

Perfluoroisobutylene-Induced Acute Lung Injury and Mortality are Heralded by Neutrophil Sequestration and Accumulation

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Abstract: Perfluoroisobutylene-Induced Acute Lung Injury and Mortality are Heralded by Neutrophil Sequestration and Accumulation: Hemei WANG, *et al.* Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences—Perfluoroisobutylene (PFIB) is a highly toxic and potentially life-threatening pneumoedematogenic agent that is usually encountered in case of fire or industrial accidents. The mechanisms by which the toxicity of PFIB are mediated remain unclear. To investigate the role of neutrophil/polymorphonuclear leukocytes (PMN) in the pathogenesis of PFIB-induced acute lung injury (ALI), mice and rats were exposed to a sublethal concentration of PFIB (130 mg/m³ and 140 mg/m³, respectively) for 5 min in a flow-past whole-body chamber. The general pattern for the time-course of the increase in lung PMN infiltration as measured by lung myeloperoxidase (MPO) assay was shown to be rather similar to that of the lung injury indexed by both the total protein increase in the bronchoalveolar lavage fluid (BALF) and lung wet-to-dry weight ratio, except for the earlier start and the maximal time-point of the increase in lung PMN infiltration. When neutropenia was obtained by cyclophosphamide pretreatment, death of the mice induced by an over-LCt₅₀ dose of PFIB (at 190 mg/m³ for 5min) dropped dramatically, and this was very well supported by observations in the BALF total protein analysis, histopathological as well as ultrastructural studies. These results confirmed that PFIB inhalation-induced ALI and mortality are heralded by the influx of PMNs into the lung.
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Perfluoroisobutylene (PFIB) is a highly toxic compound that can result from combustion of polymerized fluorocarbon compounds such as Teflon (polytetrafluoroethylene) which is widely used in industry and life in general. PFIB can also be generated as a byproduct of tetrafluoroethylene production^{1, 2}. Brief inhalation exposure to low concentrations of PFIB can result in severe lung injury characterized by permeability pulmonary edema, incapacitation and even death, and no specific antidote or successful therapeutic measures are yet available^{3, 4} because the mechanisms by which the toxicity of PFIB are mediated remain unknown.

The currently available data on the mechanisms of PFIB-induced acute lung injury (ALI) and edema are small in number, and can be simply summarized as follows:

1. The working hypothesis that hydrogen fluoride (HF)⁵ may play a role, which has been proved impractical by the detailed research of Lehnert and his coworkers⁶.
2. The working hypothesis that several reactive intermediate species (RIS) may play roles, which is supported mainly indirectly by electron paramagnetic resonance (EPR)/Spin trapping studies on the chemistry of PFIB nitrene and nitroso spin traps⁷ and by observation of a great reduction in the levels of endogenous thiols in laboratory animals after PFIB inhalation. Furthermore, pretreatment with buthionine sulfoximine (BSO), a tool drug that can decrease endogenous thiol levels significantly, or such drugs as N-acetylcysteine were observed to be capable of either making the animals more susceptible to PFIB intoxication or effectively protecting against lethal doses of PFIB inhalation respectively⁸, but direct evidence concerning the exact role of the RIS and its types resulted in calls for further clarification.
3. The observation by Lehnert *et al.*¹⁰ that the accumulation of blood monocytes and PMNs in the lung's capillary bed was one of the early responses to PFIB inhalation. Enlightened by their observation

and the pathogenesis of Acute Respiratory Distress Syndrome (ARDS) studies, in which lung neutrophil/polymorphonuclear leukocytes (PMN) sequestration and activation play a key role⁹, we speculate that PMN may also play a role in PFIB-induced ALI.

In order to verify the above speculation experimentally, in the present study we first confirmed the results of Lehnert and his coworkers¹⁰ by demonstrating that there is a significant increase in neutrophil infiltration into the lungs 1h after a 5 min PFIB (130 mg/m³) exposure with a peak at 20 h and a sustained high level for as long as 48 h as seen in lung myeloperoxidase (MPO) activity measurement, the time-course of which was shown to be similar to that of the lung injury indexed by both the total protein increase in the bronchoalveolar lavage fluid (BALF) and the wet-to-dry lung weight ratio. To determine the important role played by PMNs, we have also examined the effects of neutrophil depletion on PFIB-induced mouse mortality, increases in the BALF total protein concentration, and pathological changes as detected by histopathological and ultrastructural examinations of rats.

Materials and Methods

Animal housing and acclimation

Pathogen-free male Sprague-Dawley rats weighing 180–220 g and pathogen-free male Kunming mice (18–22 g) were used in this study. The rats were obtained from the Institute of Medical Experimental Animals, the Chinese Academy of Medical Sciences (Beijing, PRC), and the mice were from the Center of Medical Experimental Animals, the Academy of Military Medical Sciences (Beijing, PRC). The animals were housed in quiet, humidified, clean rooms with a light-cycle of 12 h/12 h for 1 week before use. Food and water were available *ad libitum* except while they were in the exposure chamber. All the animal experiments were done in accordance with the Guide for Animal Experimentation at the Chinese Academy of Medical Sciences, Beijing, P. R. China.

Animal exposure to PFIB

PFIB was obtained from Shanghai Institute of Organic Fluorine Materials at a purity of 98%. The 5 min LC₅₀ values for PFIB inhalation in rats and mice have been determined to be within the range of 240–320 mg/m³ and 160–180 mg/m³ respectively, in a similar flow-past whole-body exposure apparatus described by Lehnert *et al.*¹⁰ except that the tail gases from the exposure chamber are conducted into a flask containing 200 ml of 5% K₂MnO₄ solution and that PFIB concentrations were monitored by means of a gas chromatograph equipped with an electron capture detector (Perkin Elmer, USA). These data on LC₅₀ values for PFIB in our lab correspond to those reported previously¹¹.

Determination of PFIB doses employed in mice and rats

We determined PFIB doses in mice and rats out of the following considerations: (1) They should be below LC₅₀ and allow us to accomplish a comparatively comprehensive observation (as long as 72 h after exposure) without causing any severe loss due to death of both mice and rats in the experiment period. (2) The lung pathological changes induced in the mice and rats should be comparable. In order to satisfy the second consideration, a semiquantitative histopathological study described by Lehnert *et al.*¹⁰ was adopted. Because there were the data accumulation concerning the PFIB toxicology in mice and rats in our lab, here we just directly reviewed and arranged the data related to the determination of PFIB doses employed in the present studies.

Overview of objectives and approaches

In the first part of the present studies, we observed the time-courses of the lung wet-to-dry weight ratio and MPO activity in mice as well as the BALF total protein concentration in rats after the animals were exposed to sublethal doses of PFIB (for the exact doses see Results).

In the second part, we examined the effects of PMN depletion on PFIB-induced lung toxicity (CPA/PFIB group). Mouse survival was followed for up to 7 d after five-minute exposure to 190 mg PFIB/m³, an over-LC₅₀ dose. Analyses of lung MPO activity, the wet-to-dry weight ratio of the mice, assays of the BALF total protein concentration, and histopathological and ultrastructural examinations in the rats were performed at 24 h after the PFIB inhalation, because the time-course studies showed that the injurious effects of PFIB were approximately the most severe at this time (see Results).

Neutrophil (PMN) depletion

Rats and mice were made neutropenic by giving cyclophosphamide (CPA, Sigma Chemical Co.) intraperitoneally by the same procedures as previously reported^{12, 13}. In order to assess the effectiveness of CPA on circulating neutrophil depletion in our animals, blood samples were taken from the particularly assigned groups of animals. Total leukocyte counts were performed with a hemocytometer (Coulter Electronics, Hialeah, FL, USA). Differential counts were determined by a certified laboratory technologist, in a blinded fashion, by review of 500 consecutive cells on a Wright-Giemsa stained blood smear at 1,000 × magnification.

Lung myeloperoxidase activity assay

MPO activity was measured by the method described by Sheridan *et al.*¹⁴, with minor modifications. Briefly, lungs isolated from mice were rinsed, blotted dry, and frozen at -70°C. After weighing, frozen lung was homogenized, centrifuged for 20 min at 25,000 g and

4°C, and the pellets were resuspended in 50 mmol/L potassium phosphate buffer, pH 6.0, with 0.5% hexadecyltrimethylammonium bromide (HTAB). Samples were frozen at -70°C until the MPO activity assay was performed. After being measured, frozen samples were sonicated (UP 200s Ultrasonic Processor, Germany), incubated in a 60°C water bath for 2 h, centrifuged and assayed for MPO activity in a hydrogen peroxide/o-dianisidine buffer at 460 nm. The results are expressed as units of MPO activity per gram of lung tissue.

Lung wet-to-dry weight ratio

Mice were anesthetized by an intraperitoneal injection of pentobarbital (50 µg/g) and exsanguinated via abdominal aorta transection. The tracheae and lungs were then excised en bloc and cleared of all extrapulmonary tissue. Total lung wet weight was determined and the specimens were then dried in an oven at 80°C for 24 h and reweighed to determine the dry weight, then the lung wet-to-dry weight ratio was calculated.

Bronchoalveolar lavage fluid collection and its total protein concentration assay

Rats were anesthetized by injecting urethane (1.25 g/kg) intraperitoneally. After the onset of adequate anesthesia, the animals were exsanguinated via the abdominal aorta and their tracheae were exposed. Each animal's trachea was cannulated with a blunt, 18-gauge needle that was secured with silk ligature. BALF collection was performed with 6 ml PBS by means of a 10 ml syringe. The infusion and aspiration of the PBS in each lung were repeated 4 times. The lavage fluid was recovered (average fluid recovery was 5.4 ml), centrifuged at 1000g, 4°C, for 10 min. The supernatants were removed and stored at -70°C until the total protein concentrations were determined by the method of Lowry et al.¹⁵.

Histopathological studies

Rats specifically used for histopathological studies were deeply anesthetized with an intraperitoneal injection of urethane (1.25 g/kg). After exsanguinations via the carotid arteries, the lungs were excised and submerged in 10% formalin in phosphate-buffered saline for 48 h. After fixation, each left lobe was sliced for sectioning by the method described elsewhere¹⁰. Paraffin sections, 4 µm thick, routinely stained with hematoxylin and eosin, were examined by light microscopy.

Ultrastructural studies

Rats specifically used for ultrastructural studies were anesthetized with urethane, the lungs were collapsed by diaphragm puncture, and 4–6 ml of cold fixative (3% glutaraldehyde in 0.1 M phosphate buffer) was instilled via the trachea. The tracheae were then ligated and the

lungs were excised and submerged in cold fixative to fix overnight. A 2-to 3-mm slice through the central portion of the left lung perpendicular to the long axis was removed and further cut into 1-to 2-mm cubes. The lung fragments were then postfixed in 1% buffered osmium tetroxide (pH 7.2) and embedded in Poly-bed 812 epoxy resin. Semithin epoxy sections, 1 µm thick, differentiated with methylene blue, azure II, and basic fuchsin, and viewed by light microscopy, were used to select areas for ultrathin sectioning. Ultrathin sections, 60–90nm thick, were nonspecifically stained with uranyl acetate and lead citrate. Electron photomicrographs were made with a Philips CM 120 transmission electron microscope at 80 kv.

Statistical analysis

Data are shown as the mean ± SEM. Unless particularly indicated, a t-test or one-way analysis of variance followed by Dunnett's test was used to detect differences between groups. Mortality data were obtained with groups of 16 mice and compared by Chi-square test. The semiquantitative histopathological data were compared by the Mann-Whitney nonparametric test for unpaired data.

Results

PFIB doses employed in mice and rats

Mice and rats exposed to 130 and 140 mg PFIB/m³, respectively, for 5 min, both displayed a mortality of 1 out of 10 and obvious pathological changes in lungs.

Table 1 shows the result of semiquantitative histopathological evaluation of lungs in mice and rats after five-minute exposure to 130 and 140 mg PFIB/m³ respectively. It can be seen from Table 1 that there is no significant difference between the two species in the lung pathological changes (p>0.05, Mann-Whitney nonparametric test for unpaired data).

Based on the above results, five-min exposure to 130 mg PFIB/m³ for mice and five-min exposure to 140 mg PFIB/m³ for rats were employed in the following experiments.

PMN depletion

Intraperitoneal injection of CPA into rats caused a 92.3% reduction in the peripheral PMN count from 1,799 ± 744 PMN/mm³ before treatment with CPA to 141 ± 142 PMN/mm³ 5 d after the first dose of CPA (n=8, p<0.001, detected by paired t test). Three days after the administration of CPA to mice, the peripheral PMN count dropped from 1,264 ± 452 PMN/mm³ in the untreated group to 422 ± 176 PMN/mm³ in the CPA-treated animals, which had a 66.6% decrease (n=8, p<0.001). No animals showed any signs of illness resulting from PMN depletion.

Table 1. Histopathological evaluation of lungs in mice and rats after five-minute exposure to 130 mg PFIB/m³ and 140 mg PFIB/m³, respectively

Animal	n	Distribution	Severity	Intensity
Edema Fluid				
Mice	5	2.5 ± 0.1	2.3 ± 0.2	3.0 ± 0.0
Rats	5	2.7 ± 0.2	2.9 ± 0.1	2.9 ± 0.2
Fibrin				
Mice	5	2.1 ± 0.1	2.5 ± 0.3	2.4 ± 0.1
Rats	5	2.0 ± 0.0	2.6 ± 0.1	2.1 ± 0.2
PMN				
Mice	5	3.0 ± 0.0	2.2 ± 0.1	2.1 ± 0.1
Rats	5	3.2 ± 0.1	2.5 ± 0.2	2.3 ± 0.1
Macrophages				
Mice	5	3.1 ± 0.2	2.3 ± 0.1	2.5 ± 0.3
Rats	5	3.3 ± 0.1	2.4 ± 0.1	2.2 ± 0.3
Hemorrhage				
Mice	5	2.8 ± 0.1	2.5 ± 0.2	2.4 ± 0.4
Rats	5	3.0 ± 0.0	2.8 ± 0.1	2.8 ± 0.1
Vascular Congestion				
Mice	5	3.5 ± 0.3	3.8 ± 0.2	
Rats	5	3.3 ± 0.1	4.0 ± 0.0	

Time-courses of PFIB inhalation-induced changes in lung wet-to-dry weight ratio and MPO activity in mice as well as BALF total protein concentration in rats

Inhalation of PFIB (130 mg/m³) for 5 min led to a slow increase in the lung wet-to-dry weight ratio in mice with no significant increase in the first 20 h, a drastic increase at 24 h, which is also the peak time, and a slow recovery at 48 h (Fig. 1A). But the lung MPO activity of mice, an index that has been found to be much more sensitive than that of quantitative histology¹⁶⁾, responded very quickly. It had increased significantly as early as 1 h, reached the maximum at about 20 h, and returned to normal by about 60 h after the exposure (Fig. 1B). When rats were exposed to PFIB (140 mg/m³) for 5 min, the concentration of total protein in BALF, an important index that is commonly used to evaluate lung injury, was observed to increase significantly as early as 2 h after the exposure, reach the maximum at 24 h and remain very high even at 72 h (Fig. 1C).

These results demonstrated that PFIB inhalation can result in a rapid and sustained lung PMN accumulation measured by lung MPO activity, furthermore, the time-course was shown to be comparatively similar to that of the lung injury indexed by both the BALF total protein content and lung wet-to-dry weight ratio, suggesting that lung PMN accumulation heralded the PFIB inhalation-induced lung injury and edema. To further clarify this, we investigated the effect of PMN depletion on PFIB-induced pulmonary damage.

Table 2. Effect of PMN depletion on PFIB-induced mortality in mice

Group	n	death	survival	mortality (%)
Control	16	0	16	0*
CPA	16	0	16	0*
PFIB	16	10	6	62.5
CPA/PFIB	16	1	15	6.25*

Pathogen-free male mice were made neutropenic with the method described in the text. 3 d after CPA pretreatment, the mice were exposed to PFIB (190 mg/m³) for 5 min. Mortality was evaluated 7 d after PFIB exposure. *P<0.02 versus PFIB group by Chi-square test.

Effects of PMN depletion on PFIB inhalation-induced toxicity

Table 2 shows the effect of PMN depletion on mice mortality after five-minute exposure to 190 mg PFIB/m³. During the seven days of observation, no mice died or showed any signs of illness in the control or CPA groups. PMN depletion caused by CPA pretreatment dramatically decreased the PFIB-induced mortality in mice (p<0.02).

While the PMN depletion in mice with CPA caused a nearly total blockade of the increase in lung MPO activity induced by a 5 min exposure of mice to PFIB (130 mg/m³) (Fig. 2A), the lung wet-to-dry weight ratio remained

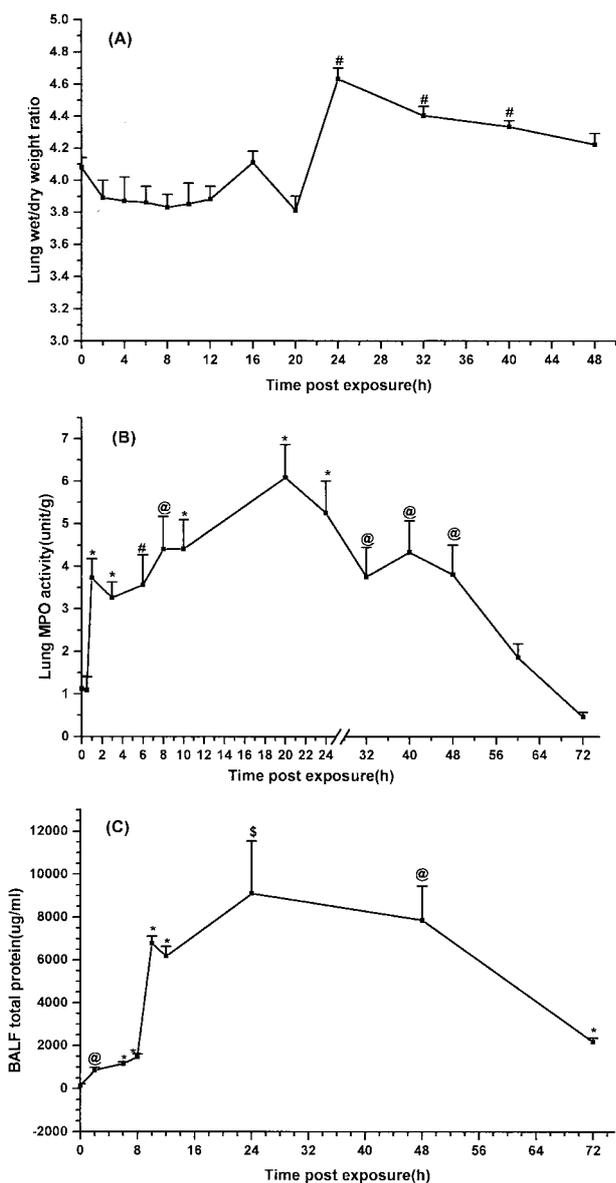


Fig. 1. Time course of PFIB inhalation-induced changes in lung wet-to-dry weight ratio (A), lung MPO activity (B) in mice, and BALF total protein concentration (C) in rats. Lung wet-to-dry weight ratio (n=6) and MPO activity (n=8) was measured at various times after the mice were exposed to 130 mg/m³ of PFIB for 5 min. The total protein concentration in BALF (n=4) was determined at 2, 6, 8, 10, 12, 24, 48 and 72 h after the rats were exposed to 140 mg/m³ of PFIB for 5 min. \$p<0.05; #p<0.01; @p<0.005; *p<0.001 compared with the control.

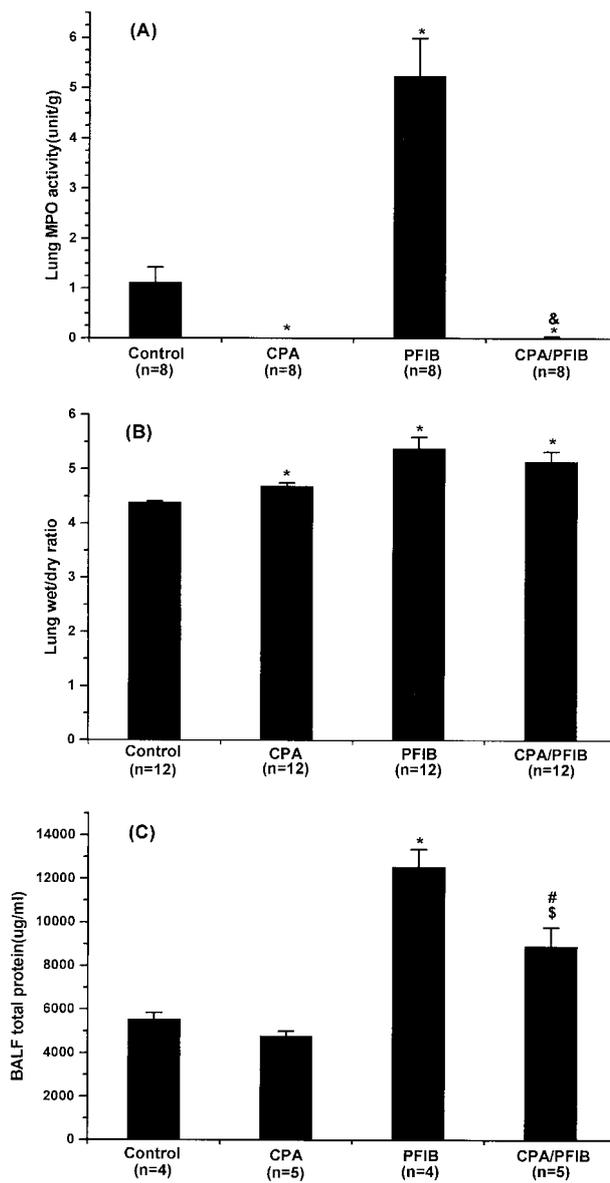


Fig. 2. Effects of PMN depletion on PFIB-induced increases in lung MPO activity (A), wet-to-dry weight ratio (B) and total protein concentration in BALF (C). As indicated in Methods and Materials, the lung MPO activity and the wet-to-dry weight ratio were measured in mice exposed to 130 mg/m³ of PFIB for 5 min, and the total protein concentration in BALF was measured in rats exposed to 140 mg/m³ of PFIB for 5 min. The animals were made neutropenic by the methods described in the text. The parameters were determined at 24 h post exposure to PFIB. \$p<0.05; *p<0.001 versus control. #p<0.05; &p<0.001 versus PFIB.

unchanged (Fig. 2B). Unlike the effect of PMN depletion on PFIB-induced lung edema, PMN depletion with CPA can significantly decrease BALF total protein content, suggesting the protective effect of PMN depletion on PFIB-induced lung injury (Fig. 2C).

When compared with the normal pulmonary architecture in the control (Fig. 3A) and CPA (Fig. 3B) groups, severe lung damage caused by PFIB inhalation was characterized by diffuse vascular congestion,

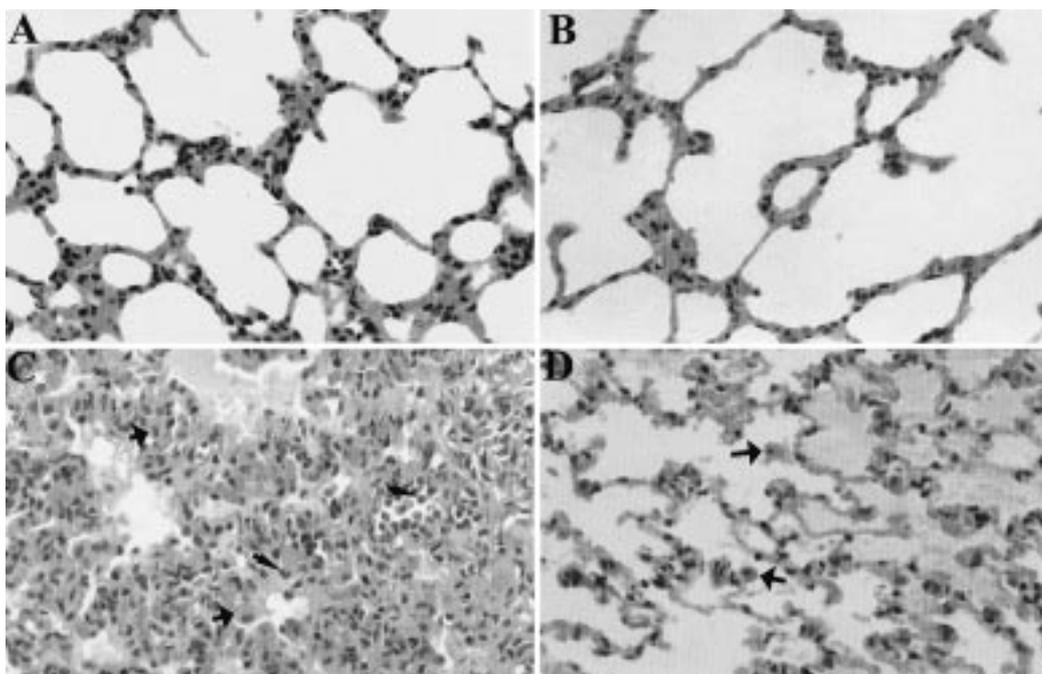


Fig. 3. Light photomicrographs of paraffin-embedded and hematoxylin and eosin-stained sections of representative lungs from rats killed at 24 h post exposure to PFIB (140 mg/m^3 for 5 min). The Control (A) and CPA (B) groups show normal or essentially normal pulmonary architecture. The PFIB group (C) shows diffuse interstitial and intra-alveolar edema, alveolar hemorrhage, hyaline membrane formation, and cellular infiltration composed mainly of macrophages (\blackrightarrow) and PMNs (\blacktriangleright). The CPA/PFIB group (D) shows modest congestion, interstitial and intra-alveolar edema, and macrophages (\blackrightarrow) accumulation. Fewer PMNs can be seen. Original magnification: $\times 66$.

interstitial and intra-alveolar edema, alveolar hemorrhage, and the accumulation of abundant fibrin and inflammatory cells (mainly PMNs and macrophages) in the alveoli. Typical hyaline membrane can also be seen in this group (Fig. 3C). These PFIB inhalation-induced lung injuries were significantly alleviated by CPA pretreatment in that the CPA/PFIB group showed slight to moderate septal thickening, intra-alveolar edema, fibrin deposition and macrophage accumulation in the alveolar spaces, and fewer PMNs can be observed in this group (Fig. 3D).

Ultrastructural studies revealed no pathological changes in the control or CPA pretreatment groups (data not shown). Lung ultrasections from the PFIB-exposed rats had severe damage to the alveolar structure. Excessive vesiculation and the formation of blebs in endothelial and epithelial cells were the striking features. Some of the epithelial cells had undergone exfoliation and many alveoli contained exudated erythrocytes, fibrin, inflammatory cells (PMNs and macrophages), cell fragments and other amorphous materials (Fig. 4A–4C). These pathological changes were noticeably less in the CPA/PFIB group in that the alveolar structure was essentially intact, and that inflammatory cells and extravasated erythrocytes could not be easily found,

although vacuoles and blebs in endothelial and epithelial cells, perivascular edema, and membrane-bound residues in some alveoli could still be seen (Fig. 4D).

Discussion

Severe lung damage induced by PFIB inhalation is speculated to be mediated by HF to pneumocytes^{5, 10}, oxidative stress^{8, 17} and other mechanisms, but none can fully account for all the toxicological effects of PFIB. PMNs are commonly thought to be involved in the pathogenesis of several forms of lung injury, including adult respiratory distress syndrome (ARDS)¹⁸, emphysema¹⁹, interstitial fibrosis²⁰, pulmonary oxygen²¹ and phosgene²² toxicities, by releasing damaging enzymes and RIS, etc. Supported by the observations by Lehnert *et al.* that the accumulation of blood monocytes and PMNs in the lung's capillary bed was one of the early responses to PFIB, and as the time post exposure is prolonged, the cellular infiltration composed mainly of macrophages and PMNs was a prominent feature of the injurious response¹⁰, the current studies have shown that PMN accumulation in the lung heralded and played an important role in PFIB inhalation-induced ALI and edema. PFIB inhalation resulted in a rapid and sustained

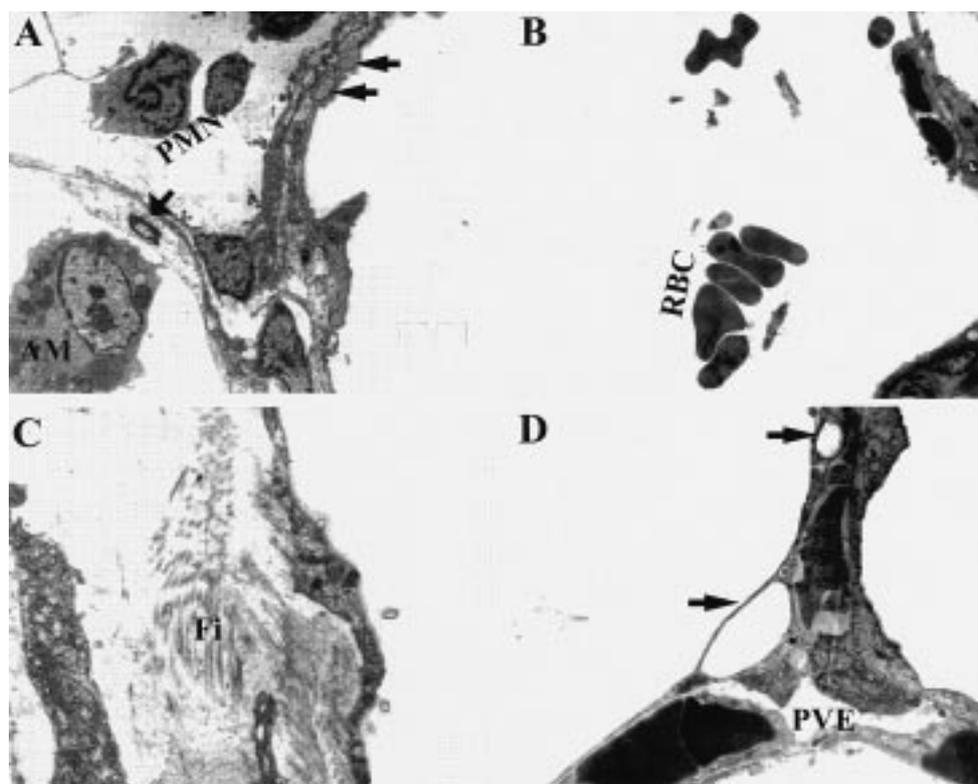


Fig. 4. Representative electron photomicrographs of lungs from rats killed at 24 h post exposure to PFIB ($140 \text{ mg/m}^3 \times 5 \text{ min}$). (A)–(C): PFIB group. Vacuoles and Blebs in epithelial and endothelial cells (\blackrightarrow), type I epithelial necrosis (\blackrightarrow), accumulation of macrophages (AM) and PMNs as well as cell fragments in the alveoli (A, original magnification: $\times 3,000$); The appearance of extravasated erythrocytes (RBC) and cell fragments in the alveolus (B, original magnification: $\times 3,000$); Excessive fibrin (Fi) in the alveolus (C, original magnification: $\times 13,000$). (D): CPA/PFIB group. Vacuoles or blebs in the type I epithelial cell (\blackrightarrow) and perivascular edema (PVE) are shown (D, original magnification: $\times 6,300$).

lung PMN accumulation. Both the start (1 h) and the maximal time (20 h) for the increase in the lung MPO activity occurred earlier than those of the BALF total protein concentration (2 h and 24 h after the exposure, respectively) and the lung wet-to-dry weight ratio (24 h being both the start and maximal time). A nearly total elimination of PFIB-induced PMN sequestration in the animal lung by CPA pretreatment significantly alleviated the toxicity of PFIB inhalation in terms of both mortality and lung injury as shown by BALF total protein concentration assay, histopathological as well as ultrastructural examinations. It comes as no surprise that CPA pretreatment was ineffective for PFIB inhalation-induced pulmonary edema when indexed by the lung wet-to-dry weight ratio since CPA *per se* is reported to be edematogenic²³.

In confirmation of an important role of PMNs in PFIB-induced lung injury, evidence that cannot be neglected includes: (1) Some of the toxic effects of PFIB on lung are still observed in the PMN depletion (CPA/PFIB) group

from both the BALF total protein concentration assay and histopathological/ultrastructural examination in the current study, which is not surprising because previous studies have suggested that PFIB may be directly toxic to lung epithelial and endothelial cells^{10, 17, 24}. (2) Based on the fact that macrophages can generate toxic oxygen products, proteases, arachidonic acid metabolites, platelet-activating factor (PAF) and cytokines, all of which are involved in the pathogenesis of ARDS²⁵, it seems that macrophages might also be involved in the initiation of PFIB-induced pulmonary damage. That substantial macrophage accumulation can still be observed in alveoli of the animals from PFIB and CPA/PFIB groups by both histopathological and ultrastructural examination of the present study might be used as one of the proofs of this aspect. (3) 5-fluorouracil, another PMN depleting agent that is proved to have no edematogenic effect²⁶, could not achieve any protective effect against PFIB inhalation-induced lung edema in terms of the lung wet-to-dry weight ratio increase either (unpublished data). Thus

PMN is not the only contributor in PFIB inhalation-induced lung damage and edema. Roles played by other effector cells, such as monocytes and macrophages need to be further clarified.

In conclusion, the current study confirmed that the neutrophil sequestration and accumulation in the lung is one of the key factors in PFIB inhalation-induced acute lung injury and mortality, which suggests that successful therapeutic intervention for this occupational disease should target, at least partially, either successful prevention of the PMN sequestration in the lung and/or the inhibition of lung damaging enzymes (such as elastase) and other effective factors (e.g. reactive oxygen species, ROS) from the sequestered PMNs, even though how PFIB initiates lung PMN accumulation and how the PMNs mediate the pulmonary damage are still issues which deserve detailed study.

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