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The Protective Effects of Preventive Atomisation Inhalation of Edaravone on the Lung Tissues of Rats with Smoke Inhalation Injury

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Abstract

Objective: To investigate the protective effects of the atomisation inhalation of edaravone on the lung tissues of rats with smoke inhalation injury. **Methods:** Forty male Sprague-Dawley (SD) rats were randomly divided into four groups of ten rats each: normal control group (group A), normal saline atomisation group (group B), edaravone aerosol group (group C) and edaravone atomisation prevention group (group D). Barring group A, the groups were used to create a model of severe smoke inhalation injury. However, before developing the model, group D rats were made to inhale edaravone (3.6 mg/mL) for 10 min. Six hours following smoke inhalation injury, abdominal artery blood samples were centrifuged, the lung tissue homogenate was prepared and carotid artery blood samples were used for blood gas analysis and oxygenation index (PaO_2/FiO_2) calculation. The levels of tumour necrosis factor alpha (TNF-α), interleukin (IL) 6 and IL-10 in serum and the levels of cysteine protease 3 (caspase-3), malondialdehyde (MDA), myeloperoxidase (MPO) and superoxide dismutase (SOD) in lung tissues were examined. The wet-dry ratio (W/D) and water content of the lung tissue were calculated, and the TUNEL method was used to determine the rate of lung tissue apoptosis in each group. Tissue specimens were obtained from the partial lung for histopathological examination. **Results:** Compared with those in group A, the water content of the lung tissue, the rate of lung tissue apoptosis, W/D and the caspase-3, TNF-α, IL-6, IL-10, MDA and MPO levels were significantly greater in other groups ($P < 0.05$), whereas the levels of SOD and PaO_2/FiO_2 were lower ($P < 0.05$). Compared with those in group B, the levels of W/D, the water content of the lung tissue, the rate of lung tissue apoptosis and the

levels of caspase-3, TNF-α, IL-6, MDA and MPO were significantly low ($P < 0.05$) and the levels of IL-10, SOD and PaO_2/FiO_2 were significantly high in groups C and D ($P < 0.05$). The expression of the aforementioned factors was more evident in Group D ($P < 0.05$). Histopathological examination revealed that groups C and D had greater levels of inflammatory granulocytes than group B. This was more evident in group D. **Conclusions:** The inhalation of edaravone can reduce smoke inhalation-induced lung injury. This may be related to the inhibition of apoptosis, the reduction of peroxidation injury and the production/release of inflammatory mediators/free radicals. It exerts a remarkable preventive effect.

Keywords

Edaravone, Rats, Prevention, Smoke Inhalation Injury

1. Introduction

Inhalation injury is a critical and severe burn injury that affects both the respiratory tract and lung parenchyma, mostly owing to smoke and/or heat, and often leads to serious consequences owing to its inside onset and atypical symptoms [1]. Edaravone exhibits antioxidant and lipid peroxidation inhibitory effects. When administered abdominally and intravenously, it can reduce nerve cell apoptosis and membrane peroxidation and alleviate lung injury caused by various factors [2] [3] [4] [5]. Since edaravone easily penetrates biofilms, aerosol inhalation can directly act on the target organs [6] [7]. In this study, a rat model with smoke inhalation injury was made to inhale edaravone aerosols from July 2019 to April 2020 to study its protective effects on the lung of the model.

2. Material and Methods

2.1. Materials

2.1.1. Experimental Animals

Male Sprague-Dawley (SD) rats, 60 days old and weighing approximately 200 g, were purchased from the animal experiment centre of the Beijing Academy of Military Medical Sciences. The rats were fasted and provided free access to drinking water 12 h before the experiment.

2.1.2. Main Reagents

Edaravone (Shuanghe Pharmaceutical (Shangqiu) Co., LTD., API, Batch number: National Drug Approval WORD H20130053, Purity: 99.7%), tumour necrosis factor alpha (TNF-α), interleukin (IL) 6, IL-10 and caspase-3 enzyme-linked immunosorbent assay (ELISA) kits were purchased from US R&D company. Myeloperoxidase (MPO), malondialdehyde (MDA), superoxide dismutase (SOD) and Coomassie protein detection kits were purchased from Nanjing Jiancheng Company, and TUNEL positive control preparation kits were purchased from

Nanjing KGI Biotechnology Development Co., LTD.

2.1.3. Instrument

Eppendorf 5702 centrifuge, Leica paraffin slicer, ABL-800 blood gas analyser (Denmark leidu), PARI BOY N series atomiser (Germany Bairi Co., LTD.) and KD-BM Biological tissue embedding machine (Zhejiang Kedi Instrument Equipment Company) were obtained for the experiments.

2.2. Experimental Methods

2.2.1. Grouping and Modelling of Animals

Forty male SD rats were randomly divided into four groups: normal control (group A), normal saline atomisation (group B), edaravone atomisation (group C) and edaravone atomisation prevention (group D) groups, with ten rats in each group. According to reference [8], ketamine hydrochloride (100 mg/kg) was administered intraperitoneally to anaesthetise the rats, and a sterile towel was used on the neck and chest for fur disinfection. A central incision was made in the neck and chest, a tracheotomy was performed and a tracheal catheter was inserted. Mechanical ventilation was performed by connecting the threaded tube of a ventilator with a type 4601 small animal ventilator, with a respiratory rate of 80 times/min and a tidal volume of 10 mL/kg. The inhaling-breathing ratio was 1:2, and the inhaled oxygen concentration (FiO_2) was 30%. The airway pressure was dynamically monitored (P_{aw}) by the pressure conversion joint, which was connected to the three-way tube connected to the threaded tube. After the P_{aw} was stabilised for 20 min, rats in groups B, C and D were prepared as previously mentioned [9]: The sawdust in the smoke generator was loosened and smoked for 15 min. The air inlet valve and fan in the injury room were opened. When the smoke alarm was detected, the exhaust valve and fan were closed.

2.2.2. Animal Intervention Methods

The time and dose of drug intervention were set for rats in each group by referring to the literature [10]:

1) Group A rats did not inhale any drugs;

2) At 30 min after the injury, group B and C rats were atomised and made to inhale isotonic sodium chloride solution (jet velocity: 0.1 mL/min) and 3.6 mg/mL edaravone (solvent: isotonic sodium chloride solution, jet velocity: 0.1 mL/min) for 10 min, once for 1 h, four times in total;

3) group D rats were atomised and made to inhale 3.6 mg/mL edaravone (solvent: isotonic sodium chloride solution, jet velocity: 0.1 mL/min) for 10 min before injury (pre-treatment) and 3.6 mg/mL edaravone for 10 min after injury (treatment) for 30 min, once for 1 h, thrice in total.

Mechanical ventilation was continued throughout the experiment, and the P_{aw} was maintained at baseline during injury and drug intervention. No injury or death occurred in rats during administration. This experiment was approved by the Medical Ethics Committee of the Affiliated Hospital of Chengde Medical

College, and the animal disposal method met the animal ethics standards.

2.2.3. Specimen Collection

A blood gas needle was used to extract 1 mL of carotid blood at 6 h after the injury for blood gas analysis. A total of 5 mL of abdominal aortic blood was collected and centrifuged. The supernatant was collected and stored at −20˚C for IL-6, IL-10 and TNF-α analysis. After the animal was sacrificed, the lung tissue was removed rapidly, and the moisture content and wet-dry ratio (W/D) of the anterior and middle lobes of the right lung were measured. The posterior lobe of the right lung was fixed with 4% formaldehyde for 24 h and subjected to pathologic examination and TUNEL staining. Caspase-3, MDA, MPO and SOD were stored in the left lung tissue at −70˚C.

2.2.4. Index Detection and Methods

1) Oxygenation index, lung tissue moisture content and W/D blood samples were used to perform arterial blood gas analysis within 30 min to calculate the oxygenation index (PaO_2/FiO_2). The frontal lobe and middle lobe of lung tissue specimens were immediately dried with filter paper, and the wet weight was weight, following which the specimen was heat-treated at 80˚C for 48 h, and the dry weight was weighed. The W/D of the lung was calculated; moisture content = (wet weight − dry weight)/wet weight × 100%.

2) Serum IL-6, IL-10 and TNF-α were thawed for double-antibody sandwich ELISA. The kit instructions were followed, and the KIT reader showed the optical density (OD) at 450 nm. The concentrations of IL-6, IL-10 and TNF-α (in pg/mL) were calculated according to the standard curve equation and OD value.

3) Lung tissue containing caspase-3, MDA, MPO and SOD were thawed, cut into pieces and pulverised. Cold isotonic sodium chloride solution at a ratio of 1:9 was then added to prepare the tissue homogenate. The protein concentration in the homogenate was measured using the Coomassie assay. The OD values of caspase-3, MPO, MDA and SOD were detected at 450, 460, 532 and 550 nm by ELISA, adjacent anisidine hydrogen supply, thiobarbituric acid and xanthine oxidase methods, respectively. The concentrations of caspase-3 (μmol/mL), MPO (U/mg), MDA (U/g) and SOD (nmol/mg) in tissues were calculated.

4) To prepare a haematoxylin-eosin (HE) staining section of lung tissues, the right posterior lobe fixed with 1%/2% formaldehyde was dehydrated, embedded, sectioned and observed under a light microscope after HE staining.

5) Apoptosis rate in lung tissues. The fixed right lung tissue was sectioned, stained with TUNEL, soaked in the permeable solution, treated for inducing infiltration on ice for 2 min and washed thrice with phosphate-buffered saline (PBS). Subsequently, a deoxyribonucleotide terminal transferase reaction solution was added. The mixture was incubated at 37˚C for 60 min in a wet box and washed thrice with PBS, and enhancement of apoptotic cell fluorescence was observed. Five non-overlapping high magnification fields (×400) were randomly selected for each rat, and the total number of cells and apoptotic cells were counted.

The apoptosis rate of lung tissue cells = number of apoptotic lung tissue cells/total number of lung tissue cells ×100%.

2.3. Statistical Treatment

Counting data in this experiment were expressed in terms of the number of cases and percentage, and measurement data were processed using SPSS 20.0 in the form of mean ± standard deviation (±S). F test was used to compare the differences in each index among groups, and the SNK-Q test was used to compare the differences among groups. P< 0.05 indicated statistical significance.

3. Results

3.1. Lung Biopsy

Microscopic observation revealed that the alveolar cavity structure of group A rats was clear, complete and devoid of inflammatory cell infiltration (Figure 1). In group B rats, the alveoli were dilated or atrophied to varying degrees and contained numerous red blood cells and inflammatory cells (Figure 2). The Alveoli of group C rats were dilated or atrophied to a certain extent, and the infiltration

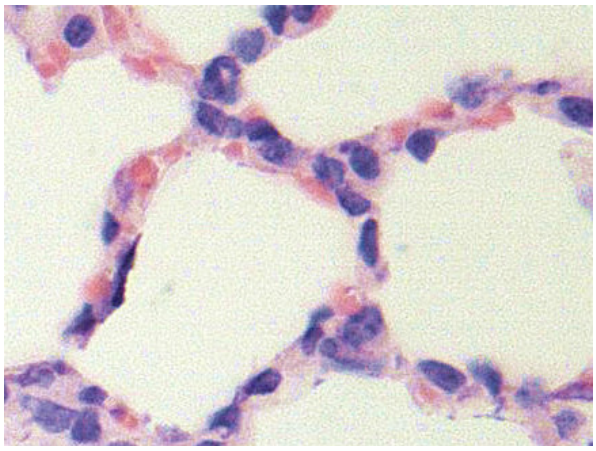

Figure 1. Pulmonary tissue slices of group A (HE ×400).

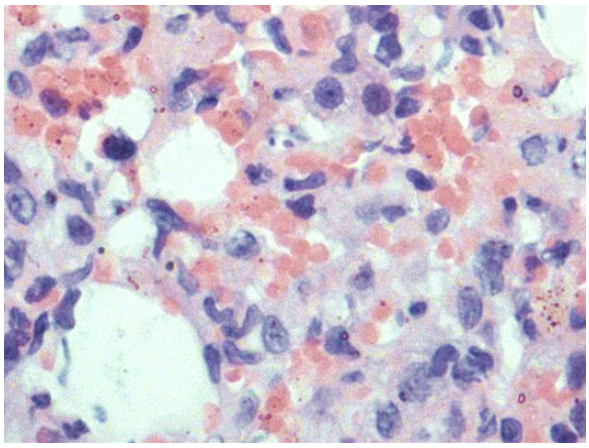

Figure 2. Pulmonary tissue slices of group B (HE ×400).

of a certain number of inflammatory cells was observed in the alveoli; however, the degree of infiltration was lesser than that in group B rats (Figure 3). Group D rats had the same alveolar size, and the infiltration of red and white blood cells was the least (Figure 4).

3.2. Comparison of the PaO_2/FiO_2, Lung Tissue Moisture Content and W/D of Rats in Each Group

Compared to that in group A rats, the PaO_2/FiO_2 in rats from groups B, C and D were significantly lower, whereas the lung water content and W/D were significantly higher (P< 0.05). Compared to that in group B rats, the PaO_2/FiO_2 in rats from groups C and D were significantly higher, whereas the lung water content and W/D were significantly lower (P< 0.05). Compared to those in group C rats, the abovementioned changes were more obvious in group D rats (P< 0.05; Table 1).

3.3. Comparison of Serum TNF-α, IL-6 and IL-10 Levels in Each Group

Compared with those in group A rats, the TNF-α, IL-6 and IL-10 levels in rats

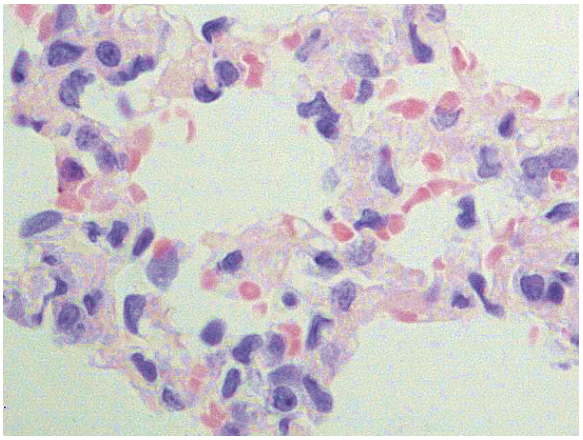

Figure 3. Pulmonary tissue slices of group C (HE ×400).

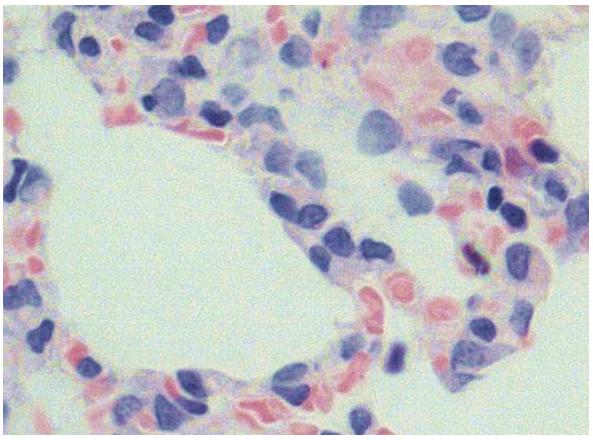

Figure 4. Pulmonary tissue slices of group D (HE ×400).

from groups B, C and D were significantly higher (P< 0.05). Compared with those in group B rats, the IL-10 levels in rats from groups C and D were significantly higher, whereas the TNF-α and IL-6 levels were significantly lower (P< 0.05). Compared with those in group C rats, the abovementioned changes were more obvious in group D rats (P< 0.05; Table 2).

3.4. Comparison of the MDA, MPO and SOD Levels in the Lung Tissues of Rats from Each Group

Compared with those in group A rats, the MDA and MPO levels in rats from groups B, C and D were significantly higher, whereas the SOD levels were significantly lower (P< 0.05). Compared with those in group B rats, the SOD levels in rats from groups C and D were significantly higher, whereas the MDA and MPO levels were significantly lower (P< 0.05). Compared with those in group C rats, the above changes were more obvious in group D rats (P< 0.05; Table 3).

3.5. Comparison of the Apoptosis Rate and Caspase-3 Content in Lung Tissues

Compared with those in group A rats, the apoptosis rate and caspase-3 content

Table 1. Comparison of the arterial oxygenation index, water content in lung tissues and wet-dry ratio of rats in each group ($\overline{x} \pm s$).

Group	n	PaO_2/FiO_2 (mmHg)	Moisture content of lung tissue (%)	W/D (g/g)
A	10	419.33 ± 10.19	76.52 ± 0.08	4.27 ± 0.14
B	10	291.17 ± 6.29[a]	88.85 ± 0.02[a]	8.42 ± 0.15[a]
C	10	336.32 ± 5.21[ab]	81.52 ± 0.03[ab]	6.11 ± 0.13[ab]
D	10	384.11 ± 5.05[abc]	78.41 ± 0.05[abc]	4.41 ± 0.12[abc]

ANOVA, F test, PaO_2/FiO_2: F = 2.588, P< 0.05; Lung tissue moisture content: F = 5.799, P< 0.05; W/D: F = 2.416, P< 0.05; Pairwise comparison of SNK-Q test, compared with group A, [a]P< 0.05; Compared with group B, [b]P< 0.05; Compared with C group, [c]P< 0.05. W/D, wed-dry ratio; PaO_2/FiO_2, arterial oxygenation index.

Table 2. Comparison of the TNF-α, IL-6 and IL-10 levels in the sera of rats in each group ($\overline{x} \pm s$).

Group	n	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)
A	10	107.79 ± 5.95	139.36 ± 6.03	117.96 ± 4.50
B	10	365.74 ± 10.41[a]	348.75 ± 6.11[a]	245.64 ± 6.85[a]
C	10	275.15 ± 9.14[ab]	204.28 ± 4.09[ab]	286.28 ± 8.14[ab]
D	10	167.47 ± 7.06[abc]	150.22 ± 4.19[abc]	433.14 ± 10.57[abc]

ANOVA, F test, TNF-α: F = 2.270, P< 0.05; Il-6: F = 3.093, P< 0.05; Il-10: F = 3.377, P< 0.05; Pairwise comparison of SNK-Q test, compared with group A, [a]P< 0.05; Compared with group B, [b]P< 0.05; Compared with C group, [c]P< 0.05.

Table 3. Comparison of the MDA, MPO and SOD levels in the lung tissues of rats in each group ($\overline{x} \pm s$).

Group	n	MDA (nmol/mg)	MPO (U/g)	SOD (U/mg)
A	10	2.32 ± 0.33	13.19 ± 0.11	360.63 ± 6.13
B	10	6.52 ± 0.06[a]	70.84 ± 0.72[a]	103.84 ± 2.15[a]
C	10	4.01 ± 0.05[ab]	42.71 ± 0.38[ab]	151.72 ± 3.05[ab]
D	10	2.49 ± 0.45[abc]	24.46 ± 1.03[abc]	309.25 ± 3.83[abc]

ANOVA, F test, MDA: F = 1.727, P< 0.05; MPO: F = 3.929, P< 0.05; SOD: F = 3.388, P< 0.05; Pairwise comparison with the SNK-Q test, compared with group A, [a]P< 0.05; Compared with group B, [b]P< 0.05; Compared with C group, [c]P< 0.05.

Table 4. Comparison of apoptosis rate and caspase-3 content in the lung tissues of rats in each group ($\overline{x} \pm s$).

Group	n	Apoptosis rate of lung tissue (%)	Caspase-3 (μmol /ml)
A	10	0.49 ± 0.03	5.49 ± 1.04
B	10	20.54 ± 0.71[a]	31.38 ± 1.15[a]
C	10	17.68 ± 0.41[ab]	18.87 ± 1.11[ab]
D	10	11.01 ± 0.47[abc]	9.07 ± 0.88[abc]

ANOVA, F test, apoptosis rate in lung tissue: F = 9.582, P< 0.05; Caspase-3: F = 7.168, P< 0.05; Pairwise comparison using the SNK-Q test, compared with group A, [a]P< 0.05; Compared with group B, [b]P< 0.05; Compared with C group, [c]P< 0.05.

in the lung tissues of rats from groups B, C and D were significantly higher (P< 0.05); Compared with those in group B rats, the apoptosis rate and caspase-3 content in the lung tissues of rats from group C and D were significantly lower, and the change in group D was more obvious (P< 0.05; Table 4).

4. Discussion

In recent years, with the improvement in burn diagnosis and treatment, the success rate of rescue for patients with burns has increased; however, effective interventions for patients with inhalation injuries are lacking. After inhalation injury, numerous inflammatory mediators, cytokines and oxygen free radicals are released, which promote the apoptosis of lung cells and cause severe lung injury [11]. Lung injury increases vascular resistance, decreases lung compliance and oxygenation capacity and causes pulmonary oedema [12] [13] [14]. In this study, the lung tissue moisture content, W/D and oxygenation index were used as indicators to evaluate pulmonary oedema and microvascular injury in response to lung tissue inflammation [15]. In the injury group, the alveolar inflammatory cells were infiltrated, the lung water content and W/D increased significantly and the PaO_2/FiO_2 decreased, suggesting that the smoke inhalation injury model was successfully established. The aforementioned changes were significantly reversed after edaravone intervention, and the improvement was more significant

in group D. This finding indicates that nebulised edaravone inhalation can reduce pulmonary oedema, oxygenation and inflammatory cell infiltration, and the earlier the application, the more effective it is. The specific mechanism of action requires further study.

TNF-α promotes the chemotaxis and phagocytosis of circulating polymorphonuclear leukocytes (PMNs), increases the release of bone marrow leukocytes and induces the synthesis and secretion of cytokines, such as IL-1 and IL-6 [16]. Il-6 upregulates the levels of other cytokines, forming cellular networks that amplify and sustain local and systemic inflammatory cascades. Il-6, IL-1β, TNF-α and other inflammatory mediators and cytokines act on the lungs, which can cause abnormal blood flow ratios and impair tissue perfusion and oxygenation [17]. Therefore, TNF-α and IL-6 can better reflect the severity of tissue injury and indicate multi-organ dysfunction. They can be used as reliable indicators in the evaluation of the therapeutic impact on lung injury [18]. IL-10 is produced and released after lymphocyte activation, which can inhibit the synthesis and secretion of TNF-α and IL-6 by PMNs and downregulate the expression of the major histocompatibility complex to protect organ tissues [19]. In this study, the IL-10 levels were measured to indirectly indicate the body's anti-inflammatory ability. The serum levels of IL-6 and TNF-α in group B rats were higher, whereas those in group C rats were lower than those in group B rats, and those in group D rats were lower than those in group C rats. In group A rats, the aerosol inhalation of edaravone inhibited inflammatory factors and improved anti-inflammatory factors in smoke inhalation injury; the prophylactic effect was good.

SOD, a natural oxygen-free radical scavenger in the body, can inhibit chemotaxis and terminate inflammatory response pathways, which is an important indicator of the body's antioxidant capacity. The interaction of inflammatory cells increases the activity of lung MPO and intensifies the oxidation process in lung tissues, whereas the MDA content indicates the level of oxygen free radicals in the body and the severity of free radical damage in tissue cells [20]. In this experiment, the levels of MPO and MDA in group B rats increased, whereas the SOD level decreased and disrupted the oxidation/antioxidant balance. Groups C and D rats had lower levels of MPO and MDA and higher levels of SOD compared to Group B rats. The findings from group C rats suggest that early or prophylactic aerosol inhalation of edaravone exerts a significant antioxidant effect on smoke inhalation injury.

Apoptosis is the pathological basis underlying tissue injury, and the development of inhalation lung injury is closely related to lung tissue apoptosis [21]. Various inflammatory mediators, cytokines and free radicals can release cytochrome C and other apoptosis promoters, activate the caspase family and cause apoptosis of numerous cells [22]. Caspase-3 acts as an executor of apoptosis after activation, marking the irreversible stage of apoptosis [23]. In this study, the apoptosis rate and caspase-3 level in the lung tissues of group B rats were higher than those of group A rats, the aforementioned indexes in group C and D rats

were significantly lower than those in group B rats, and the degree of reduction was more evident in group D rats. The findings from group C rats revealed that the aerosol inhalation of edaravone can reduce the apoptosis of lung cells caused by inhalation injury, which may be related to the caspase-3 signalling pathway, and preventive administration for inducing an anti-apoptotic effect is more effective.

In conclusion, aerosol inhalation of edaravone is believed to exert a protective effect on smoke inhalation lung injury. The mechanism may be related to the attenuation of the inflammatory response and peroxide damage and inhibition of apoptosis. The earlier the medication is administered, the more effective it would be.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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