Original Article
Edaravone attenuates smoke inhalation injury in rats by the Notch pathway

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Abstract: Objective: To explore the protective effect of inhaled edaravone (EDA) on inflammation, oxidative stress (OS), and pulmonary function (PF) in rats after smoke inhalation injury (SII), as well as its mechanisms. Methods: Twenty-four rats were designated as group A (model group), group B (EDA control group), group C (low-dose group) and group D (high-dose group) (n=6 for each group). SII models were induced in all groups. After successful modeling, rats in each group were treated accordingly. After 6 hours of modeling, assessments of PF, oxygenation index (OI), inflammatory cytokine expression, oxidative stress index (OSI), wet/dry weight ratio (W/D), total lung water (TLW), and the expression of Notch markers were carried out. Results: Compared with group A, the remaining groups had higher peak respiratory velocity (PEF), forced expiratory volume in the first second (FEV1), FEV1/forced vital capacity (FVC) and OI, as well as lower W/D and TLW; levels of serum superoxide dismutase (SOD), malondialdehyde (MDA), tumor necrosis factor-α (TNF-α), and interleukin (IL)-6 decreased, and those of serum myeloperoxidase (MPO) and IL-10 increased. Levels of PEF, FEV1, FVC, OI, MPO, and IL-10 were higher in group A than in groups C and D, and those of W/D, TLW, SOD, MDA, TNF-α, and IL-6 were lower. Levels of Notch markers NICD, Hes1 and Hes5 were downregulated in groups B, C, and D, and in group B were lower than those in groups C and D. Conclusion: Inhaled EDA is able to alleviate inflammation and OS and effectively improve PF in rats after SII, possibly by inhibiting the Notch pathway.

Keywords: Edaravone, Notch pathway, smoke inhalation injury

Introduction

It is reported that millions of people worldwide experience burns every year, and ~300,000 die [1]. Smoke inhalation injury (SII) is a major cause of death in fires. Smoke contains various harmful components that result in damage to the respiratory tract and lungs [2, 3]. Although constant advances have been achieved in the treatment of SII, yet there is no standard therapy [4]. Therefore, SII remains a great challenge to clinicians.

Edaravone (EDA) is a novel free radical scavenger developed by Mitsubishi Pharma (Japan), and listed in 2001 [5]. EDA can eliminate free radicals in patients with acute cerebral infarction, protect against lipid peroxidation, and inhibit oxidative damage in the brain, vascular endothelium and nerve cells, thereby relieving cerebral edema and infarction induced by ischemia [6, 7]. Excessive release of free radicals, cytokines, and other inflammatory mediators is a trigger for the aggravation of SII [8]. Therefore, as a free radical scavenger, EDA may be effective in treating SII. There has been evidence that EDA has therapeutic effects on SII in rats [9], however, the mechanism has not been discussed in depth. Notch pathway is highly conserved and participates in many physiologic and pathologic processes [10]. Also, it is activated in smoke-induced lung injury in rats [11], and its inhibition leads to the alleviation of inflammation and injury of the lungs following mechanical ventilation [12]. These indicate the significant role of Notch pathway in lung injury. Therefore, we hypothesize that EDA...
may have a protective effect in SII by regulating the Notch pathway.

The present study aims to estimate the effect of EDA in SII, and to identify the relationship between EDA and Notch pathway. Our findings suggest that EDA is able to alleviate inflammation and oxidative stress (OS) and effectively improve pulmonary function (PF) in rats after SII, apparently achieved by inhibiting the Notch pathway.

Materials and methods

Animal selection

Male SD rats (Vital River, Beijing, China), weighing 250-300 g, were housed at a room temperature of 21-26°C and a relative humidity of 51-57%, with a free diet and natural light exposure. Ethics approval of this animal experiment was granted by our hospital.

Rat grouping and treatment

Grouping: Twenty-four SD rats were allocated into four groups: group A (model group), group B (EDA prevention group), group C (low-dose group) and group D (high-dose group) (n=6 for each group). Treatment: (1) Modeling: A smoke generator filled with white pine sawdust was turned on to generate smoke for 15-20 minutes. Afterwards, the rats anesthetized by intraperitoneal injection of 100 mg/kg ketamine were put into the smoke box for 5 minutes and taken out. After 2 minutes, they were put into the smoke box again. In group B, white pine sawdust was not used, and other steps were the same as described above. (2) Intervention: The rats in group B were allowed to inhale 0.5 mg/ml atomized EDA for 30 minutes before the modeling. After the modeling, the rats were placed in a ventilated place for 30 minutes until their full restoration of consciousness and breath. The rats in group C inhaled 0.5 mg/ml atomized EDA for 30 minutes; those in group D were given 1 mg/ml atomized EDA for 30 minutes; while those in group A received an equal volume of distilled water. No deaths were reported during modeling and intervention; and rats were allowed to move freely within 6 hours after injury.

Outcome measures

Six hours after injury, PF indexes, including peak respiratory velocity (PEF), forced expiratory volume in the first second (FEV1) and FEV1/forced vital capacity (FVC), were assessed with an animal pulmonary function tester (Ranger Apparatus Co., Ltd., Shanghai, China, RZ-flexi). In addition, arterial blood gas analysis was performed with the Roche Cobasb analyzer.

Blood sampled from the femoral artery was centrifuged (1500×g, 4°C, 10 minutes) to collect sera. Enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of inflammatory cytokines (TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; IL-10) and oxidative stress indexes (OSI) (MDA: malondialdehyde; SOD: superoxide dismutase; MPO: myeloperoxidase).

The rats were sacrificed for tissue sampling. The left lung was weighed, and wrapped in tin foil and placed in a drying oven at a constant temperature of 65°C for 72 hours. After weighing, the wet/dry weight ratio (W/D) and total lung water (TLW) were calculated. Any gross pathologic changes in lung tissues were monitored. The posterior lobe of the right lung was fixed in 4% neutral-buffered formaldehyde for 24 hours, then paraaffin-embedded, sectioned (4 μm), and observed under a light microscope after conventional H&E staining.

Levels of Notch markers NICD, Hes1, and Hes5 were quantified by western blotting (WB). Total protein was extracted by radioimmunoprecipitation assay (RIPA; Thermo Scientific, USA), and the concentration was detected by bicinchninic acid assay (BCA; Thermo Fisher, USA). Polyacrylamide gel electrophoresis (10%, 120 v) was carried out on 40 μg sample, and then the protein was moved to a polyvinylidene difluoride (PVDF) membrane (Life Technologies, USA), which was sealed with 5% skim milk powder at 37°C for 2 hours. After the addition of primary antibodies against NICD (1:1500), Hes1 (1:1500), Hes5 (1:1500) and β-caten (1:1500) (Abcam, USA), the membrane was sealed again overnight at 4°C. Following the removal of those primary antibodies, HRP-conjugated secondary antibody (1:4000, Abcam, USA) was added and incubated at 37°C for 2 hours. Next, the membrane was subjected to 3 PBS rinses for 5 min each. After visualization by enhanced chemiluminescence, gray values were analyzed to calculate relative levels of target proteins.
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**Statistical methods**

SPSS 21.0 (IBM Corp, Armonk, NY, USA) and GraphPad 7 were employed for data processing and graphing, respectively. Between-group comparisons of continuous data were performed with independent samples t test, and multiple-group comparisons were analyzed by one-way ANOVA, followed by a Tukey HSD test. Significant differences were determined at P < 0.05.

**Results**

**Comparison of PF and oxygenation index (OI)**

Levels of PEF, FEV1, FEV1/FVC, and OI in group A were lower than those in other groups (P < 0.05), and in groups C and D were lower than those in group B (P < 0.05) (Figure 1).

**Comparison of oxidative stress index (OSI)**

Compared with group A, the remaining groups presented with increased serum MDA and SOD levels, and decreased MPO levels (P < 0.05). However, compared with group B, the levels of serum MDA and SOD in groups C and D decreased, and those of MPO increased (P < 0.05) (Figure 2).

**Comparison of inflammatory cytokine expression**

Compared with group A, levels of serum TNF-α and IL-6 increased in other groups, and those of IL-10 decreased (P < 0.05). Opposite results were obtained in the comparison between group B and groups C and D (P < 0.05) (Figure 3).

**Comparison of TLW and W/D**

The lung W/D and TLW in group A were higher than those in the remaining groups (P < 0.05), and in group B were lower than those in groups C and D (P < 0.05) (Figure 4).

**Histopathologic findings of lung tissue**

The findings of histopathologic examination revealed that in group A, a moist surface and ecchymoses were seen, with damaged alveolar structure and irregularly distributed alveoli. A large number of neutrophils infiltrated, and the alveolar wall was found congested and thickened. The structure of lung tissues of rats in group B was normal, and alveoli remained intact and regularly distributed, with no extravasation or infiltration of red and white blood cells in the cavity. In group C, the congestion and edema of lung tissues were markedly reduced, with clear alveolar structure, and very few red and white blood cells in the cavity. In group D, reduced congestion and edema of lung tissues, irregular size, and shape of alveoli, inhibited leukocyte infiltration, and a slightly improved distribution of alveoli was also observed (Figure 5).

**Comparison of levels of Notch markers**

The levels of NICD, Hes1, and Hes5 in group A were upregulated compared to other groups (P < 0.05), and in group B they were downregulated compared to groups C and D (P < 0.05) (Figure 6).
Discussion

Through a series of investigations, this study proposed that inhaled EDA can effectively reduce the inflammation and oxidative stress (OS) in smoke inhalation injury (SII), as well as improve the pulmonary function (PF) of rats. Also, inhaled EDA is able to reduce the expression of Notch markers NICD, Hes1, and Hes5 in lung tissues.

Heat and smoke frequently result in quick activation of macrophages and large release of inflammatory cytokines, leading to aggravation of SII [13]. In addition, the toxicity of smoke activates oxygen free radicals in lung tissues, thereby inducing OS and damaging the airway epithelium and vascular endothelium [14]. Therefore, excessive oxidative and inflammatory responses have a significant negative impact on SII. EDA is a new type of free radical scavenger that utilizes electron transfer, which has been widely studied in recent years; moreover, it has been reported to associate with reduction of neutrophil infiltration and chain reaction of lipid peroxidation, inhibition of the generation of lipid free radicals, and suppression of the release of pro-inflammatory cytokines [15, 16]. Thus, we believe that EDA may effectively protect the lung from inhalation injury. In the present stu-
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Figure 5. Histopathologic findings in lung tissue. A. H&E staining image of lung tissues in group A. B. H&E staining image of lung tissues in group B. C. H&E staining image of lung tissues in group C. D. H&E staining image of lung tissues in group D.

Figure 6. Comparison of levels of Notch markers. A. Expression and protein profile of NICD in each group. B. Expression and protein profile of Hes1 in each group. C. Expression and protein profile of Hes5 in each group. Note: *P < 0.05 vs. group A; **P < 0.05 vs. group B.

Inhaled EDA downregulated levels of serum TNF-α, IL-6, MDA, and SOD, and upregulated levels of IL-10 and MPO. This indicates that inhaled EDA contributes to the alleviation of inflammation and OS in rats after SII. Afterwards, we found that inhaled EDA significantly improved PF, OI, TLW, and W/D, as well as inhibited alveolar expansion and inflammatory cell infiltration, suggesting a protective effect of inhaled EDA against SII, and that its pre-inhalation may achieve greater efficacy.

As a classical signal pathway, the Notch pathway has been confirmed to participate in various physiologic processes, such as embryo development, cell proliferation, differentiation and death [17, 18]. Also, its activation is strongly associated with inflammation and oxidative stress. Specifically, Notch pathway regulates the secretion of inflammatory cytokines in mouse basophils [19]; its inhibition relieves oxidative stress injury of cardiomyocytes following hypoxia/reoxygenation [20]. Moreover, blocking the Notch pathway in which is activated by SII is able to effectively attenuate SII [11, 21]. The decreased expression of Notch markers (NICD, Hes1, and Hes5) in SII rats shown in this study reveals that EDA may play a role in treating SII by inhibiting the Notch pathway.

Overall, while the present study verified the favorable role of inhaled EDA in reducing inflammation and OS and improving PF in SII possibly by inhibiting Notch pathway,
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still, there were limitations. First, we have not analyzed the therapeutic effect of EDA at the cellular level. Secondly, as far as we know, EDA can regulate NF-κB, Nrf2/HO-1, and JAK/STAT pathways [22-24], but we still have failed to explore whether the regulation of EDA in SII can be achieved through other pathways. We will address these limitations to supplement our conclusions in further research.

To sum up, inhaled EDA is able to alleviate inflammation and OS and effectively improve PF in rats after SII, possibly by inhibiting the Notch pathway.

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Disclosure of conflict of interest

None.

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