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Protective effects of methyl palmitate against silica-induced pulmonary fibrosis in rats

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ABSTRACT

Silicosis is one of the most prevalent chronic occupational pulmonary diseases worldwide. The present study aimed to investigate the effects of methyl palmitate on silica-induced lung fibrosis in rats and explore the possible mechanisms. Male Sprague–Dawley rats were divided into 3 groups: group I served as control and group II served as positive control where rats were intranasally instilled with a single dose of silica suspension (50 mg in 0.1 ml saline/rat). Rats of group III received methyl palmitate (300 mg/kg, I.P. three times per week at alternating days) for 60 days after instillation of silica. At the end of the treatment period, animals were sacrificed then biochemical parameters and histopathology were assessed. Treatment with methyl palmitate attenuated silica-induced lung inflammation and fibrosis, as it significantly decreased lactate dehydrogenase (LDH) activity and the accumulation of the inflammatory cells in the bronchoalveolar lavage fluid (BALF). Methyl palmitate significantly reduced collagen deposition as indicated by a decrease in lung hydroxyproline content and marked attenuation in silica-induced lung fibrosis in histopathological results. In addition, methyl palmitate significantly increased superoxide dismutase (SOD) and reduced glutathione (GSH) activities with a significant decrease in the lung malondialdehyde (MDA) content. Methyl palmitate also reduced silica mediated overproduction of pulmonary nitrite/nitrate (NO_2^-/NO_3^-). Importantly, methyl palmitate decreased the level of the inflammatory cytokine tumor necrosis factor-alpha (TNF-α) in the lung. Taken together, these results demonstrate the potent protective effects of methyl palmitate against silica-induced lung fibrosis. This effect can be attributed to methyl palmitate's ability to counteract the inflammatory cells' infiltration and hence reactive oxygen species (ROS) generation and regulate cytokine effects.

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1. Introduction

Silicosis is among the oldest chronic occupational pulmonary diseases worldwide. It occurs due to the inhalation of crystalline silica (silicon dioxide) which is found as a component of rock or sand. Persons who are exposed to silica as sandblasters, rock miners, quarry workers and stonecutters are considered high risk groups. Silicosis is characterized by inflammation and fibrosis of the lung [1,2]. After inhalation of silica particles, silica particles are engulfed by alveolar macrophages, which are activated and release inflammatory mediators as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), lipid mediators, oxygen-derived free radicals, various reactive oxygen species (ROS) and fibrogenic cytokines [3,4]. These released cytokines activate inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO) [5]. The free radicals released by the activated macrophages produce an increase in lipid peroxidation and damage to pulmonary structure. Recently, the role of ROS and oxidative damage in silica-induced pulmonary inflammation, fibrosis, and carcinogenesis has been extensively studied. Silica-induced ROS formation led to the depletion of reduced glutathione (GSH) and impairment of the antioxidant system, which, in turn, exacerbates the oxidative damage in silica-exposed cells [2,6]. Increased levels of ROS/reactive nitrogen species (RNS) [7] and cytokines [4] can be used as indicators of silicosis. Another important indicator of the cytotoxicity of silica is lactate dehydrogenase (LDH) leakage. LDH leakage from cells indicate the penetration of particles into the cells and cell membrane damage [8]. It has been well documented that LDH level (as a marker of necrosis) in the cell medium elevated after the cells were exposed to nanoparticles [9].

Methyl palmitate is an endogenous naturally occurring fatty acid methyl ester [10]. This compound is reported to have the ability to inhibit Kupffer cells which are the resident macrophages of the liver playing an important role in regulating the inflammatory process as

Abbreviations: BALF, Bronchoalveolar lavage fluid; LDH, Lactate dehydrogenase; ROS, Reactive oxygen species; MDA, Malondialdehyde; SOD, Superoxide dismutase; GSH, Reduced glutathione; NO_2^-/NO_3^-, Nitrite/nitrate; iNOS, Inducible nitric oxide synthase; TNF-α, Tumor necrosis factor-alpha.

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it inhibits the secretion of TNF-α and NO [11,12]. Recently, methyl palmitate has been shown to possess potent anti-inflammatory and anti-fibrotic effects through multiple pathways. It prevented lung fibrosis induced by bleomycin in rats as it attenuated the severity of oxidative stress and inflammatory response by decreasing the expression of nuclear factor-κB (NF-κB) and subsequent proinflammatory cytokine production [13]. The present study was undertaken to test the potential protective effects of methyl palmitate treatment against the development of silica-induced pulmonary fibrosis and to investigate the possible mechanism(s).

2. Materials and methods

2.1. Drugs and chemicals

Crystalline silica was generously supplied by U.S. Silica Company (Berkeley Springs, West Virginia, USA). Methyl palmitate (Sigma-Aldrich, St. Louis, MO, USA) was kindly provided by *Dr. Ebtehal El-Demerdash*, and was dissolved in corn oil with vortex. All other chemicals and bio-chemicals used in this study were of high analytical grade.

2.2. Experimental animals

Male Sprague Dawley rats (150–200 g) were purchased from "Egyptian Organization for Biological Products and Vaccines", Giza, Egypt. The animals were housed (4 per cage) in an air-conditioned room maintained at 25 ± 2 °C with a regular 12 h light/12 h dark cycle. All procedures involving the animals were conducted in accordance with the protocol approved by the committee of animal experimentation of the Faculty of Pharmacy, Mansoura University.

2.3. Experimental design

2.3.1. Silica preparation and injection

The silica dust was suspended in saline (50 mg in 0.1 ml saline/rat) [14], then 20,000 IU penicillium was added to the silica suspension. The suspension was shaken vigorously by a vortex shaker, prior to the administration. The animals were lightly anesthetized with diethyl ether [15], and then were given silica by intranasal instillation [16]; normal animals received an equal volume of sterilized saline instead of silica suspension.

2.3.2. Animal groups

Rats were divided into three groups (n = 8): 1) control; 2) silica and 3) silica + methyl palmitate. Rats were administered methyl palmitate intraperitoneally (300 mg/kg) three times per week at alternating days [13,17]. The dosage regimen of methyl palmitate was selected based upon previous studies which reported that this dosage scheme can produce marked antioxidant, anti-inflammatory and even potent antifibrotic effects [13]. Drug treatment continued for an experimental period of 60 days while animals of the control and silica groups received the vehicle intraperitoneally once daily. At the end of the treatment period, the rats were anesthetized by inhalation of diethyl ether, and the chest was opened and the trachea with the heart–lung package was excised from the thorax then the left main bronchi were clamped. A cannula was inserted into the trachea in situ, and the right lung was lavaged with sterile 0.9% saline. The volume of saline used for bronchoalveolar lavage (BAL) was 6 ml and bronchoalveolar lavage fluid (BALF) was collected for subsequent analysis. After BALF collection, the right lung tissues were rapidly removed, snap frozen in liquid nitrogen, and stored at −80 °C until subsequent analysis. Before the experiment, the lung tissue samples were weighed and homogenized (1:10, w/v) in 0.1 M phosphate buffer (pH 7.4) in an ice bath. The homogenate was centrifuged at 3000 *g* for 20 min at 4 °C, using a cooling centrifuge (Damon/IEC Division, Model: CRU-5000, Needham, Mass., USA). Biochemical assays were performed on the supernatant of the lung homogenate.

2.4. Measurement of lung wet/dry weight (W/D) ratio

The wet left upper lung was excised, blotted dry and weighed to calculate "wet" weight, and then placed in an oven at 80 °C for 24 h to calculate the "dry" weight. The lung W/D ratio was calculated to evaluate tissue edema.

2.5. Bronchoalveolar lavage fluid (BALF)

BALF fractions were centrifuged (4000 rpm, 10 min, 4 °C) using cooling centrifuge to collect the cell pellet for the total cell count determination. The supernatants of the BALF were stored at −80 °C until required for determination of protein content and LDH activity.

2.6. Measurement of cell counts, total protein and LDH activity in BALF

2.6.1. Total cell count

The cell pellets obtained after centrifugation of the BALF were resuspended in 100 μl of saline, centrifuged onto slides and stained for 8 min with Wright–Giemsa staining. Differential cell count was carried out through quantification of the slides for neutrophils and lymphocytes by counting a total of 200 cells/slide at 40× magnification.

2.6.2. Protein content

The total protein concentration was measured using a commercial kit (Thermo Scientific, Rockford, USA) in the supernatant of BALF.

2.6.3. LDH activity

The LDH activity was assessed using a commercial kit (Human Gesllschaft fur Biochemica und Diagnostica, Germany). In brief, the reaction mixture consisted of NADPH (0.8 mmol/l), and sodium pyruvate (1.5 mmol/l) and TRIS buffer (50 mmol/l, pH 7.4) was added to the sample. The changes in absorbance were recorded at 340 nm and enzyme activity was calculated and expressed in U/l.

2.7. Histopathological examination of lung

The left lower pulmonary lobe was harvested and flushed with phosphate buffered saline then fixed in 10% neutral-buffered formalin for 24 h, embedded in paraffin wax, and sectioned (6 μm) and stained with hematoxylin–eosin (H&E) and Masson's trichrome. The tissues were examined under a microscope in a random order and without knowledge of animal or group.

The structural alterations of tissue were assessed based on the degree of cellular proliferation, alveolar wall thickening, inflammatory lesions and collagen deposition or fibrosis. Such changes were graded in terms of severity and distribution. The grading system adopted is as follows and was utilized for each group of animals [14].

> *For severity of lesions*: 0 = nothing/zero, 1 = marginal, 2 = slight, 3 = moderate, 4 = severe, 5 = very severe. *For distribution of lesion over the tissue*: 0 = absent, 1 = rare/occasional (10% of the lung area), 2 = sparse/limited (10–25% of the lung area), 3 = moderate (25–50% of the lung area), 4 = extensive/widespread (50–75% of the lung area), 5 = very extensive/predominant (over 75% of the lung area).

2.8. Hydroxyproline assay

To assess the degree of collagen deposition, hydroxyproline content of the lung was determined by the colorimetric method as described previously [18] using base rather than acid hydrolysis for the dissolution of

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