Original Article
Lung injury via oxidative stress in mice induced by inhalation exposure to rocket kerosene

Bingxin Xu, Chenglin Li, Jianying Wang, Jihua Wu, Shaoyan Si, Zhiguo Liu, Jianzhong Li, Jianzhong Zhang, Yan Cui

1Special Medicine and Experimental Research Center, 2Department of General Surgery, 3Epidemic Prevention Team, 4Department of Pathology, 306 Hospital of PLA, Beijing 100101, China

Received March 4, 2015; Accepted April 21, 2015; Epub May 1, 2015; Published May 15, 2015

Abstract: Rocket kerosene (RK) is a new rocket propellant. Toxicity occurs if a high level of RK is inhaled. To study the toxicity of RK in lung and the mechanisms of RK-induced lung injury, a total of 72 male ICR mice (1.5 months, adult) were randomly assigned to the RK exposure group (RKEG) and normal control group (NCG). Mice were whole-body exposed to room air or aerosol of 18000 mg/m$^3$ RK for 4 hours. Histopathological analysis was performed to evaluate the pulmonary lesions. Oxidative stress was assessed by assay of MDA, SOD, GSH-PX and TAOC. Inflammatory response was estimated by detecting inflammatory cell counts, TNF-α and IL-6 protein levels in serum. The results showed that after 2 to 6 hours of RK exposure, pulmonary vascular dilatation, congestion and edematous widening of the alveolar septum were noted. After 12 to 24 hours post-exposure, diffuse hemorrhage in alveolar space were found, along with the progressive pulmonary vascular dilatation and edematous widening of alveolar septum. During 3 to 7 days of RK-exposure, inflammatory cells were scattered in the lung tissue. The pathological alterations of the lung were alleviated after 14 days post-exposure, and showed significant improvement after 21 days post-exposure. After 30 days of RK exposure, the pathological changes in the lung tissue were nearly recovered except the local thickening of the alveolar wall. Compared with NCG, RK inhalation produced a significant increase of MDA levels and a significant decrease of SOD, GSH-PX and TAOC activity in the lung after 2 hours post-exposure ($P < 0.05$). There were significant increases of TNF-α and IL-6 protein levels in serum of mice in RKEG after 2, 6 and 12 hours and 1, 4 and 7 days post-exposure compared with NCG ($P < 0.05$). TNF-α protein levels had a sharp increase after 4 days of exposure. IL-6 protein level was increased at early phase of experiment and then gradually decreased along with the prolonged course of exposure. Considering that the RK-induced lung injury was through the oxidative stress, inhibition of oxidative stress after RK exposure may be urgently needed.

Keywords: Rocket kerosene, lung injury, inhalation, oxidative stress

Introduction

Rocket kerosene (RK) is a new rocket propellant which was developed in recent years. The chemical compositions of RK are not fixed by their specification; however, they are bounded by specification requirement such as density, aromatics, boiling range and freezing point. It was found that almost a half of RK components were bicyclo-paraffins, followed by monocyclo-paraffins, iso-alkanes, n-alkanes, oxygen-containing compounds, alkenes, tricyclo-paraffins and aromatics. In RK vapour, more than half of the components were bicyclo-paraffins, followed by monocyclo-paraffins, alkanes, oxygen-containing compounds and alkenes [1]. Aspiration of RK may cause respiratory irritation and toxicity may occur if a high level of RK is inhaled. Up to date, however, there are no comprehensive studies on RK toxicity in the lung. This study was conducted to determine the acute toxicity of the inhaled RK on male ICR mice with the targeted exposure levels of 18000 mg/m$^3$ and mainly to assess the effects of PK inhalation on respiratory histomorphology and oxidative status.

Materials and methods

Animals and inhalation exposures

The study was approved by the Hospital Ethics Committee. Male ICR mice (6 wk-old, 18-22 g) were purchased from Vital River laboratories (Beijing, China). The mice were housed in a spe-
Lung injury and inhalation exposure to rocket kerosene

Specific pathogen-free (SPF) facility and fed with laboratory chow and ad libitum water. After a minimum 7 days of acclimation, the mice were randomly allocated into two groups with each containing 36 mice: (1) normal control group (NCG), in which mice inhaled fresh air and (2) rocket kerosene experimental group (RKEG), in which mice inhaled RK (provided by China Academy of Space Technology). Mice in the normal control group and 18000 mg/m$^3$ RK exposure group were exposed to room air or aerosol of 18000 mg/m$^3$ RK by whole-body continuous dynamic inhalation for 4 hours (air flow rate: 2 m$^3$·h$^{-1}$, temperature: 25°C, humidity: 50%). The whole-body exposure chambers (Hope-Med Co. Ltd. Beijing, China) were constructed of stainless steel with a nominal internal volume of 300 L that maintains a constant throughput air. With this exposure test, subjects were exposed while free to move about in groups.

Collection of blood sampling

Blood samples were collected from mice by retroorbital bleeding before slaughter. Disodium EDTA was used as anticoagulant for 50 μl of blood. Another set of 1 ml of blood samples collected into test tubes were allowed to clot. Blood serum was separated by centrifugation at 3000 rpm, for at 4°C and stored at -20°C.

Histopathological analysis

The mice were anesthetized by intraperitoneal injection of chloral hydrate. The samples of the lungs were excised, sectioned transversely or longitudinally, and fixed in 10% neutral-buffered formalin. The sections were then embedded in paraffin and stained with hematoxylin and eosin for microscopic evaluation at a magnification of ×100. The extent of tissue injury was evaluated by two certified pathologists who knew nothing about the nature of the groups being assessed.

Assay of T-AOC, SOD, MDA and GSH-PX

Lung tissue samples were homogenized in cold saline at 4°C. The tissue homogenate was 10% (w/v). Samples were centrifuged at 800 × g for 10 min at 4°C and the supernatant was used to biochemical measurements. Total (Cu-Zn and Mn) super oxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px), total antioxidant capacity (TAOC) and malondialdehyde (MDA) levels in lung tissue were determined by the test kits provided by Nanjing Jiancheng Bioengineering Institute. For SOD, the principle of the method was based on the inhibition of nitro blue tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was expressed as U/mg protein. The activity of GSH-Px was determined by quantifying the catalyzed reaction rate of GSH per minute on the base of its catalysis. One unit of GSH-Px activity was defined as 1.0 μM GSH oxidized to oxidized glutathione (GSSG) per milligram of protein per 5 min at 37°C after exclusion of non enzymatic reaction. GSH-Px activity was expressed as U/mg protein. The TAOC was measured by the method of ferric reducing/antioxidant power assay. TAOC activity was expressed as U/mg protein. For MDA, the principle of the method was based on the reaction of MDA with thiobarbituric acid at 90-100°C. Levels of MDA were expressed as nmol/mg protein. The analyses

Figure 1. Histopathological changes of the lungs of mice after RK inhalation (H&E, original magnification, ×100). A: Normal lung; B-J: Lungs of mice after 2, 6 and 12 hours and 1, 4, 7, 14 and 21 days of RK exposure respectively.
were performed with a UV 1800 spectrophotometer (Shimadzu, Japan). Protein concentration was measured by the Lowry method [2].

**Evaluation of inflammatory cells**

The white blood cells (WBC) and differential leucocyte counts including lymphocyte (LYM), neutrophile granulocyte (NEUT) and monocytes (MO) were measured with an automated cell counter (Sysmex KX-21, Japan).

**Assay of TNF-α and IL-6**

All samples were analyzed by flow cytometry using the Cytometric Bead Array. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were determined by the test kits provided by Becton, Dickinson and Company. The protocol for analysis was designed by biosciences that were the manufactures of the reagent. 50 µl of the mixed capture beads were vortexed and then added to the appropriate assay tubes. A 50 µl of the mouse cytokine standard dilutions were then added to the control assay tubes. 50 µl of each test sample was measured into the test assay tubes and 50 µl of detection reagent added to the assay tubes. The assay tubes were then incubated for 2 hours at room temperature protected from direct exposure to light. 1 ml of wash buffer was added to each assay tube and centrifuged at 200 x g for 5 minutes. The supernatant was carefully aspirated and discarded from each assay tube. 300 µl of wash buffer was added to each assay tube to re-suspend the bead pellet. The samples were then ready for analysis on a flow cytometer (FACScalibur, Becton, Dickinson and Company). Each sample was vortexed for 5-10 seconds immediately before analyzing on the flow cytometer. Cytokine levels were measured from standard curves calibrated from reagent standards using the flow cytometer’s software and the values were in pg/ml.

**Results**

**General observation and histopathologic findings**

Lung tissue sections from the NCG displayed normal structure and no pathologic changes under light microscope (Figure 1A). After 2 to 6 hours of RK exposure, pulmonary vascular dilatation, congestion and edematous widening of the alveolar septum were noted (Figure 1B, 1C). After 12 to 24 hours post-exposure, diffuse hemorrhage in alveolar space were found and the alveolar cavities were filled with erythrocytes and inflammatory cells, along with the progressive pulmonary vascular dilatation and edematous widening of alveolar septum (Figure 1D, 1E). During 3 to 7 days of RK-exposure, lymphocytic, neutrophile granulocyte and mononuclear cell infiltration were scattered in the lung tissue (Figure 1F, 1G). The pathological alterations of the lung including edematous widening of alveolar septum, pulmonary vascular dilatation and infiltration of inflammatory cell were alleviated after 14 days post-exposure (Figure 1H) and showed significant improvement after 21 days post-exposure (Figure 1I). After 30 days of RK exposure, the pathological changes in the lung tissue were nearly recovered except the local thickening of the alveolar wall (Figure 1J).

**Influence on oxidant stress**

Compared with NCG, RK inhalation produced a significant increase of MDA levels (1.18 ± 0.04 nmol/mg protein vs. 0.98 ± 0.15 nmol/mg protein, P < 0.05) and a significant decrease of SOD activity (4.40 ± 0.29 U/mg protein vs. 5.21 ± 0.38 U/mg protein, P < 0.05). RK inhalation also produced a significant decrease in the GSH-Px activity in the lung tissue (56.83 ± 9.70...
Lung injury and inhalation exposure to rocket kerosene

U/mg protein) when compared with the NCG (73.26 ± 12.69 U/mg protein, P < 0.05). A significant decrease in the TAOC activity in the lung tissue of RK inhaled animals was also noticed (1.19 ± 0.10 U/mg protein) when compared with the NCG (1.33 ± 0.11 U/mg protein, P < 0.05) (Table 1).

Effect of RK on inflammatory cells

After RK exposure, animal’s WBC, LYM, MO and GR were fluctuated significantly (Figure 2). Compared with NCG, WBC counts of RKEP were decreased significantly by 67%, 39% and 55% (3.6 × 10^9/L, 1.6 × 10^9/L and 3.2 × 10^9/L) after 6, 12 and 24 hours post-exposure, respectively (P < 0.05) and increased significantly by 206% (11.9 × 10^9/L, P < 0.05) after 4 days (Figure 2A). Counts of LYM were significantly decreased by 53%, 38% and 45% (2.17 × 10^9/L, 1.28 × 10^9/L and 2.32 × 10^9/L, P < 0.05) after 6, 12 and 24 hours, respectively (Figure 2B). Counts of MO were significantly decreased by 500% (4.0 × 10^9/L, P < 0.05) after 4 days (Figure 2C) and counts of GR were significantly increased by 426% and 177% after 2 hours and 4 days (Figure 2D).

Effect of RK on serum cytokines

There were significant increases of TNF-α and IL-6 protein levels in serum of mice in RKEG after 2, 6 and 12 hours and 1, 4 and 7 days post-exposure compared with NCG (P < 0.05) (Figure 3A, 3B). TNF-α protein levels had a sharp increase after 4 days of exposure. IL-6 protein level was increased at early phase of experiment and then gradually decreased along with the prolonged course post-exposure.

Discussion

Rocket kerosene (RK) is a new rocket propel-lant. Although its chemical compositions are not fixed by their specification, it was found that almost half of RK components was bicyclo-paraffins and more than half of RK vapour were bicyclo-paraffins [3]. Aspiration of RK may cause respiratory irritation and toxicity may occur if a high level of RK is inhaled. Our study revealed that inhalation exposure to RK could induce animal’s lung injuries and the pathological changes included neutrophil infiltration, aggravated lung vascular permeability and edema, increased MDA levels, decreased SOD, GSH-Px and TAOC activities in the lung tissue.
The increased serum levels of pro-inflammatory cytokines such as TNF-α and IL-6 at early phase of the experiment further displayed the harmful effects of RK on pulmonary tissues.

The acute lung injury is usually characterized by increased capillary permeability, interstitial and alveolar edema, release of inflammatory mediators and extensive neutrophil infiltration [4]. According to reports in the literature, the exposure to petroleum hydrocarbons could impair the type II pneumocytes resulting in a decreased production of surfactant and consequent alveolar collapse, ventilation-perfusion mismatch and hypoxemia. This ultimately leads to hemorrhagic alveolitis, interstitial inflammation, intra-alveolar hemorrhage and edema, hyperemia, bronchial necrosis and vascular necrosis [5, 6]. Our experimental results showed that there were significant increases of TNF-α and IL-6 protein levels in serum of mice in RKEG after 2, 6 and 12 hours and 1, 4 and 7 days post-exposure. TNF-α protein levels had a sharp increase after 4 days post-exposure and IL-6 protein levels was increased at early phase of experiment. The pathological alterations of the lung including edematous widening of alveolar septum, pulmonary vascular dilatation and infiltration of inflammatory cells were found obviously during the early phase of experiment. The pathological alterations of the lung including edematous widening of alveolar septum, pulmonary vascular dilatation and infiltration of inflammatory cells were found obviously during the early phase of the experiment and then gradually alleviated and got improvement along with the prolonged course of post-exposure. We concluded that RK inhalation induced lung injury by direct erosion and indirect damage through inflammatory response, which is one of the most important features of the pathological process of RK induced lung injury [7]. Among the pro-inflammatory cytokines, IL-6 and TNF-α are considered to contribute to the initiation and extension of the inflammatory process [8, 9].

Oxidative stress is a threat to well-being. Recent studies reported that excessive oxidative stress has a proven role in the pathogenesis of lung injury [10-13]. Oxidative stress could initiate pro-inflammatory effects in macrophages and bronchial epithelial cells [14-16]. The antioxidant defense system via oxidation, dismutation and hydrolysis protects normal biological functions of tissues and cells against the harmful effects of superoxide free radicals and are thus natural endogenous protection against mitochondrial generation of reactive oxygen species [10, 11]. This implies that insult of the antioxidant defense system results in loss of cell and tissue integrity. It may be due to the leakage of fluid into the extravascular space [12]. This could explain the edema and hemorrhagic spots of lung tissue seen in this study following exposure to RK. The reduction of TAOC in this study also suggested that the antioxidant capacity was decreased upon RK inhalation. The antioxidant enzymes SOD and GSH-Px serve as a primary line of defense in destroying the free radicals produced by oxidative stress. SOD reduces the radical superoxide (O2-) to form hydrogen peroxide (H2O2) and oxygen (O2), then, GSH-Px works simultaneously with the protein glutathione to reduce H2O2 and ultimately produce water (H2O). MDA is one of the most frequently used indicators of lipid peroxidation. This study documents the effects and possible mechanisms of RK on lung tissue. The results showed that exposure of experimental mice to RK triggered the oxidative stress and

**Figure 3.** Serum TNF-α and IL-6 protein levels of mice inhaled RK. A: TNF-α protein levels; B: IL-6 protein levels. *P < 0.05 vs. NCG group.
the increased MDA level with decreased SOD and GSH-Px suggest that exposure to RK could cause oxidative stress to pulmonary tissue by consuming the protective free radical scavengers. Findings in this study are similar to the case report by Prasad et al [13], which documented that aspiration of kerosene led to bilateral hemorrhagic pleural effusion.

In conclusion, we have demonstrated that inhalation exposure of mice to RK, similar to human occupational exposure, could induce lung toxicity, and that the oxidative stress and inflammatory response may be the underlying mechanisms. All results collectively suggested that the inhalation exposure to RK could induce the adverse effects on lung in mice, which might increase the risk of the pulmonary disease. Further study is needed on validating additional markers of oxidative stress and inflammation. Considering the multiple ways in which human exposure to RK, inhibition of oxidative stress after RK exposure is urgently needed.

Acknowledgements

This work is supported by the Programs of Science and Technique Foundation of Logistic Department of GED, No. CZZ11C004.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yan Cui, Department of General Surgery, 306 Hospital of PLA, 9 North Anxiang Road, Chaoyang District, Beijing 100101, China. Tel: +86 10-66356106; Fax: +86 10-64876056; E-mail: dryancui@163.com

References