Acute exposure to crotonaldehyde induces dysfunction of immune system in male Wistar rats

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ABSTRACT — Crotonaldehyde is a ubiquitous air pollutant in the environment. It is reported to be harmful to the biosystems in vivo and in vitro. The exposure to crotonaldehyde irritates the mucous membranes and induces edema, hyperemia, cell necrosis, inflammation, and acute respiratory distress syndrome in the lungs. However, the effects of crotonaldehyde on the immune system have not been reported. In the present study, 6-8 weeks old male Wistar rats were exposed to crotonaldehyde by intratracheal instillation at doses of 4, 8, and 16 $\mu$L/kg body weight (b.w.). The general damage in the animals was investigated; the cell counting and the biochemical analysis in the peripheral blood were tested. Furthermore, we investigated the functions of alveolar macrophages (AMs), the alterations of the T-lymphocyte subsets, and the cell composition in the bronchoalveolar lavage fluid (BALF). We found that the activities of the animals were changed after exposure to crotonaldehyde, the cellular ratios and the biochemical components in the peripheral blood were altered, the ratio of mononuclear phagocytes decreased, and the ratios of lymphocytes and granulocytes elevated significantly in BALF. Meanwhile, crotonaldehyde altered the ratio of the T-lymphocyte subsets, and the phagocytic rates and indices of AMs increased obviously. In conclusion, crotonaldehyde induces dysfunction of immune system in male Wistar rats.

Key words: Crotonaldehyde, Male Wistar rats, Immune system

INTRODUCTION

Crotonaldehyde ubiquitously exists in the environment. Most of the human activities involving long carbon chain rupture result in its production. Crotonaldehyde is mainly formed from the incomplete combustion of organic materials (Lipari \textit{et al.}, 1984), and it can be found almost everywhere, especially in cigarette smoke, automatic exhaust (Feron \textit{et al.}, 1991), and also cooking oil fumes. Cooking oil fumes have been implicated as the reason for higher rate of lung cancer in Chinese women (Xue \textit{et al.}, 2016; Zamora \textit{et al.}, 2016). Crotonaldehyde commonly exists in chemical plants producing tocopherol, sorbic acid or $\alpha$-butanol (IARC, 1995), where occupational exposure to crotonaldehyde is probable for workers (Huang \textit{et al.}, 2015). It can be produced naturally during the storage and transportation of foodstuffs (IARC, 1995). Crotonaldehyde can also be formed endogenously from lipid metabolism. It works as an initiator or end product of lipid peroxidation (Kawaguchi-Niida \textit{et al.}, 2006; Pan and Chung, 2002; Esterbauer \textit{et al.}, 1991; Chung \textit{et al.}, 1999, 1996). Furthermore, it can be produced during metabolism of the carcinogens $N$-nitrosopyrrolidine and 1, 3-butadiene (Sharer \textit{et al.}, 1992; Filser \textit{et al.}, 2001; Elfarra \textit{et al.}, 1991; Duescher and Elfarra, 1992, 1993; Cheng and Ruth, 1993).

Crotonaldehyde is reported to be harmful to animals and cell lines. Crotonaldehyde is a quite toxic irritant...
which causes acute mortality by inhalation, subcutaneous injections or intraperitoneal exposure in experimental animals (Skog, 1950). It is a strong irritant to mucous membranes (Bainova and Madzhunov, 1984; Steinhagen and Barrow, 1984; IARC, 1995), including oral mucosa, tracheal mucosa (Dalhann and Rosengren, 1971), eyes, skin, especially the respiratory system. The odor of crotonaldehyde vapor above 15 ppm is strong, at concentrations above 1,000 ppm, a central nervous system (CNS) effect is evident during exposure as well as pulmonary irritation post-exposure (Rinehart, 1967). The response to repeated injection of crotonaldehyde intraperitoneally into mice indicated an acquired tolerance against the irritant in terms of lactate dehydrogenase (LDH) activity (Warholm et al., 1984). Crotonaldehyde induces edema, hyperemia, cell necrosis, and inflammation in the lungs (Skog, 1950; Li et al., 2017); furthermore, research by André et al. (2008) showed that neurogenic inflammation induced by cigarette smoke was mediated by crotonaldehyde. Occupational exposure to crotonaldehyde induces acute respiratory distress syndrome in human beings (Huang et al., 2015). The immune system mediates the generation and development of many diseases (Desforges et al., 2016), and it may be the target of crotonaldehyde. Unfortunately, little is known about the alterations of immune system induced by acute occupational exposure to highly concentrated crotonaldehyde.

In the present study, a relatively high dose of crotonaldehyde was selected to simulate the atmosphere of the acute occupational exposure; male Wistar rats were selected to be exposed to crotonaldehyde because of the susceptibility of the respiratory system, and the effects of crotonaldehyde on the immune system were preliminarily investigated. An advanced exposure method of intratracheal instillation was adopted without direct mechanical injury to the animals (Li et al., 2017). The general damage in the rats was evaluated. To illustrate the change of immune system related to acute pulmonary damage, alteration of the immune system was detected in a week post-exposure in the aspects of T lymphocytes and alveolar macrophages (AMs). Our research will be helpful for the risk assessment of crotonaldehyde.

MATERIALS AND METHODS

Reagents and antibodies

Crotonaldehyde (Labor Dr. Ehrenstorfer-Schäfers, Augsburg, Germany). Latex beads (1.0 μm mean particle size, Sigma, St. Louis, MO, USA). Fluorescein isothiocyanate (FITC) conjugated mouse anti-rat cluster of differentiation (CD) 3+ antibody (BD Biosciences, Franklin lakes, NJ, USA), Peridinin-Chlorophyll-Protein complex (PerCP) conjugated mouse anti-rat CD4+ antibody (BD Biosciences), Phycoerythrin (PE) conjugated mouse anti-rat CD8+ antibody (BD Biosciences).

Animals

Male Wistar rats, 6-8 weeks of age, were purchased from Vital River Laboratory Animal Co. (Beijing, China), and maintained in a caged house equipped with specific pathogen-free (SPF) isolation facility. The environment was set at 22°C with a 12/12-hr light/dark cycle. The animals were provided with standard rodent chow and water ad libitum. All experiments were conducted in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals.

Anesthesia methods

Two anesthesia methods were used for selection in the present study: in the first one, rats were anesthetized with ether in an anesthetic cylinder, and then administered crotonaldehyde; in the second one, chloral hydrate was used to inhibit convulsions, with 0.5-1.0 mL 5% chloral hydrate in normal saline (NS) injected intraperitoneally in each animal to produce mild anesthesia, and then the animals were anesthetized with ether prior to administration of crotonaldehyde. The method with higher exposure dose of crotonaldehyde was selected for following experiments.

Animal preparation

The animals were divided randomly into 5 groups: blank control group, negative control group, low-, medium- and high-dose groups. Animals were anesthetized by administering chloral hydrate intraperitoneally prior to exposure to ether, and then intratracheally instilled 200 μL of crotonaldehyde at different concentrations. Animals were not treated in the blank control group (Ctrl); rats were anesthetized and exposed to equal volume of normal saline in the negative control group (NS); animals were anesthetized and exposed to 4 μL/kg, 8 μL/kg or 16 μL/kg b.w. (1μL/kg = 0.8495 mg/kg) crotonaldehyde in the low-, medium- or high-dose group. The variance between NS group and the experiment groups demonstrates the effect of crotonaldehyde. Animals were maintained in SPF barrier system. All the animals were labeled. At 1 d, 3 d and 7 d after administration of crotonaldehyde, equal number of rats from each group were sacrificed to detect general damage and related immune alterations.
Crotonaldehyde exposure

Intratracheal instillation was selected to administer crotonaldehyde. Briefly, anesthetize each animal with chloral hydrate and ether, place it supine, prop open its mouth wide with a tweezer, insert a tube from the mouth, through the epiglottis, and into the trachea. Crotonaldehyde dissolved in 200 μL NS was sprayed into the trachea and lung, and the tube was pulled out immediately.

Blood routine tests and biochemical analysis

Normal saline containing 20% ethyl urethane was injected intraperitoneally to anesthetize each rat. The femoral artery was exposed, and 20 μL of blood was collected for detecting in a full-automatic blood cell counter system (Perlong, Nanjing, China). Ten milliliters of blood were collected into an evacuated coagulation-promoting tube, stored at 4°C for 30 min, and centrifuged at 3,000 revolutions per minute (rpm). The supernatant was used for biochemical analysis with a blood biochemical analyzer (Perlong).

Analysis of T lymphocyte subsets

We added 100 μL of heparin to a flow tube before 100 μL of peripheral blood from each rat was added, and then added FITC conjugated CD3+ antibody, PerCP conjugated CD4+ antibody, and PE conjugated CD8+ antibody 1 μL, respectively. We incubated the tubes at 37°C for 30 min, added 2 mL of FACS Lysing Solution (BD Biosciences) to each, stored them at room temperature (RT) for 15 min to lyse the red blood cells (RBC), centrifuged at 3,000 rpm for 5 min, washed with phosphate buffer solution (PBS) twice, fixed with 300 μL of 2% paraformaldehyde, and detected with a flow cytometer (BD FACSCalibur, BD Biosciences). The data were analyzed with FlowJo software (Treestar, Ashland, OR, USA).

Cell count in bronchoalveolar lavage fluid (BALF)

We anesthetized the animals with 20% ethyl urethane in normal saline intraperitoneally, released the blood thoroughly, dissected the rat and separated its trachea. Five milliliters of normal saline were lavaged into the lung each time for three times. All the lavage fluid was collected and centrifuged to obtain the cell pellet, and we adjusted the number of cells to 10⁶/mL, and centrifuged 200 μL of suspended cells onto a smear with a cytopsin. The smear was fixed in formaldehyde for 15 min, stained with Giemsa staining solution for 5 min and observed with a microscope (Olympus, Tokyo, Japan).

Detection of phagocytosis of macrophages in BALF

Cells in BALF were obtained and suspended to 10⁶/mL, and 1 mL of cell suspension was transferred into each well in a 24-well culture clusters. The clusters were placed in an incubator (Thermo Scientific, Waltham, MA, USA) for 2 hr to make the macrophages attach to the plates, and the other cells were removed. A sufficient number of fluorescent latex beads were added to each well, and incubated another 2 hr. Extra beads were removed. We observed the macrophages under a microscope (Olympus) and took pictures. The cells were collected and fixed, the fluorescence intensity was detected with a FACSCalibur machine (BD Biosciences), and the phagocytic rates and indices of alveolar macrophages were calculated.

Statistical analysis

Data were expressed as mean ± standard deviation (SD), and significance between groups was determined with SPSS software (SPSS 13.0, USA) by using one-way analysis of variance (ANOVA) followed by least significant difference (LSD). p < 0.05 was considered to be statistically significant between groups.

RESULTS

Administration of chloral hydrate improved the doses of crotonaldehyde

A proper dose of crotonaldehyde is crucial to evaluate the toxicological effects in animals. As is shown in Table 1A, the highest dose of crotonaldehyde promising 100% survival of animals anesthetized with ether was 4 μL/kg b.w., and the survival rate significantly decreased...
to 60% or 40% when the dose of crotonaldehyde was elevated to 6 μL/kg or 8 μL/kg. We observed that convulsions might be the reason behind rat deaths. The usage of the convulsion inhibitor chloral hydrate obviously enabled the dose of crotonaldehyde to be raised while ensuring no deaths. As is shown in Table 1B, the 100% survival dose of crotonaldehyde was elevated to 16 μL/kg b.w. However, the dose region inducing death was still narrow. Administration of 20 or 24 μL/kg b.w. of crotonaldehyde made the survival rate decrease sharply to 50% or 40%.

Crotonaldehyde exposure attenuated the body weight increase of animals

The body weight increase of animals in each group was calculated and is shown in Fig. 1. Administration of 16 μL/kg b.w. of crotonaldehyde attenuated the increase in body weight obviously in 1 d after administration. At 3 d after administration of crotonaldehyde, there was no significant variance between the experiment groups and the NS group. No statistical significance was observed between groups at 7 d post exposure to crotonaldehyde.

Crotonaldehyde changed the cell components in the peripheral blood

To evaluate the damage from crotonaldehyde, the routine blood test and biochemical analysis of the peripheral blood in rats were conducted first. The number of platelets (PLT) decreased obviously in the 16 μL/kg group (Fig. 2A), and the number of eosinophils (EO) increased significantly in the 8 and 16 μL/kg groups (Fig. 2B), while the other cells, including lymphocytes and basophils, showed no significant alterations (data not shown). Changes in PLT and EO in blood occurred at 7 d post exposure to crotonaldehyde. They exhibited delayed alterations compared with body weight increase. The
results of biochemical analysis are shown in Table 2. We found that compared with the NS group, globulin (GLB) in serum increased to 23.82 ± 4.08 g/L in the high-dose group, causing decrease in the ratio of albumin to GLB (A/G); total bilirubin (TBIL) and direct bilirubin (DBIL) decreased at 7 d after administration; and the concentrations of triglyceride (TG), cholinesterase (CHE), cholesterol (CHO), and Ca²⁺ increased significantly. The concentrations of aspartate transaminase (AST), blood usea nitrogen (BUN), glucose (GLU), high density lipoprotein (HDL), creatinine (Cr), and LDH increased prior to decreasing, or decreased and then increased (data not shown). The other elements in serum, including alanine transaminase (ALT), alkaline phosphatase (ALP), indirect bilirubin (IBIL), creatine kinase (CK), and uric acid (UA), showed no significant changes (data not shown).

Table 2. Biochemical analysis of peripheral blood in rats exposed to crotonaldehyde.

<table>
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<td>1.19 ± 0.48</td>
<td>1.27 ± 0.97</td>
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* Statistically significant from the NS group

Crotonaldehyde decreased the ratio of CD4⁺/CD8⁺ subset in lymphocytes

The strategy for gating cells in the process of analyzing T lymphocyte subsets is shown in Fig. 3A. Briefly, the white blood cells (WBC) with higher forward scatter (FSC) and lower side scatter (SSC) were first separated, of which the T lymphocytes with the character of CD3⁺ were grouped, and finally the subsets of CD4⁺ or CD8⁺ in CD3⁺ lymphocytes were gated. The representative results of the T lymphocyte subsets are shown in Fig. 3B. The quantitative results of the T lymphocyte subsets are shown in Fig. 4. We found that the proportion of CD3⁺ T lymphocytes in total lymphocytes in the peripheral blood had an increasing trend at 1 d and 3 d after administration of crotonaldehyde, but not all the experiment groups showed statistically significant differences compared to the NS group. The CD4⁺ subset in CD3⁺ T lymphocytes decreased significantly in the 8 and
16 μL/kg groups while the CD8+ subset increased obviously at 1 d post-exposure, which induced decrease of the ratio of CD4+/CD8+ subset.

Crotonaldehyde changed the ratios of cells in BALF

The results of numbering and classification of the cells in BALF are shown in Fig. 5. Figure 5A shows that almost all the cells in BALF were mononuclear phago-
cytes in the control and NS groups. Administration of crotonaldehyde induced a sharp decrease of the ratio of mononuclear phagocytes at 1 d after exposure. However, it recovered quickly, and only the high-dose group had significant decreases at 3 d after exposure. No statistical variance between groups was observed at 7 d after exposure. The rate of lymphocytes elevated significantly and then decreased gradually (Fig. 5B). The ratios of neutrophils, eosinophils, and basophils increased at 1 d post-exposure, and then decreased to the normal levels (Fig. 5C-5E). Crotonaldehyde increased the total number of the cells in BALF at 1 d post-exposure (Fig. 5F), but no significant alteration was observed at 3 d and 7 d post-exposure (data not shown). The number of mononuclear phagocytes did not change significantly (data not shown); it was the increase of lymphocytes and granulocytes that resulted in the decrease in the ratio of phagocytes.

**Crotonaldehyde enhanced the phagocytosis of macrophages in BALF**

The phagocytosis function of macrophages was investigated and the results are shown in Fig. 6. Figure 6A shows that crotonaldehyde promoted the phagocytic rates and indices in alveolar macrophages. With the exposure to crotonaldehyde, more macrophages were involved in phagocytosis and more latex beads were endocytosed into cells. The quantification of phagocytic rate (Fig. 6B) showed that it increased in a dose-dependent manner, and
decreased gradually with the elongation of time. The flow cytometry results of the phagocytic indices at 1 d after exposure are shown in Fig. 6C. The mean fluorescence intensity of a single cell was calculated and is shown in Fig. 6D. The results indicate that more beads were endocytosed into a macrophage, and the mean fluorescence intensity of each cell was elevated with the increase of crotonaldehyde doses.

Fig. 5. Effects of crotonaldehyde on the ratios of cells in BALF in rats at different times post-exposure. Cells in BALF were obtained and stained with Giemsa staining solution as described in Materials and Methods. The ratios of mononuclear phagocytes and lymphocytes are shown in 5A and 5B, respectively. The ratios of neutrophils, eosinophils, and basophils are shown in 5C-5E. Some granulocytes were not detected in certain groups, so they are listed as 0 in the figures. The total number of cells in BALF at 1 d post-exposure is shown in 5F. * p < 0.05 compared with NS group.
DISCUSSION

Crotonaldehyde is ubiquitously distributed in the environment. Being structurally an α, β-unsaturated aldehyde, crotonaldehyde possesses high reactivity with nucleophilic sites in DNA and proteins. Many reports in vitro have shown that crotonaldehyde regulates the immune functions in different cell lines (Warholm et al., 1984; Moretto et al., 2009; Facchinetti et al., 2007). However, the exact role it plays in in vivo experiments has not been reported. In the present study, we investigated the general damage and related alterations of the immune system in male Wistar rats exposed to crotonaldehyde. We found that crotonaldehyde altered the cell count and biochemical components in the blood of animals. It changed the ratios of mononuclear phagocytes, lymphocytes, and granulocytes in BALF. Furthermore, crotonaldehyde altered the rate of T lymphocyte subsets and augmented the activities of AMs by improving the phagocytic rates and indices significantly. Collectively, crotonaldehyde induced the dysfunction of the immune system in male Wistar rats.

In the present study, intratracheal instillation was selected to administer crotonaldehyde with the reason that it possessed the advantages of exposing accurate doses of crotonaldehyde directly to the lungs of rats. The high dose of crotonaldehyde used in the present study was 16 μL/kg b.w., which is comparative to that in previous research (Jha et al., 2007). During the selection of highest

Fig. 6. Crotonaldehyde improved the phagocytosis function of macrophages in BALF. (A) The pictures of the phagocytosis of latex beads in macrophages. The pictures were taken with the magnification of 200 ×. The phagocytic rates were calculated and are shown in 6B. The analysis of fluorescence intensity in different groups with flow cytometer at 1 d post-exposure is shown in 6C, and the phagocytic indices were calculated and are shown in 6D. * p < 0.05 compared with NS group.
dose of crotonaldehyde, convulsions resulted in numerous rat deaths. The results were consistent with findings from Rinehart and Skog. Rinehart (1967) showed that crotonaldehyde vapor induced evident CNS effect during exposure and pulmonary irritation post-exposure. While Skog (1950) found that subcutaneous administration of larger doses of crotonaldehyde induced intense excitation, tremors and convulsions in rats. The results were confirmed by significantly elevated crotonaldehyde doses and reduced mortality with the usage of chloral hydrate.

Crotonaldehyde attenuated the increase of the body weight of animals (Fig. 1), which supported that crotonaldehyde was an irritant toxicant, and its administration limited routine activities of animals. What needs to be emphasized is that 4 µL/kg crotonaldehyde slightly but significantly improved the increase at 1 d post-exposure, indicating that low doses of crotonaldehyde might promote the activities of animals. It could be further evidence supporting the exciting effect of crotonaldehyde. Crotonaldehyde changed the body weight increase, blood cell numbering and biochemical components. The attenuation of body weight increase was temporary, and it recovered from 3 d post-exposure, which is similar to acrolein (Ong et al., 2012). The decrease of PLT and increase of EO in peripheral blood were delayed alterations, indicating some other injury or recovery activities were still persistent in the organisms. PLT are potent immune modulators and effectors. They play roles in regulating immune function and inflammation. The decrease of PLT suggests that crotonaldehyde might acutely impair the PLT function, and it further indicates that inflammation might be increased in rats (Jenne and Kubes, 2015). Chung et al. (1986) found that crotonaldehyde in the liver tissues of F344 rats induced lesions, with the majority of the foci were of the EO consisting of enlarged eosinophilic cells with large nuclei. These results indicate that EO probably plays a central role in the development of crotonaldehyde-induced diseases. The increase of EO is consistent with above results. EO is the regulator of immune and inflammato-

AMs play important roles in maintaining pulmonary innate immune responses (Miyata and van Eeden, 2011). Crotonaldehyde was reported to play a crucial role in regulating the activities of the AMs (Facchinetti et al., 2007). In the present study, we found that crotonaldehyde activated AMs characterized by augmented phagocytosis rates and indices. This indicates that crotonaldehyde enhanced the innate immune system in the lungs of rats. These results are consistent with some results in vitro. For example, crotonaldehyde mediated macrophage activation and pulmonary inflammation induced by cigarette smoke (Facchinetti et al., 2007), and promoted the release of pro-inflammation cytokines in macrophages and epithelial cells (Facchinetti et al., 2007; Moretto et al., 2009). But meanwhile, some opposite conclusions have been reported. Yang et al. (2013) found that crotonaldehyde suppressed the immune function in AMs. Related research about acrolein showed that acrolein accelerated pulmonary inflammation by elevating the numbers of macrophages and neutrophils in BALF, and the production of several related cytokines including IL-1α, IL-1β, IL-6, tumor necrosis factor (TNF), interferon (IFN)-γ, and monocyte chemotactic protein 1 (MCP-1) (Ong et al., 2012). Some other studies found that acrolein inhibited the gene expression and production of IL-2, IL-10, IFN-γ and TNF-α in T cells (Lambert et al., 2007). Even one study reported that no alterations of immunotoxicity were observed in rats exposed to acrolein at atmospheric concentrations (3 ppm) (Sherwood et al., 1986). Collectively, no consensus is obtained about the immune effect of crotonaldehyde in vitro, and the controversy probably results from the different concentrations, exposure periods or cell lines used in different studies. Combined with the results that immune cells were recruited into the BALF, our results supported that crotonaldehyde activated the immune function of AMs in the lungs.

T lymphocytes primarily execute the adaptive immunity in the organisms (Sopori, 2002). CD4+ and CD8+ T cells are two major subsets of T lymphocytes (Tanaka and Taniuchi, 2014). We found that crotonaldehyde did not change the total number of T cells in peripheral blood, but significantly altered the ratio of CD4+/CD8+ T cells subsets. CD8+ T cells work as the effectors of immuni-

ity. It plays a role in either promoting or preventing the development of autoimmune disease (Gravano and Hoy-
er, 2013). CD4+/CD8+ ratio is an important index of immuno-function. It has been used clinically to evaluate patients' immunity (Yin et al., 2015). Results showed that crotonaldehyde decreased the ratio of CD4+/CD8+, which indicated that crotonaldehyde induced the dysfunction of adaptive immunity. Further research will be need-
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ed to conclude that the immune function is promoted or inhibited.

There are still limitations existing in this paper. For instance, the levels of cytokines and immunoglobulin are important to evaluate the immune responses, but they were not detected with the main reason that we focused on the responses of T lymphocytes and the phagocytosis of AMs. Further investigation will be needed. In general, we found that crotonaldehyde affected the activities of animals, induced attenuation of body weight increase in male Wistar rats. It changed the cell number and biochemical components in peripheral blood. Hyperreactivity of AMs was induced, characterized by augmented phagocytosis rates and indices; immune responses may be disordered with the altered rate of T lymphocytes. The current study provides new insight into the toxic effects of crotonaldehyde, and it will be helpful for the risk assessment of crotonaldehyde.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES


