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Original Article

Icariin attenuates carbon black-induced pulmonary inflammatory response and oxidative stress in mice

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Abstract: Particulate matter (PM) exposure is closely associated with mortality and morbidity related to pulmonary and cardiovascular diseases. Icariin has been shown to play a beneficial role in pulmonary and cardiovascular diseases. Here, we assessed the protective effect of icariin on mice exposed to ultrafine carbon black (CB), one of the main components in PM. Mice were subjected to a single intratracheal instillation of CB (50 μg), followed by icariin treatment. Twenty-four hours post-exposure, lung tissues and the bronchial alveolar lavage fluid (BALF) were collected for subsequent histopathological and molecular analyses. Instillation of CB caused significant increase in BAL neutrophils, macrophages, and lymphocytes, with concomitant elevation of total protein levels, lactic dehydrogenase (LDH) release, and proinflammatory mediators (TNF-α, IL-6, and IL-1β). Furthermore, CB increased the MDA content while decreased the activity of antioxidant superoxide dismutase (SOD) in lung tissues. Compared to CB group, icariin treatment significantly reversed these elevations induced by CB and increased the activity of SOD in lung tissues. Taken together, our results indicate that icariin exerts protective effects on CB-induced pulmonary inflammation and cytotoxicity, which might be due to its anti-inflammatory and anti-oxidant properties.

Keywords: Icariin, carbon black, lung, inflammation, oxidative stress

Introduction

Numerous epidemiological studies have shown a link between particulate matter (PM) exposure and the increased adverse health effects, especially on pulmonary and cardiovascular diseases [1-3]. PM with aerodynamic diameter less than 100 nm are commonly defined as ultrafine particles, it has been considered that the ultrafine particles exert more deleterious impacts as they have more potential to cross the pulmonary epithelium then enter the systemic circulation [4, 5]. Nevertheless, regarding the complex chemical composition of PM [6], most experimental studies have chosen some particles as surrogates for PM, which are well characterized for their physical and chemical properties. The ultrafine carbon black (CB) is one of the best studied materials in particle toxicology, and it is a core component of PM [7]. Previous studies have shown that CB exposure causes pulmonary inflammation and functional impairments in both human and experimental animals [7, 8]. Moreover, CB exposure could aggravate lung damages in several respiratory diseases, such as pulmonary fibrosis [9], allergic airway disease [10], and LPS-induced acute lung injury [11]. Therefore, we used the ultrafine CB as a model ultrafine particle for their toxicity.

Icariin, the main constituent of Epimedium extract, has been shown to exert numerous pharmacological activities, such as anti-inflam-

matory [12], antioxidant [13], antitumor [14], and cardiovascular protective properties [15]. In addition, icariin shows therapic role in respiratory diseases. For example, icariin was able to alleviate acute lung injury in LPS-treated mice [16]. Icariin exerted protective effects in ovalbumin-induced mouse asthma model [17]. Icariin also suppressed pulmonary inflammation and oxidative stress induced by cigarette smoke[18, 19]. Based on these findings, we speculate that icariin may protect the lung injury caused by ultrafine CB particle.

This study was conducted to investigate the effects of icariin intervention on mice exposed to ultrafine carbon black (CB). The evaluation of icariin on CB-induced inflammation and oxidative stress will enlight the application of icariin in PM-medicated adverse health effects.

Materials and methods

Characteristics of ultrafine CB

CB was purchased from Degussa (Printex 90; Dusseldorf, Germany), it has a primary particle diameter of 14 nm and surface area of 300 m^2/g, containing less than 1% organic and inorganic impurities. CB suspension was prepared just before using, the CB particles were suspended at a concentration of 1 mg/ml in 0.9% pyrogen-free saline.

Animals and treatment

All animal care and experimental procedures in this study were in accordance with the guidelines by the Animal Care and Committee of Harbin Medical University. BALB/c mice aged 8 weeks (n = 32) were purchased from Harbin Medical University and randomly divided into 4 groups: the control group, CB infused group, icariin 50 mg/kg treatment group, and icariin 100 mg/kg treatment group. Intratracheal instillation of CB was performed as described previously [20]. Briefly, after chloral hydrate anesthesia (350 mg/kg, intraperitoneally, i.p.), mice were intratracheally instilled with 50 μl CB solution (containing 50 μg of CB) using a bulb-headed cannula, followed by 100 μl air. Mice in control group received the same volume of vehicle only. Subsequently, mice in icariin treatment groups were subjected to intraperitoneally injection of icariin 50 mg/kg or 100 mg/kg, respectively. The dose of icariin chose here was based on previously reports that the dose of icariin was receivable and could protect against acute or chronic pulmonary inflammation in mice [16, 18]. Twenty-four hours after instillation, mice were sacrificed, the lungs and bronchoalveolar lavage fluid (BALF) were obtained for following analyses.

Pulmonary histopathological analysis

Lung tissues were harvested 24 h after CB instillation, then fixed in formalin, embedded in paraffin, and sectioned. These sections were stained with hematoxylin and eosin (H&E), pathological changes in lung tissues were observed under a light microscope (DP73, Olympus, Japan).

Bronchoalveolar lavage fluid (BALF) collection and cell counting

Bronchoalveolar lavage fluid (BAL) was performed at 24 h after CB instillation. The lung was lavaged three times with cold sterile PBS, a total volume of 2.7 ml bronchoalveolar lavage fluid (BALF) per mouse was collected and centrifuged at 1500 rpm for 10 min. After centrifugation the cell pellet was resuspended in PBS for total cell counts using a hemacytometer, and the differential cell counts were prepared using Wright-Giemsa staining method (Nanjing Jiancheng, Nanjing, China). The supernatant was harvested for total protein, and Lactate dehydrogenase (LDH) analyses.

Total protein and LDH activity measurement

Total protein in the BALF was performed by a Bradford assay according to the manufacturer's instructions (Beyotime, Haimen, China). The protein concentration was determined by comparison with a standard curve for bovine serum albumin (BSA). The activity of LDH was assessed using an assay based on monitoring the decrease of nicotinamide adenine dinucleotide (NADH) at 340 nm in a spectrophotometer (Nanjing Jiancheng).

Cytokines analysis

The concentrations of TNF-α, IL-1β, and IL-6 were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits

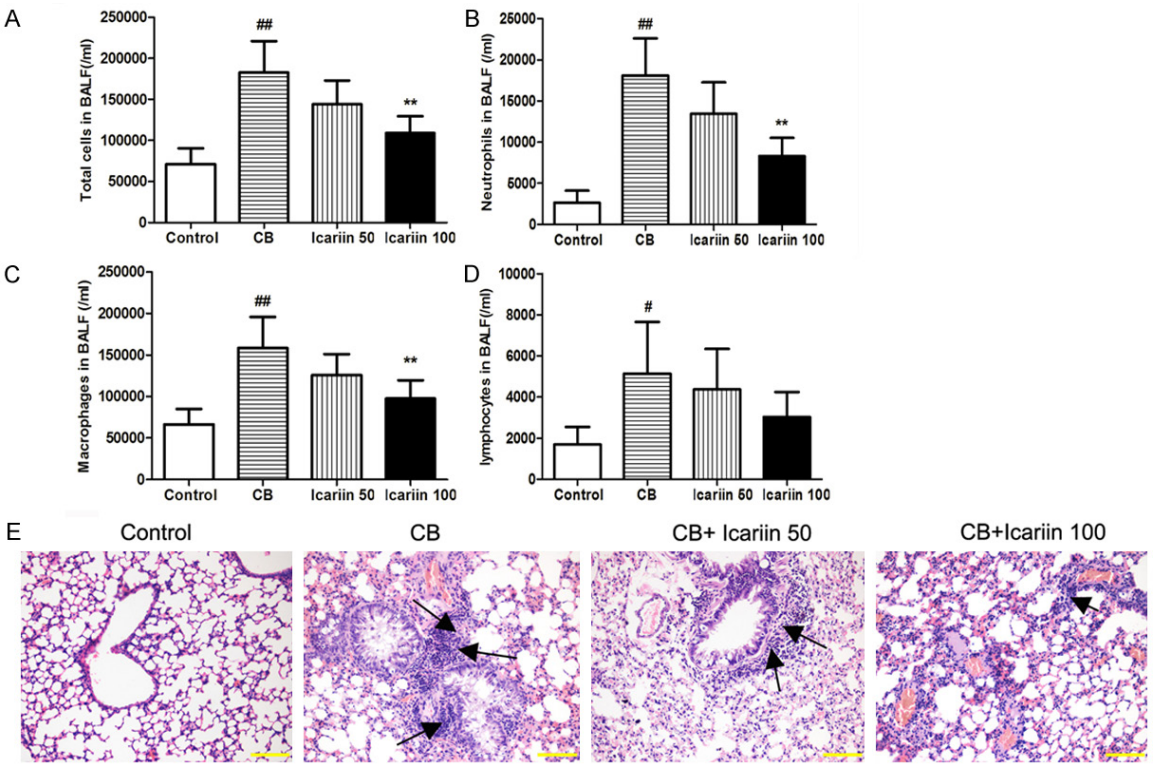

Figure 1. Effect of icariin on cell counts and pulmonary histopathologic changes in mice exposed to CB. BALF were obtained from mice in control, CB exposed, CB+ icariin (50 mg/kg), and CB+ icariin (100 mg/kg) groups. The number of total cells (A), neutrophils (B), macrophage (C), and lymphocytes (D) were measured 24 h after CB exposure. (E) Histopathological changes in lung tissues from mice of different groups were assessed by Hematoxylin and eosin staining (magnification 200×, scale bar = 50 μm). Data are shown mean ± SD of triplicate experiments. #P<0.05, ##P<0.01 represents significant differences compared with the control group, **P<0.01 represents significant differences compared with the CB group.

according to the manufacturer's instructions. Absorbance was measured at 450 nm in ELX-800 microplate reader (Biotek Instruments, Winooski, VT, USA).

MDA and SOD measurement

Lung tissue samples were homogenized, the content of malondialdehyde (MDA) and the activity of super oxide dismutase (SOD) in the homogenates were determined using commercial assay kits (Nanjing Jiancheng) according to the protocol instructions.

Statistical analysis

Data are presented as the mean ± standard deviation and processed using GraphPad Prism 5.0 software (San Diego, CA, USA). Differences among groups were performed using a one-way analysis of variance (ANOVA), and multiple comparisons were performed using the Bonferroni hoc test. Values of $P<0.05$ was considered statistically significant.

Results

Icariin treatment reduces inflammatory cell influx in BALF

Inflammatory cell counting and differential were performed by Wright-Giemsa staining. As shown in **Figure 1A-D**, CB instillation markedly increased the total cell numbers in BALF, especially in alveolar macrophages and neutrophils as compared to the control group. There was also minor increase in the number of lymphocytes in BALF after CB instillation. However, high concentration of icariin effectively reduced the cell numbers induced by CB ($P<0.01$). There was no significant difference between low concentration of icariin and CB groups ($P>0.05$). Pulmonary histologic changes revealed that mice treated with CB presented

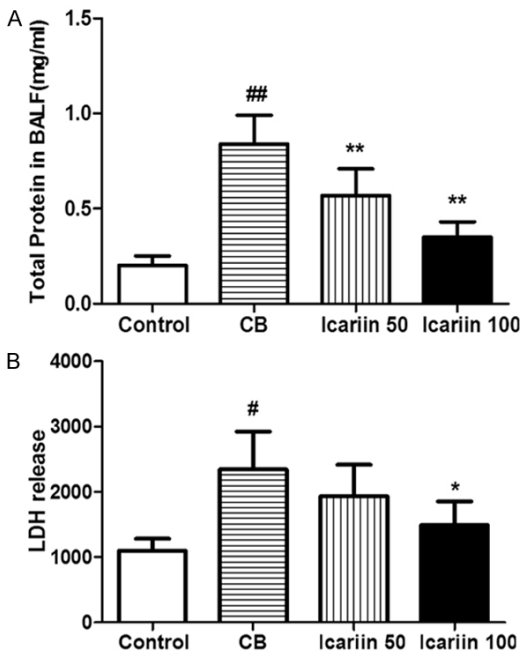

Figure 2. Icariin alleviates pulmonary injury induced by CB instillation. (A) Total protein content and (B) LDH release in the BALF were determined to assess the pulmonary epithelial permeability and cytotoxicity induced by CB exposure. Data are shown mean ± SD of triplicate experiments. #P<0.05, ##P<0.01 represents significant differences compared with the control group, *P, 0.05, **P<0.01 represents significant differences compared with the CB group.

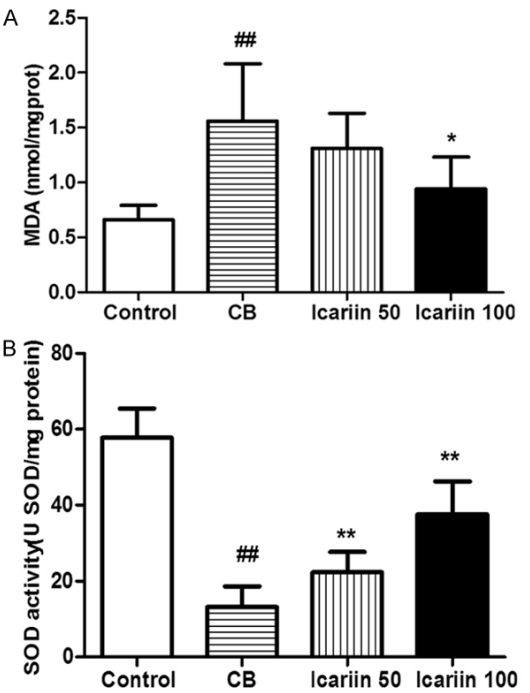

Figure 3. Icariin alleviates CB-induced oxidative stress. Mice were exposed to ufCB, followed by ICA treatment. After 24 h, (A) MDA, and (B) SOD activity in lung tissues were analyzed. Data are shown mean ± SD of triplicate experiments. #P<0.05, ##P<0.01 represents significant differences compared with the control group, **P<0.01 represents significant differences compared with the CB group.

overt histopathologic changes, such as inflammatory cells infiltration into alveolar space and interstitial. In contrast, Icariin ameliorated these pathologic changes compared with CB group (**Figure 1E**), indicating a protective effect of icariin on mice acutely exposed to CB.

Icariin alleviates pulmonary injury induced by CB

CB-induced pulmonary epithelial permeability and cytotoxicity were assessed by protein accumulation and LDH activity in the BALF. Data showed that CB instillation induced a significant increase in total protein level in the BALF; whereas, icariin dose-dependently reduced the protein content (**Figure 2A**). Results from LDH assay further revealed that CB exposure caused significant pulmonary cytotoxicity, as characterized by increased LDH levels in the CB group. However, high-dose of icariin was able to lighten the cytotoxicity induced by CB. There was no significant difference in LDH levels between CB group and low dose of icariin treatment group (**Figure 2B**).

Icariin treatment prevents CB-induced oxidative stress

MDA and SOD activity were assessed as indicators of oxidative stress. Exposure to CB caused a significant increase in MDA level and a reduction in SOD activity in lung tissues, the level of MDA was 1.56 ± 0.52 nmol/mg, SOD activity was 13.22 ± 5.38 U/mg in CB groups. The control animals presented low level of MDA (0.66 ± 0.13) and high SOD activity (57.82 ± 7.6 U/mg). After icariin treatment, the MDA content in high dose of icariin group was significantly reduced compared to that in CB group. Concurrently, the SOD activity was markedly elevated in animals with either low or high dose of icariin treatment (**Figure 3A**, **3B**).

Icariin inhibits the production of proinflammatory mediators induced by CB

The concentrations of TNF-α, IL-1β, and IL-6 were analyzed at 24 h post CB exposure. As shown in **Figure 4A-C**, CB exposure markedly increased the production of TNF-α, IL-1β, and

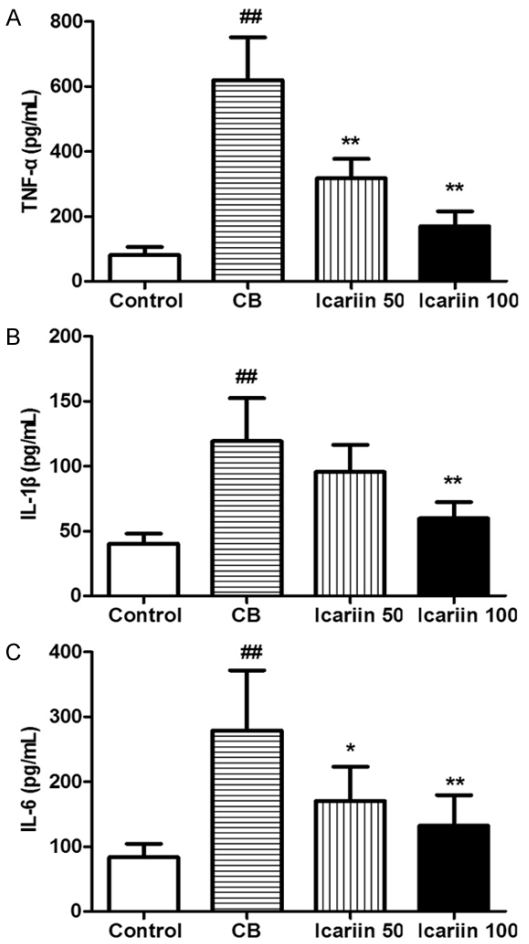

Figure 4. Icariin inhibits the production of proinflammatory mediators induced by CB. Mice were instilled intratracheally with CB (50 μg), followed by icariin treatment. 24 h after exposure, the concentrations of TNF-α (A), IL-1β (B), and IL-6 (C) were determined. Data are shown mean ± SD of triplicate experiments. ##P<0.01 represents significant differences compared with the control group, *P, 0.05, **P<0.01 represents significant differences compared with the CB group.

IL-6. Levels of TNF-α, IL-1β, and IL-6 in CB group were 7.6-, 2.9-, and 3.4- fold higher than control groups, respectively (P<0.01). Whereas, icariin (100 mg/kg) treatment significantly reduced the production of TNF-α, IL-1β, and IL-6. There was no significant statistical significance between the low dose of icariin and CB only groups.

Discussion

Previous studies have shown the anti-inflammatory and anti-oxidant roles of icariin in lung diseases. In agreement with these reports, here we demonstrated that icariin could alleviate CB-induced pulmonary inflammation and oxidative stress in mice. Icariin treatment distinctly diminished CB-induced inflammatory response, as evidenced by significant decreases in inflammatory cells influx, LDH release, and the expression of TNF-α, IL-1β, and IL-6. In addition, icariin treatment effectively reduced the products of lipid perxidation (MDA) while increased the activity of SOD. Our results indicate that icariin exerts beneficial effect on mice acute exposure to CB particle.

CB exposure can trigger multifaceted adverse impacts, with lung the main target organ. CB exposure can result in pulmonary inflammation, oxidative stress, and DNA damage [21-24]. PM-mediated inflammation is associated with inflammatory cells influx, such as neutrophils and macrophages [25]. During the process, the recruited inflammatory cells produce cytokines and reactive oxygen species, which in turn act on the immune cells and result in aggravation of inflammatory cascades [26, 27]. A grown body of literature has shown that CB exposure induces elevated levels of cytokines like TNF-α, IL-1β, and IL-6 [8, 28, 29], and the inflammatory lesions are dependent on the mass and surface of CB particle [8]. Bourdon et al previously reported that single dose instillation of CB (54 μg) could induce acute pulmonary inflammatory response, and the inflammation persistently insisted up to 28 days [30]. To characterize the inflammatory responses and lung injury induced by CB exposure, we assessed the pulmonary inflammation by determining total cell counts, cell differentials, and accumulation of inflammatory cytokines, including TNF-α, IL-1β, and IL-6 in the BALF. Pulmonary toxicity and was assessed by elevation of LDH and total protein levels in BALF. Our results showed that a single intratracheal instillation of ultrafine CB (50 μg) was feasible to produce pulmonary inflammatory lesions. The inflammatory responses included 1) increase in total cell numbers and neutrophilic influx, 2) production of TNF-α, IL-1β, IL-6, and, 3) elevation of total protein and LDH levels. Our study supports the findings of previous studies showing that ultrafine CB was highly inflammogenic [31, 32]. Icariin treatment (100 mg/kg) markedly reduced the number of neutrophils and macrophages, decreased the levels of TNF-α, IL-6, and IL-1β. These results suggest that icariin exerts anti-inflammatory role in acute inflam-

mation, which is partially through modulation of cell infiltration and inflammatory cytokines.

The oxidative stress has been identified as an important mechanism of particle toxicity [33, 34]. Lipid peroxidation is an important consequence of oxidative stress. During this event, the cell membranes were attacked by free radicals, resulted in the formation of by-products like MDA [35]. Lipid peroxidation disturbs the integrity of cellular membranes and lead to the leakage of cytoplasmic enzymes, such as LDH [36]. A large number of antioxidants such as SOD play a central role in preventing the oxidative stress [37]. Our results showed that CB exposure induced a significant elevation of MDA levels and caused reduction in SOD activity as compared with the control group. However, the elevation of MDA was abolished by icariin intervene. Furthermore, icariin treatment alleviated the levels of LDH caused by CB. The results further revealed that icariin protected against CB-induced cytotoxicity and oxidative stress in the lung of mice.

In summary, our results indicate that icariin attenuated CB-induced pulmonary damage, including inhibition of inflammatory cells infiltration, inflammatory cytokines expression, and oxidative stress. The evaluation of icariin on CB-induced inflammation and oxidative stress will enlight the application of icariin in PM-medicated adverse health effects.

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Disclosure of conflict of interest

None.

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