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OXIDATIVE STRESS IN A RAT MODEL OF COTTON SMOKE INHALATION-INDUCED PULMONARY INJURY

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Abstract

Background: Smoke inhalation injury refers to airway and lung parenchyma injury and general chemical damage caused by inhaling toxic gases and substances. The aim of this study was to explore the oxidative stress mechanism of cotton smoke inhalation-induced pulmonary injury in a rat model.

Materials and Methods: Eighteen male Sprague-Dawley rats were randomly divided into control group, 6 h group, and 24 h group (six rats in each group), which duplicated previous rat cotton smoke-inhalation injury models. Rats in 6 h and 24 h groups were euthanised at 6 h and 24 h after smoke inhalation, respectively. ELISA method was used to detect indicators in the rats' lung tissue. Quantitative iNOS mRNA and γ-GCS mRNA measurements were performed using a fluorescence PCR method.

Results: The concentrations of MDA, NO, iNOS, γ-GCS, iNOS mRNA, and the relative expression of γ-GCS mRNA in the rats' lung tissues in 6 h and 24 h groups were higher than control group ($P < 0.05$), and the concentration of NO and relative expressions of iNOS mRNA and γ-GCS mRNA in 24 h group were significantly higher than 6 h group ($P < 0.05$). The concentrations of GSH in 24 h and 6 h groups were significantly lower than control group ($P < 0.05$), and that in 24 h group was even significantly lower than 6 h group ($P < 0.05$).

Conclusion: In rats with cotton smoke inhalation-induced pulmonary injury, the increased iNOS mRNA transcription can cause increase of iNOS synthesis and promotion of NO synthesis. The increased γ-GCS mRNA transcription can cause increase of γ-GCS synthesis and but decrease of GSH concentration. The activation of the antioxidant system is insufficient to combat oxidative stress damage. So the oxidant/antioxidant system is imbalanced, leading to gradual aggravation of lung injury.

Key words: Acute lung injury, Smoke inhalation injury, Oxidative stress

Introduction

Smoke inhalation injury is the airway and lung parenchyma injury caused by hot air, steam, smoke, harmful gases and volatile chemical substances and general chemical damage caused by inhaling toxic gases and substances into the body. It is an important contributor to human mortality after fire (Toon et al., 2010). More than 30% of burn patients may have comorbid inhalation injuries. It can increase the burn-related mortality by 20% and raises the smoke inhalation/burn injury mortality rate to 30-90% (Ballard-Croft et al., 2010). In a smoke-filled environment such as a fire, the human respiratory system undergoes a series of pathophysiological changes, such as thickening of the alveolar walls, protein exudation from the alveolar spaces and septa, accumulations of inflammatory cells and cytokines, and even pulmonary oedema. Clinical manifestations include dyspnoea, hypoxemia, respiratory failure, and in severe cases, acute respiratory distress syndrome.

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Smoke-producing materials are of various types, including plant fibres, petroleum products, black powder, organic polymeric materials, and so on, which can all burn and produce smoke under certain conditions to cause smoke injury. differences in combustible type and quality, combustion time, combustion space, combustion speed, flow rate of the smoke, smoke temperature, animal species and body size, anaesthetic status, inhalation method, environment, and other factors result in widely varied animal model performances (Yamamoto et al., 2012; Belenkiy et al., 2013; Guillaumin and Hopper, 2013; Rehberg et al., 2013). Cotton combustion produces smoke that consists mainly of CO and carbon powder and relatively fewer other harmful chemicals and gases. In the researched animal models of smoke inhalation injury, most were combined with burns, and most utilised large animals, such as sheep, pigs, and dogs (Belli et al., 2011; Enkhbaatar et al., 2011). There are fewer data regarding small animal pure cotton combustion smoke inhalation models.

At present, particles and toxic substances are the ‘arch-criminals’ in lung injury pathophysiologic changes induced by smoke inhalation (Møller et al., 2010), and the interaction of these substances with the lung parenchyma may cause an inflammatory cascade reaction. Meanwhile, significantly increased pulmonary reactive oxygen species and reactive nitrogen species accelerate oxidative stress, and interventions including antioxidant and free radical scavenging could reduce lung injury (Jiang 1991; Lange et al., 2010), suggesting that enhanced oxidative stress is an important cause of damage. Oxidative stress is a key link in smoke inhalation injury. This study was designed to investigate the oxidative stress mechanism of pulmonary injury after cotton smoke inhalation in a rat model.

Materials and Methods

Animals and Grouping

Eighteen clean, healthy, adult male Sprague-Dawley rats, weight 150-250 g, were provided by the experimental animal centre of the Military Medical Science Academy of the Chinese People's Liberation Army [SCXK- (Army) -2012-0004] and bred by the experimental animal centre of the Navy General Hospital [SCXK- (Army) -2012-0012]. The rats were fed in single cage (temperature 22-24 °C, humidity 50-60%, 12h/12h light-dark cycle), free to food and water. The animals were treated according to the guidelines of the experimental animal ordinance. In accordance with the principle of randomisation, the animals were divided into a control group, 6 h group, and 24 h group, with 6 rats in each group. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Military Medical University.

Smoke Inhalation

After connecting the smoke inhalation device and preheating a tin furnace being to 300°C, 2 rats were placed into a bottle to be filled with smoke, and 2 g of cotton were weighed and placed into the tin. Immediately after, the collecting chimney was covered and a fan and timer started. When the rats exhibited cherry red-to-purple plantar skin, agitation, shortness of breath, mouth breathing, slow and deep breathing, mouth wheezing, or upon reaching the 2 min time limit, the smoke was stopped, and the bottleneck immediately opened. After inhaling air for 7 min, the rats visibly and gradually improved.

The rats in the 6 h and 24 h groups were subjected to the above steps 3-5 times, until the rats were still in a coma after inhaling air for 7 min, and then the experiment was ended. Rats in the control group were similarly placed in the bottle, but without cotton combustion smoke, and the above steps were repeated 5 times. The rats in the 6 h and 24 h groups were euthanised by an overdose of an intraperitoneal injection of pentobarbital sodium at 6 h and 24 h after leaving the smoke-filled environment, respectively, and rats in the control group were euthanised 24 h after simulated smoke inhalation.

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ELISA

The tissue homogenates from the right lower lung lobes of rats were prepared. Using from each group, malondialdehyde (MDA) concentrations were detected by a colorimetric method. The concentrations of malondialdehyde (MDA), glutathione (GSH), nitric oxide (NO), inducible nitric oxide synthase (iNOS), and gamma glutamylcysteine synthetase (γ-GCS) were detected by a double-antibody sandwich ABC-ELISA methodusing DENLEY DRAGON Wellscan MK 3 microplate reader (Thermo Fisher Scientific Inc., Vantaa, Finland). The related kits were provided by Jiamay Biotech Co., Ltd. (Beijing, China), and the tests were performed according to the manufacturer's instructions.

PCR

The PCR primer sequences for the iNOS and γ-GCS target genes were designed and synthesised by Jiamay Biotech Co. Ltd. The upstream and downstream primers for iNOS were 5'-ACACCGATTCCACTCAACTA-3' and 5'-ACCACCTGTTAGTTCAAGCC-3', respectively; the upstream and downstream primers for γ-GCS were 5'-GCATTCATTTCACCCTGTTCT-3' and 5'-ACAAAGAGCCCTGACCTAATG-3', respectively; the lengths of the PCR products were 159 bp and 132 bp, respectively, with β-actin (ComWin Biotech. Co. Ltd., Beijing, China) used as an internal control. Using an ultrapure RNA Kit (CWbio Co. Ltd, Cat#CW0581), the total RNA was extracted from tissue samples. 5 μL RNA were used to perform electrophoresis in 1% agarose gel. For the first strand, a HiFi-MMLVcDNA Synthesis Kit (CWbio Co. Ltd, Cat#CW0744) was used for reverse transcription, with UltraSYBR Mixture (with Rox) (CWbio. Co. Ltd, Cat#CW0956) used for amplification.

The amplification protocol was as follows: 95℃ for 10 min (95℃ for 15 s + 60℃ for 60 s) for 40 cycles. The Light Cycler-480II fluorescence quantitative PCR instrument (Idaho Technology, Inc., Utah, USA) was used for measurements, and the $2^{-\Delta\Delta CT}$ method was used for the relative quantitative data analysis.

Statistical Analysis

SPSS version 18.0 software was used for the data analysis, and the experimental results were compared. The data are presented as the mean ± standard deviation; the comparison used was a single-factor analysis of variance (one-way ANOVA), comparisons between each group were performed using an LSD test, and $P < 0.05$ was considered statistically significant.

Results

Weights of the Rats and Concentrations of MDA, NO, and GSH

As shown in Table 1, the average body weight of all rats was 180.80 ± 26.68 g, with no significant difference between the groups ($P > 0.05$). The concentrations of MDA in rat lung tissue homogenates in 6 h and 24 h groups were significantly higher than control group ($P < 0.05$), but there was no statistical difference in the concentration of MDA between 6 h group and 24 h group. The concentrations of NO in 6 h and 24 h groups were significantly higher than control group ($P < 0.05$), and that in 24 h group was significantly higher than 6 h group ($P < 0.05$). The concentrations of GSH in 6 h and 24 h groups were significantly lower than control group ($P < 0.05$), and that in 24 h group was significantly lower than 6 h group ($P < 0.05$).

The Concentrations of iNOS and Γ-GCS and Expressions of iNOS and Γ-GCS mRNA

As seen in Table 2, the concentrations of iNOS and γ-GCS in rat lung tissue homogenates from the 6 h and 24 h groups were significantly higher than those in the control group, but there was no significant difference between the 6 h group and the 24 h group. The relative expressions of iNOS mRNA and γ-GCS mRNA in the lung tissue homogenate of each rat as detected by

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immunofluorescence PCR in the 6 h and 24 h groups were higher than those in the control group, and those in the 24 h group were higher than in the 6 h group.

Discussion

Smoke inhalation can be divided into the following 3 main morbidity factors. The first is injury from heat (Toon et al., 2010), namely, inhaled dry or humid air may cause direct respiratory mucosa and pulmonary parenchyma injury, such as glottic oedema, and can cause asphyxia (Rehberg et al., 2009). The second is hypoxia, which is a major cause of death in fires due to hypoxia or an inhaled asphyxiant such as CO or HCN. The third is particles and toxic substances, in which particles with diameters of less than 1 μm can reach the alveolar cavities and cause direct injury to the respiratory mucosa and a widespread systemic toxic reaction.

At present, particles and toxic substances are "arch-criminals" that cause the pathophysiologic changes in lung injury induced by smoke inhalation, and the interaction of these substances and the lung parenchyma may cause an inflammatory cascade reaction, resulting in small airway pulmonary oedema, congestion, loss of vasoconstriction, pulmonary hypoxia, and imbalance of the ventilation/perfusion ratio. Oxidative stress and inflammation are 2 key links in smoke inhalation injury (Lange et al., 2012). Smoke inhalation lung injury has an acute onset and rapid progression. As a syndrome, it lacks an objective gold standard for clinical diagnosis. Previous researches (Quinn et al., 2003; Lee et al., 2005; Huang et al., 2006; Zou et al., 2009) designed a rat model of smoke inhalation lung injury. In this experimental model, when high-temperature smoke entered into the bottle through the cooling pipe, its temperature was below 30°C; thus, the damage induced by heat could be excluded. However, in the monitored smoke, the concentration of O_2 was greater than 19% and the concentration of CO was less than 0.25%, which could prevent and reduce death caused by hypoxia and acute CO poisoning in the animal model. The mortality rate was controlled at about 10% after testing and evaluation, and it was proven that it could meet the needs of experimental studies on smoke inhalation-induced pulmonary injury.

Smoke inhalation can activate the pulmonary macrophages, neutrophils, endothelial cells, and vascular smooth muscle cells to release large amounts of cytokines and chemokines, such as tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6, IL-8, and other factors, and can activate the nuclear transcription factor κB (Rehberg et al., 2009) in the cytoplasm to form the NF-κB p65 active fragment and enter into the nucleus to promote the transcription of inducible nitric oxide synthase (iNOS) mRNA. Additionally, it increases iNOS synthesis (Lange et al., 2010), causes the catalytic synthesis of peroxynitrite, and induces membrane lipid peroxidation (Enkhbaatar and Traber, 2004), which may result in membrane structure and function disorders. At the same time, iNOS can decompose arginine to produce excessive NO (Cox et al., 2009), and the latter can cause blood vessel vasodilation, and thus lead to vascular leakage and the loss of hypoxic pulmonary vasoconstriction function (Westphal et al., 2008).

This, in turn, may result in a decrease in the ventilation/perfusion ratio and pulmonary vasodilation in low-ventilation areas and further the deterioration of gas exchange in the lung. After smoke inhalation, oxidant levels may significantly increase; for example, NO can produce an increase in the reactive nitrogen species (RNS) (Rehberg et al., 2010) NO, N_2O, and SO_2. Oxide particles in smoke are also strong oxidants, and neutrophil aggregation in the lung from stimulation of the inflammatory response can release a large amount of oxygen free radicals. Hypoxia after oxygen treatment can also increase the production of oxygen free radicals. In this experiment, the detected MDA was the peroxide generated by the reaction of free radicals and biological membrane polyunsaturated fatty acids, and its content can represent the degree of oxidative stress. The results show that 6 h and 24 h after smoke inhalation, MDA increased significantly, indicating the activation of oxidative stress and the continued progression of injury. In lung tissue, the relative expression of iNOS mRNA and concentrations of iNOS and NO also were increased obviously, indicating that the iNOS-NO system plays a significant role in response to oxidative stress after smoke inhalation.

The activation of the antioxidant system after smoke inhalation plays an anti-oxidative stress function, and antioxidant enzymes (superoxide dismutase, γ-GCS, catalase, and glutathione peroxidase) and non-enzymatic antioxidants (such as glutathione, vitamin C, vitamin E, and so on) compose the antioxidant system, but oxidative stress after smoke inhalation is severe, and significantly increases active oxygen and accompanies a decrease in antioxidants (Patton and Haith, 2010), such as antioxidant redistribution to immune activity organisation, liquid dilution from resuscitation treatments, a shortage of supplements, loss of body

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fluids, and so on (Xie et al., 1997), and can lead to the relative depletion of antioxidant capacity, oxidant/antioxidant balance disorder, and the aggravation of oxidative stress damage. The γ-GCS detected in this experiment is the rate-limiting enzyme in the synthesis of GSH, and GSH is the main component of anti-oxidative stress (Kwon et al., 2011). The experimental results showed that the expression of γ-GCS mRNA and the concentration of γ-GCS in 6 h and 24 h groups were significantly increased compared to the control group, suggesting that the synthesis of the antioxidant enzyme γ-GCS can be activated after smoke inhalation. However, the concentration of GSH decreased with increasing time, suggesting that the activation of the antioxidant system after smoke inhalation is not enough to combat oxidative stress damage, and lung injuries might be further aggravated.

The experimental results show that after cotton smoke inhalation, oxidative stress was activated in the bodies of the rats, resulting in iNOS mRNA transcription activity, an increase in synthesis of the downstream iNOS protein and NO, and further aggravation of the damage. At the same time, smoke inhalation also activated the transcription of γ-GCS mRNA and γ-GCS synthesis increased, but reduced the concentration of GSH, and induced less of an antioxidant stress reaction (Patton and Haith, 2010). Oxidative/anti-oxidative balance disorder and oxidative damage were dominant, and the injury was gradually aggravated. The next step in study design will continue to observe the dynamic changes at follow-up time points, and will simultaneously conduct in-depth research on the mechanism of signal transduction and explore new directions in the treatment of smoke inhalation-induced lung injury with respect to oxidative stress.

In this study, the trends of the detection indices in the 6 h and 24 h groups were the same, and a comparison between the 6 h group and the control group revealed a difference. For the purpose of simplifying the experimental design, research designed towards the prevention and treatment of smoke inhalation injury could select a time point 6 h after the smoke inhalation, and there was not much reason to observe dynamic changes in the following time points. Meanwhile, the experimental results also revealed that after smoke inhalation, the *in vivo* oxidative stress response was activated; resulting in the activation of iNOS mRNA transcription, and increased downstream iNOS protein and NO synthesis further aggravated the injury. Simultaneously, smoke inhalation also activated the transcription of γ-GCS mRNA and increased γ-GCS synthesis, but concentrations of GSH decreased, the synthesis of anti-oxidative stress products was inadequate, the *in vivo* oxidative/anti-oxidative balance was disordered, major oxidative damage took place, and thus the injury gradually increased. These findings suggest that new directions could be explored from the standpoint of oxidative stress, such as to reduce oxidative stress and increase antioxidant capacity.

Conclusion

In rats with cotton smoke inhalation-induced pulmonary injury, the increased iNOS mRNA transcription can cause increase of iNOS synthesis and promotion of NO synthesis. At the same time, the increased γ-GCS mRNA transcription can cause increase of γ-GCS synthesis and but decrease of GSH concentration.

The activation of the antioxidant system is insufficient to combat oxidative stress damage. So the oxidant/antioxidant system is imbalanced, leading to gradual aggravation of lung injury. This study has verified the changes of oxidative stress and reduction reaction in rats after smoke inhalation injury, and has provided a basis for further studying the intervention measures. However, there are some limitations in this study. The component in the smoke causing injury is not analyzed, and the reactiveness of whole body system after injury is not investigated. These should be completed in further study.

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Conflict of Interest

All authors have no conflict of interest regarding this paper.

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