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Liver injury induced in Balb/c mice by $PM_{2.5}$ exposure and its alleviation by compound essential oils

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ABSTRACT

Accumulating evidence has suggested a strong link between exposure to air pollution and public health. In particular, inhaled airborne particulate matter < 2.5 μm in aerodynamic diameter ($PM_{2.5}$) can rapidly diffuse from the lungs to the systemic blood circulation and accumulate in the liver. In this study, we used a Balb/c mouse model to investigate liver injury caused by $PM_{2.5}$ inhalation and the anti-inflammatory and antioxidant effects of compound essential oils (CEOs) in alleviating the extent of this injury. The results of serum biochemical and histopathological analyses showed that $PM_{2.5}$ exposure induced inflammatory liver injury, meantime CEOs pretreatment attenuated $PM_{2.5}$-induced liver inflammatory injury. Western blot and *q*RT-PCR assays showed that $PM_{2.5}$ increased secretion of cytokines, however CEOs suppressed the production of IL-6 and TNF-α. Furthermore, heme oxygenase-1(HO-1) and superoxide dismutase-1(SOD-1) expression levels showed that $PM_{2.5}$ could trigger oxidative stress-mediated liver injury, whereas CEOs pretreatment might protect against $PM_{2.5}$-induced liver injury through regulation of the antioxidant system. Molecular analysis showed that the expression of TLR4, a protein which plays a key role in liver health and injury. Results showed that TLR4 was promoted by $PM_{2.5}$ but inhibited by CEOs pretreatment in $PM_{2.5}$-induced inflammatory liver injury. In addition, $PM_{2.5}$-promoted secretion of cytokines by activating TLR4/MyD88 pathway, whereas CEOs might alleviate this type of liver inflammation inhibiting the activation of TLR4/MyD88 signaling pathway.

1. Introduction

Epidemiological studies have shown that air pollution peaks are related to increased rates of morbidity and mortality in the cities of many developed and developing countries [1–4]. Particulate matter (PM) with a diameter of no more than 2.5 μm ($PM_{2.5}$), the toxic component of urban air pollution, can exert a detrimental effect on public health [1,5]. Toxicological studies have revealed that the toxicity of $PM_{2.5}$ is a result of the combined effect of particles and the adsorbed toxic pollutants, such as biological components, particles, polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs) and heavy metals [6]. $PM_{2.5}$ can migrate from the lungs to the systemic blood circulation system and eventually accumulates in the liver [1,7]. $PM_{2.5}$-contributed air pollution can cause multifarious maladaptive signaling pathways in the liver, lung, adipose tissues and blood vessels that are connected to oxidative stress, endoplasmic reticulum (ER) stress and inflammatory responses [8].

It has been reported that exposure to external oxidant substances or the failure of self-defense mechanism can induce excessive production of reactive oxygen species (ROS), which in turn can trigger the production of oxygen-free radicals and eventually lead to induction of oxidative stress [9]. Recent studies have demonstrated that oxidative stress and oxygen-free radicals are associated with the causative agents of several diseases, including liver injury, heart disease, compromised immunity, inflammation, and cancer [10,11].

Toll-like receptors (TLRs) play significant roles in liver health [12]. Furthermore, TLR-mediated signals are associated with virtually all liver diseases [13]. TLR4 is the most widely studied TLR, which plays a harmful role in hepatic injury and inflammation. TLR4 is recognized as DAMPs ("damage-associated molecular patterns"), and it is released by

Abbreviations: $PM_{2.5}$, particulate matter < 2.5 μm in aerodynamic diameter; PM, particulate matter; CEOs, compound essential oils; HO-1, heme oxygenase-1; SOD-1, superoxide dismutase-1; PAHs, polycyclic aromatic hydrocarbons; VOCs, volatile organic compounds; ER, endoplasmic reticulum; ROS, reactive oxygen species; TLRs, Toll-like receptors; DAMPs, damage-associated molecular patterns; EOs, essential oils; PVDF, polyvinylidene fluoride

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stressed cells undergoing necrosis in liver injury, including oxidative stress, alcoholic steatohepatitis, and autoimmune liver diseases [14]. In addition, deposition of $PM_{2.5}$ in the liver can trigger oxidative stress-mediated liver injury [1]. The DAMPs generated by stressed liver cells undergoing necrosis can activate TLR4 signaling. TLR4 and its co-receptors may be expressed in high level, which might induce the production of chemokines and pro-inflammatory cytokines, consequently exacerbating liver inflammation and injury [14]. Myeloid differentiation factor 88 (MyD88), a downstream signal adapter protein of TLR4, is indispensable for cytokine release in response to TLR ligands [15,16]. In TLR4-signaling, MyD88 can induce the production of inflammatory cytokines through activating NF-κB1 [17]. Consequently, the activated TLR4/MyD88/NF-κB1 signaling pathway can also participate in the liver inflammatory response.

Essential oils (EOs) are complex mixture separated from fruit, stem, leaf and flower through distillation, extrusion or solvent extraction [6,18]. According to recently available scientific evidence, EOs isolated from medicinal plants have numerous applications in human health [19,20]. The primary constituents of EOs are terpenes, monoterpenes and sesquiterpenes [21,22], which have antioxidant, anti-inflammatory, antifibrogenic, anti-tumor activities respectively, therefore, these compounds can protect the liver against multiple diseases [23–27]. Although the effect of EOs on liver diseases caused by other factors (e.g., drugs and organic toxicants) has been investigated, few studies have focused on the protective effects of EOs on the liver under condition of oxidative stress and inflammatory injury induced by $PM_{2.5}$.

In this study, we used a Balb/c mouse model to study the priming of liver oxidative stress and inflammatory response by $PM_{2.5}$ in the blood circulation. In addition, we also evaluated the anti-inflammatory and antioxidant functions of CEOs (compound EOs formulated from Spruce, Mint, Frankincense and Eucalyptus EOs) in the mice that were exposed to $PM_{2.5}$ to determine if CEOs could offer protection against $PM_{2.5}$ induced liver diseases.

2. Materials and methods

2.1. Animal model

All animal experimental procedures were approved by the Animal Care and Use Committee of Dalian Medical University, and the experiments were carried out in accordance with the guidelines and principles of the National Institutes of Health Guide for the Care and Use of Experimental Animals. Male Balb/c mice (6–8 weeks old) weighing an average of 18–22 g were obtained from Changsheng Biotechnology Ltd (Shenyang, China). All the animals were housed under controlled laboratory conditions (temperature 23 ± 2 °C; 60% humidity; 12/12 h light: dark cycle) with free access to food and water.

2.2. Chemical reagents

Four types of EOs, each from a different plant (spruce, mint, frankincense and eucalyptus) and with a purity of 100%, were mixed together to yield a mixture designated as CEOs [6]. Different EOs were chosen on the basis of aromatherapy formulations, which have been shown to be clinically effective. All the EOs were provided by Absolute Aromas Ltd (4 Riverwey Alton GU34 2QL England). Rabbit polyclonal antibodies against TNFα, TLR4, HO-1 were purchased from Proteintech Group (Chicago, IL, USA). Rabbit anti-GAPDH monoclonal antibody was obtained from Minnesota (USA). HRP-conjugated goat anti-rabbit IgG was obtained from Proteintech Group (Chicago, IL, USA). The polymerase chain reaction (PCR) primers of IL-6, TNFα, TLR4, MyD88, NF-κB1, HO-1, SOD-1 and GAPDH as well as cDNA reverse transcription and real-time PCR kits were acquired from Takara Biotech (Otsu, Shiga, Japan). PVDF membrane was purchased from Millipore Corp. (Billerica, MA, USA), and Trizol was purchased from Invitrogen (Carlsbad,CA, USA).

2.3. $PM_{2.5}$ collection and preparation procedure

$PM_{2.5}$ was collected from February to April 2013 in Langfang, Hebei Province of China. $PM_{2.5}$ was collected and analyzed according to a previous study [6]. Detached $PM_{2.5}$ was then vacuum-freeze dried, weighed and stored at -20 °C [28]. To establish the mouse model, a suspension of $PM_{2.5}$ with a concentration of 10 mg/ml was prepared in sterile saline solution. The suspension of $PM_{2.5}$ was sonicated and votexed before each intratracheal instillation. Sonication was performed in an ultrasonic bath set at 28 Hz.

2.4. Experimental design

Ninety-six Balb/c mice were randomly divided into four groups (control, $PM_{2.5}$, $PM_{2.5}$ + saline, and $PM_{2.5}$ + CEOs groups), with each group consisting of 24 animals. In the control group, each animal was given 50 μl sterile saline on day 0 and day 2 via tracheal perfusion. In the $PM_{2.5}$ group, each animal was given 50 μl $PM_{2.5}$ suspension on day 0 and day 2 via tracheal perfusion. In the $PM_{2.5}$ + saline group, each animal was given 50 μl $PM_{2.5}$ suspension on day 0 and day 2 by tracheal perfusion, but these animals were also exposed to 200 μl sterile saline by static inhalation for 30 min per day one day before $PM_{2.5}$ perfusion and until they were sacrificed. In the $PM_{2.5}$ + CEOs group, the animals were treated as in the $PM_{2.5}$ + saline group, except that the 200 μl sterile saline also contained 2 drops of CEOs. Mice from each group were sacrificed and anesthetized with Nembutalon on day 3, day 7 and day 14. The liver and serum were collected and stored at -80 °C for later analysis.

2.5. Analysis of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

After anesthetization, blood was extracted from the heart of the animal and incubated for 30 min at 37 °C. The sample was then centrifuged at 1000 × *g* for 20 min and the supernatant (the serum fraction) was collected. The levels of ALT and AST in the serum were detected using an automatic biochemical analyzer (type 8020 A, URIT).

2.6. Pathological examination

A portion of the collected liver was cut into small pieces and fixed in 10% formaldehyde solution. They were dehydrated in a series of increasing concentrations of alcohol and dimethyl benzene, and then embedded in paraffin and sliced at 6–7 mm. After deparaffinization, the slices were stained with hematoxylin andeosin (H&E) and examined by a light microscope (Olympus BX-41, Tokyo, Japan).

2.7. RNA extraction and cDNA synthesis

Total RNA was extracted from the liver homogenate made from the remaining portion of the liver using Trizol reagent under the manufacturer's instruction. Complementary DNA was synthesized from the total liver RNA (1 μg) using a RrimeScript RT reagent with gDNA eraser kit (RR047 A; Takara) under the manufacturer's instruction.

2.8. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR was performed according to instructions provided in the SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (RR820 A; Takara). Each gene was assayed in duplicate. All the primers used are listed in Table 1. The amplification reaction was carried out using a TP800 Thermal Cycler Dice (Takara; Japan). The levels of target gene mRNAs were normalized against the level of GAPDH mRNA via the $2^{-\triangle\triangle Ct}$ method.