects of strawberry supplementation on $PM_{2.5}$-mediated atherosclerosis. Future studies, which concentrate on TNF-α or IFN-γ signaling and other

mechanistic pathways of $PM_{2.5}$-mediated atherosclerosis using modified models, will be critical to elucidate the effects of strawberry supplementation on this event.

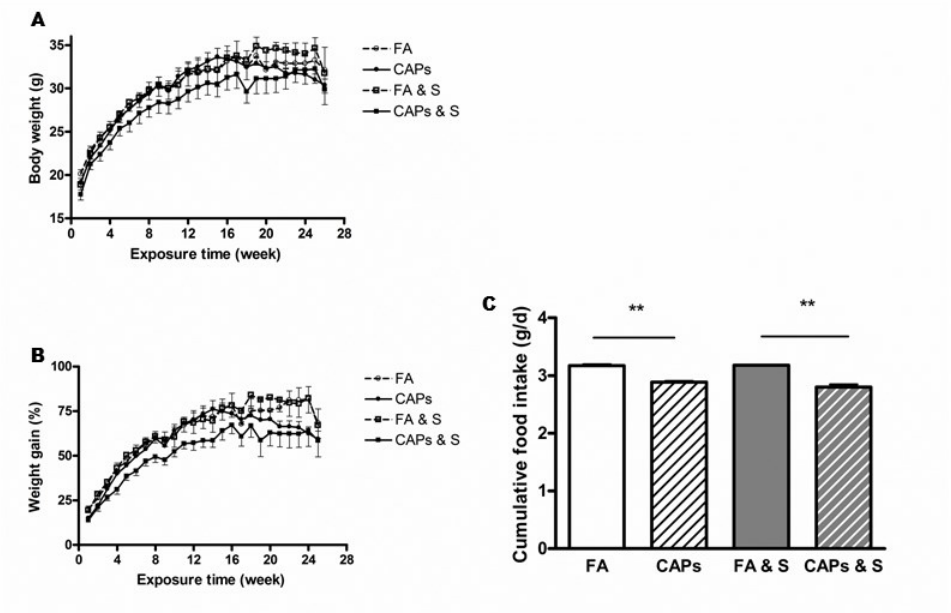

Figure 14. Body Weights and Food Consumption. (A) The curves of body weights during the exposure. (B) The percentages of body weights gained during the exposure. (C) Cumulative food intakes during the exposure. FA: filtered air; and CAPs: concentrated ambient particles; FA & S: filtered air plus strawberry supplementation; CAPs & S: concentrated ambient particles plus strawberry supplementation. ** $p < 0.01$ vs. FA group.

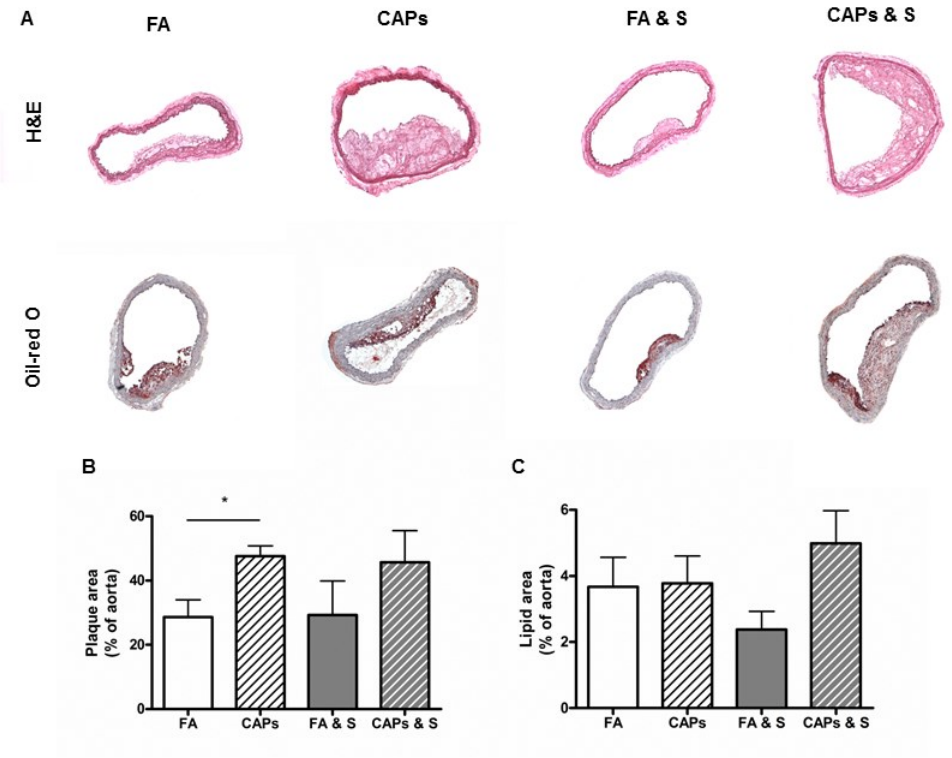

Figure 15. Effects of Strawberries on Plaque Development after $PM_{2.5}$ Exposure. (A) Representative images of H & E staining of abdominal aortic sections and Oil-red O staining of aortic arch sections. (B) Plaque area was analyzed from H & E staining. (C) Lipid area was analyzed from Oil-red O staining. H & E staining: hematoxylin-eosin staining; FA: filtered air; and CAPs: concentrated ambient particles; FA & S: filtered air plus strawberry supplementation; CAPs & S: concentrated ambient particles plus strawberry supplementation. * $p < 0.05$ vs. FA group.

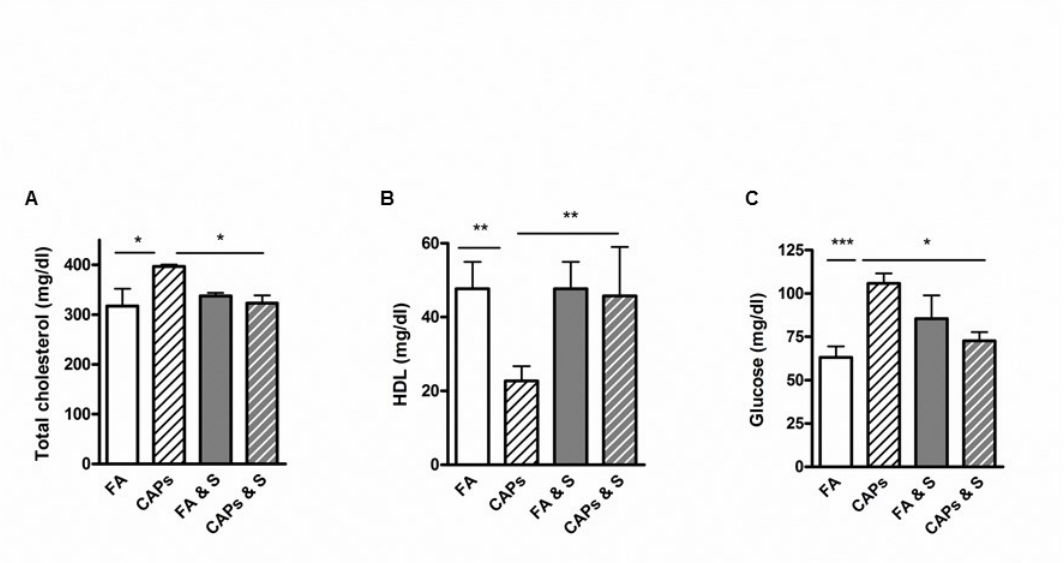

Figure 16. Effects of Strawberries on Serum Lipids and Glucose after $PM_{2.5}$ Exposure. (A) The concentration of serum cholesterol. (B) The concentration of serum HDL. (C) The concentration of fasting glucose. HDL: high-density lipoprotein; FA: filtered air; and CAPs: concentrated ambient particles; FA & S: filtered air plus strawberry supplementation; CAPs & S: concentrated ambient particles plus strawberry supplementation. * $p < 0.05$ vs. FA group; ** $p < 0.01$ vs. FA group.

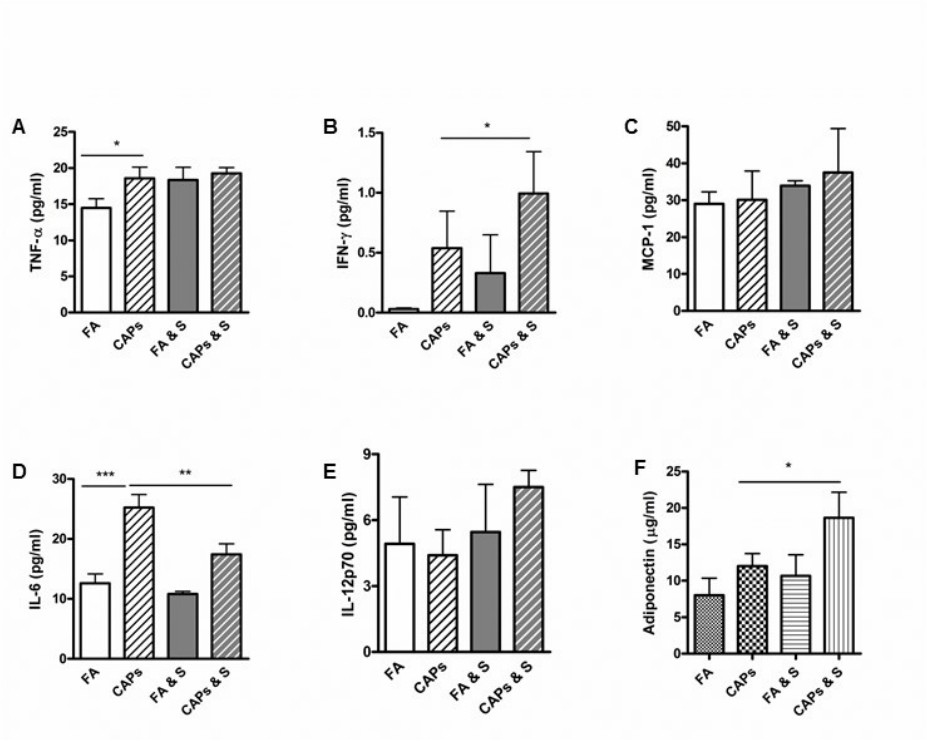

Figure 17. Effects of Strawberries on Plasma Levels of Inflammatory Cytokines after $PM_{2.5}$ Exposure. (A) The concentration of plasma TNF-α. (B) The concentration of plasma IFN-γ. (C) The concentration of plasma MCP-1. (D) The concentration of plasma IL-6. (E) The concentration of plasma IL-12. (F) The concentration of plasma adiponectin. TNF: tumor necrosis factor; IFN: interferon; MCP: monocyte chemoattractant protein; IL: interleukin; FA: filtered air; and CAPs: concentrated ambient particles; FA & S: filtered air plus strawberry supplementation; CAPs & S: concentrated ambient particles plus strawberry supplementation. ** $p < 0.01$ vs. FA group.

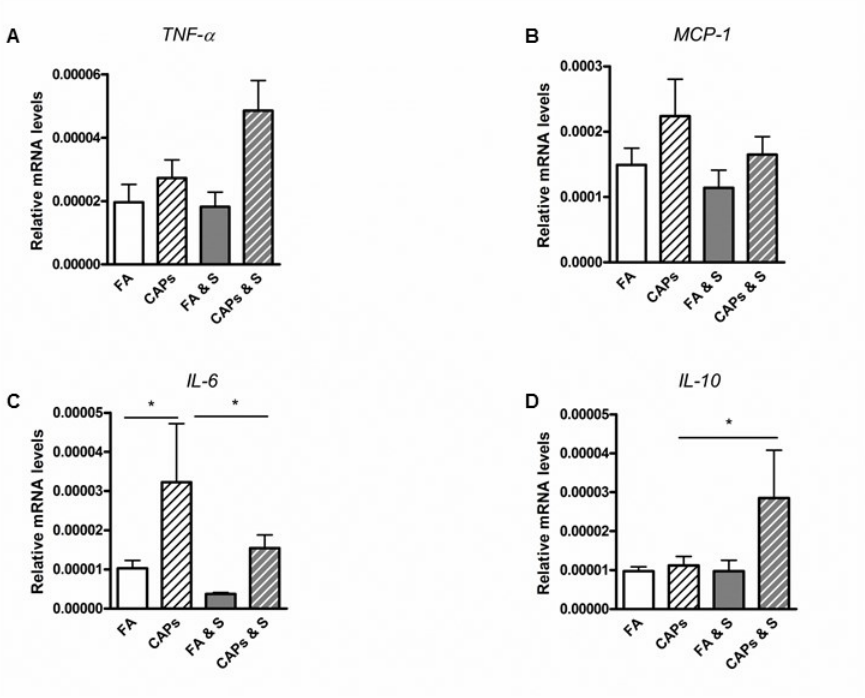

Figure 18. Effects of Strawberries on Relative mRNA Levels in Lung after $PM_{2.5}$ Exposure. (A) TNF-α mRNA level. (B) MCP-1 mRNA level. (C) IL-6 mRNA level. (D) IL-10 mRNA level. TNF: tumor necrosis factor; MCP: monocyte chemoattractant protein; IL: interleukin; FA: filtered air; and CAPs: concentrated ambient particles; FA & S: filtered air plus strawberry supplementation; CAPs & S: concentrated ambient particles plus strawberry supplementation. * $p < 0.05$ vs. FA group.

Chapter 8: Conclusions and Future Studies

This study demonstrates that our exposure system "OASIS" is suitable for monitoring the local $PM_{2.5}$ levels. The data collected via this system reveal the annual level and seasonal trend of $PM_{2.5}$ in Columbus, Ohio. Although there are some fluctuations, the levels of $PM_{2.5}$ in Columbus are below EPA standards. In addition, our data indicate that black carbon could be used as another marker to monitor local air pollution. Future studies of tracking emission sources and determining chemical-specific toxicological responses will help clarify the relationship between the compositions of $PM_{2.5}$ and human health effects.

In addition, our data show that "OASIS" only concentrates the ambient $PM_{2.5}$ without changing its chemical compositions, indicating that this system is reliable for mimicking the effects of air pollution in real world. Using genetic mouse model, this study provides compelling evidence supporting that long-term $PM_{2.5}$ exposure potentiated atherosclerosis and changed the lipid profile. We also identified TNF-α and IL-6 as the key inflammatory mediators in this process. These findings lead us to explore the protective effects of anti-inflammation using natural nutrients such as strawberry on the development of atherosclerosis after long-term $PM_{2.5}$ exposure.

Our data indicate that strawberry supplementation can improve serum lipid profile and reduce inflammation in response to long-term $PM_{2.5}$ exposure. However, the morphometric data show no effects of strawberry supplementation on $PM_{2.5}$-mediated

atherosclerosis. To elucidate the underlying mechanisms, several future studies will be needed. First, a relative moderate model involving one or two risk factors for atherosclerosis will be used to investigate the benefits of strawberries on cardiovascular function. Second, the effects of strawberries on the key cytokines, e.g. TNF-α and IFN-γ, will be robustly evaluated in response to long-term exposure to $PM_{2.5}$. Finally, other mechanistic pathways of $PM_{2.5}$-mediated atherosclerosis will be evaluated to explore the potential of strawberries.

The effects of cytokines on $PM_{2.5}$-mediated atherosclerosis remain largely unknown. Since most of cytokines have a very short-half life *in vivo*, it will be significant to monitor the dynamics of cytokines at different time-points, thereby evaluating their effects on atherosclerosis development. Cytokine-mediated signaling pathways will also be detected in future studies, such as TNF-α-activated NF-κB pathways. In addition, other significant molecules involving in development of atherosclerosis will be detected after $PM_{2.5}$ exposure. For instance, CD36, a fatty acid translocase, can bind to oxidized LDL and regulate LDL aggregation, contributing to atherosclerosis. Whether $PM_{2.5}$ exposure influences the level of this molecule remains unclear. Data generated from immunoblots or real time-PCR will be helpful to clarify this question.

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