Cell Injuries of the Blood-Air Barrier in Acute Lung Injury Caused by Perfluoroisobutylene Exposure

Ge Meng#, Jian Zhao#, He-Mei Wang, Ri-Gao Ding, Xian-Cheng Zhang, Chun-Qian Huang and Jin-Xiu Ruan

Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, P.R. China

Abstract: Cell Injuries of the Blood-Air Barrier in Acute Lung Injury Caused by Perfluoroisobutylene Exposure: Ge Meng, et al. Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, PR China—Objectives: To investigate the complete process of cell injuries in the blood-air barrier after perfluoroisobutylene (PFIB) exposure. Methods: Rats were exposed to PFIB (140 mg/m³) for 5 min. The pathological changes were evaluated by lung wet-to-dry weight ratio, total protein concentration of bronchoalveolar lavage fluid and HE stain. Ultrastructural changes were observed by transmission electron microscope. Apoptosis was detected by in situ apoptosis detection. Changes of actin in the lung tissue were evaluated by western blot assay. Results: No significant pulmonary edema or increased permeability was observed within the first 4 h post PFIB exposure. However, inflammatory cell infiltration and alveolar wall thickening were observed from 2 h. Destruction of the alveoli constitution integrity, edema and protein leakage were observed at 8 h. The injuries culminated at 24 h and then recovered gradually. The injuries culminated at 24 h and then ameliorated. The number of apoptotic cells abnormally increased at 30 min post PFIB exposure. Some injuries were similar to apoptosis. Compared with control, more serious injuries were observed in PFIB-exposed rats after 30 min. At 8 h, some signs of cell necrosis were observed. The injuries culminated at 24 h and then ameliorated. Western blot analysis revealed that the level of actin in the lung showed no significant changes within the first 4 h post PFIB exposure. However, it decreased at 8 h, reached a nadir at 24 h, and then recovered gradually. Conclusions: The pathological processes were in progress persistently post PFIB exposure. The early injuries probably were the result of the direct attack of PFIB and the advanced injuries probably arose from the inflammatory reaction induced by PFIB.

Key words: Acute lung injury, Alveolar epithelial cell, Apoptosis, Perfluoroisobutylene, Pulmonary microvascular endothelial cell

Dysfunction of the blood-air barrier has recently been identified as a focus for the study of acute lung injury (ALI) induced by lipopolysaccharide (LPS), platelet activating factor (PAF), thrombin, ischemia and reperfusion and mechanical ventilation etc. The blood-air barrier is composed of the pulmonary alveoli epithelial barrier, the extracellular matrix and the pulmonary microvascular endothelium barrier. It is responsible for gas exchange between the circulatory system and the alveolar space. Alveolar type I epithelial cells (AT-I), alveolar type II epithelial cells (AT-II) and pulmonary microvascular endothelial cells (PMVEC) are the main cells of the blood-air barrier. It is well-known that increased permeability of the blood-air barrier in ALI, in which fluid, protein, macrophage and leukocytes move into the alveolar air space through the interstitial space, is the general basis for the formation of the pulmonary edema. In some cases of severe ALI, patients die in the early stage of pulmonary edema. If the injuries of the blood-air barrier are not repaired quickly, pulmonary fibrosis eventually forms, and it is the most serious complication in the advanced ALI. Along with the changes of structure and function, if left uncontrolled, ALI will result in excessive apoptosis or necrosis. Among the ALI models, the toxic gas inhalation model has been extensively used by researchers in recent years.
Ge Meng, et al.: PFIB-Induced Impairments of Lung Blood-Air Barrier

because this model is common in chemical accidents and results in rapid ALI. Perfluoroisobutylene (PFIB), a newly discovered chemical (chemical structure: \((\text{F}_2\text{C}_3\text{H}_3)_2\text{CF}_2\)), is a potential pneumoedematogenic gas. It is usually produced as the main by-product in the fluoropolymer industry and is a hazard to human beings in chemical industry accidents, fire disasters and other emergencies. PFIB inhalation can cause acute lung injury or acute pulmonary edema, and even death\(^{16, 17}\). Unfortunately, no specific antidote or successful therapeutic measures are currently available. The mechanisms of ALI induced by PFIB remain largely unclear. Findings of other laboratories and ours have shown that aggregation, sequestration of polymorphonuclear leukocytes (PMN) and excessive inflammatory reaction are the decisive factors \(^{18-21}\). The blood-air barrier is probably the main target of attack in ALI induced by PFIB. Moreover, because of smaller molecular weight and stronger electrophilicity, PFIB can directly attack the blood-air barrier in the early period post PFIB exposure, however, literature on the changes of the blood-air barrier post PFIB exposure are rare. In the current paper, we will describe the complete process of cell injuries in the blood-air barrier post PFIB exposure.

Materials and Methods

General

1) Animals

Eighty specific pathogen-free male Wistar rats (180–220 g, 8 wk old) were used in this study. They were all obtained from the Center of Medical Experimental Animals, the Academy of Military Medical Sciences (Beijing, P. R. China). The animals were housed in quiet, humidified, clean rooms with a light-cycle of 12 h/12 h for 1 wk before use. They were fed with laboratory pellet food and tap water \text{ad libitum} except when they were in the exposure chamber. All the animal experiments were performed in accordance with the Guidelines for Animal Experiments of the Chinese Academy of Medical Sciences, Beijing, P. R. China.

2) Doses of PFIB for animal exposure

PFIB was obtained from the Shanghai Institute of Organic Fluorine Materials at a purity of 98%. A head-nose exposure apparatus was used to expose rats to PFIB, as previously described\(^{19}\). The PFIB exposure dose was 140 mg/m\(^3\) × 5 min.

3) Treatment of specimens

The rats were randomly divided into a control group and PFIB exposure groups (30 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h and 72 h post PFIB exposure) with 8 rats in each group. At sacrifice, they were anesthetized by 25% ethylcarbamate (0.5 ml/100 g bw, i.p.) and exsanguinated via abdominal aorta transection. The right lungs were clamped by hemostatic forceps. Each animal’s trachea was cannulated with a blunt needle secured with silk ligature. Bronchoalveolar lavage fluid (BALF) collection was performed with 5 ml phosphate buffered saline (PBS) that was infused and aspirated in the left lung 3 times. The lavage fluid was recovered (average fluid recovery was 4 ml), and centrifuged (1,000 g, 4°C) for 10 min. The supernatants were removed and stored at –70°C until the total protein concentrations were assayed by the method of Lowry\(^{20}\).

A part of the lobus apicalis of the right lungs were used for ultrastructural studies, the rest were stored in liquid nitrogen and used for western blot analysis. The lobus cardiacus was used for paraffin sections, the lobus diaphragmaticus for assay of the lung wet-to-dry weight ratio and the accessory lobus for frozen sections. The paraffin sections were used for conventional pathohistological studies and \text{in situ} apoptosis detection of lung tissue.

Experimental methods

1) Assay of the lung wet-to-dry weight ratio and the total protein concentration of the BALF assay

The assay methods were described in our previous paper\(^{19}\), and are described here in briefly. Assay of the lung wet-to-dry weight ratio: the lobus diaphragmaticus was weighed to obtain the wet weight then dried in an oven at 80°C for 24 h to obtain the dry weight and the lung wet-to-dry weight ratio was calculated as: \(\frac{\text{mass}_{\text{wet lungs}}}{\text{mass}_{\text{dry lungs}}}\). The total protein concentration of BALF assay: 0.1 ml BALF was added to 1.9 ml \(\text{Na}_2\text{SO}_4\) (22.2%), and 0.5 ml of the mixture was collected after being vortexed. Then, 9 ml \(\text{NaOH}\) (0.5%) was added to each sample, and 0.3 ml Folin phenol was added after standing for 30 min. Finally the OD value was recorded at 500 nm after 10 min using 0.5 ml tyrosine (0.2 mg/ml) as the standard. Total protein concentration was calculated as: \(C_{\text{total protein}}=\frac{\text{OD}_{\text{total protein sample}}}{\text{OD}_{\text{standard}} } \times 6.4\). The increased lung wet-to-dry weight ratio was used as an indicator of pulmonary edema and the increased total protein concentration was used as an indicator of increased permeability of the blood-air barrier.

2) Hematoxylin and eosin (HE) staining of rat lung slices

The rat lungs fixed in 10% formalin solution were sectioned (4 µm) after dehydration, cleaning and paraffin embedding. The sections were flattened, pasted and heated on blank glass slides. Histological evaluations were performed with HE staining and pathological examination.

3) Ultrastructural studies

The rat lungs were submerged in cold fixative overnight...
then cut into 1- to 2-mm cubes. The lung fragments were post-fixed in 1% buffered osmium tetroxide (pH 7.2) and embedded in Poly-812 epoxy resin. Semithin epoxy sections, 1 μm thick, differentiated with methylene blue, azure II, and basic fuchsin, were used to select areas for ultrathin sectioning under light microscopy. Ultrathin sections, 60–90 nm thick, were nonspecifically stained with uranyl acetate and lead citrate. Electron photomicrographs were taken using a Philips CM 120 transmission electron microscope at 80 kv.

4) In situ apoptosis detection of lung tissue

The Apoptag® Plus Peroxidase In Situ Apoptosis Detection Kit was used to evaluate the apoptosis of lung tissue post PFIB exposure. The paraffin sections were washed with dimethyl benzene, incubated with proteinase K (20 mg/ml, 15 min) and 3% hydrogen peroxide (5 min), then treated with TdT Enzyme (1 h, 37°C), antidigoxigenin conjugate (30 min), peroxidase substrate (3–6 min), 0.5% methyl green (10 min), followed by mounting. Same size areas were observed. The apoptosis ratio was calculated as: the number of apoptotic cells/total cells × 100%.

5) Western blot analysis

Tissue specimens were pulverized in liquid nitrogen and homogenized in ice-cold lysate buffer containing a cocktail of protease inhibitors (Complete, EDTA-free: Roche, Mannheim, Germany). The homogenates were centrifuged at 15,000 g for 1 h at 4°C and the protein was extracted and stored in liquid nitrogen until electrophoresis. Protein concentrations of the extracts were determined by the Bradford assay. Ten-microgram proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), then transferred to a nitrocellulose membrane. The membrane was washed for 10 min in Tween-20 Tris buffered saline (TTBS) [20 mmol/l Tris-Cl, 50 mmol/l dithiothreitol (DTT), 2% sodium dodecyl sulphate (SDS), 0.1% bromophenol blue and 10% glycerol] and heated in boiling water for 5 min. The proteins were separated by 10% SDS-polyacylamide gel electrophoresis (PAGE), then transferred to a nitrocellulose membrane. The membrane was washed for 10 min in Tween-20 Tris buffered saline (TTBS) [20 mmol/l Tris-HCl (pH 7.5), 0.15 mol/l NaCl, 0.05% Tween-20], then blocked with 10% skimmed milk for 1 h at room temperature. The blocked membrane was incubated with a primary antibody against actin (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 4°C. After incubation with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, dilution 1:5000; Beijing Zhong Shan-Golden Bridge Biological Technology Company, Beijing, China) for 1 h at room temperature, its was stained by ECL chemiluminescence (Amersham Life Science, Arlington Heights, IL). This experiment was repeated three times using separate samples.

6) Statistical analysis

Data are shown as the Mean ± SD. One-way analysis of variance followed by Dunnett’s test was used to detect differences between groups unless otherwise indicated.

Results

Changes of lung wet-to-dry weight ratio and total protein concentration of BALF post PFIB exposure

The lung wet-to-dry weight ratio and the total protein concentration of BALF remained unchanged within the first 4 h (30 min, 1 h, 2 h and 4 h) post PFIB exposure. They increased significantly at 8 h, peaked at 24 h, and declined thereafter. Figure 1 shows the clinical course of lung injury by PFIB, which included periods of stimulation, latency, pulmonary edema and convalescence (Fig. 1).

Lung histopathological changes post PFIB exposure

No significant pathological changes were found in HE stained lung slices within the first 1 h post PFIB exposure. However, inflammatory cell infiltration and alveolar wall thickening were observed from 2 h, and destruction of the alveoli constitution integrity and the protein leakage were observed at 8 h. The most serious injuries were observed at 24 h, and gradual recovery ensued thereafter (Fig. 2).

Ultrastructural changes of the blood-air barrier post PFIB exposure

The normal AT-I is a kind of squamous flat cell with a small and compact nucleus and a thin cytoplasm, in which organelles are rare, and mitochondrion and rough endoplasmic reticulum are occasionally observed. A few thick short microvilli are observed on the dissociative side of the cell, and connect tightly to AT-II. The earliest recognized morphologic changes were compaction and segregation of the nuclear chromatin, uniform fine granular masses that became localized on the nuclear envelope, condensation of the cytoplasm, and swollen mitochondria at 30 min post PFIB exposure. All these observations indicate apoptosis. Subsequently, more serious nuclear shrinkage, chromatin marginalization, split nucleolus, double membranes of mitochondria which had been destroyed, swollen, even broken cristae of mitochondria and a great number of vacuoli in cytoplasm were observed in temporal order of description. At 8 h, some characters of necrosis were observed; broken cells and fragments appeared in tissues. All the above-mentioned pathological changes culminated at 24 h. After 24 h, the symptoms were ameliorated (Fig. 3).

The normal AT-II is cubic, and contains abundant organelles. The lamellar body, which can be dyed black by osmic acid, is the most significant diagnostic characteristic of AT-II. The microvilli of AT-II began to fall off at 30 min post PFIB exposure. Later, microvilli
fell off more in greater numbers. The most dramatic changes were observed at 24 h, followed by a gradual amelioration. The pathological changes of nuclear chromatin and mitochondria of AT-II were similar to those of AT-I (Fig. 4).

The normal PMVEC is flat-like and contains various organelles. Swollen basal laminae were observed at 30 min post PFIB exposure. Later, broken basal laminae were observed. The maximum changes were observed at 24 h, followed by a gradual amelioration. The pathological changes of nuclear chromatin and mitochondria pathological changes of PMVEC were similar to those of AT-I and AT-II (Fig. 5).

Apoptosis of lung tissue post PFIB exposure

The number of apoptotic cells at 30 min post PFIB exposure exceeded normal. The number significantly increased at 8 h vs that within the first 4 h post PFIB exposure. The greatest number of apoptotic cells was seen after which they gradually declined in number (Fig. 6).

Changes of actin in the blood-air barrier post PFIB exposure

Western blot analysis revealed that the level of actin in the blood-air barrier had no significant changes within the first 4 h post PFIB exposure. However, it decreased at 8 h, and the minimum level was seen at 24 h, and thereafter gradually recovered (Fig. 7).

Discussion

In this paper, we focused on the complete process of
cell injuries in the blood-air barrier post PFIB exposure. No significant pulmonary edema or increased permeability were found within the first 4 h post PFIB exposure. However slight inflammatory cell infiltration and alveolar wall thickening were observed at 2 h in HE stained lung slices and ultrastructural injuries of the blood-air barrier were observed at 30 min. Pulmonary edema and increased permeability were observed at 8 h, peaked at 24 h, and then recovered. These results indicate that the pathological process develops persistently post PFIB exposure, although there was no significant pulmonary edema during the early period of PFIB exposure. The early period is known as the latency period in the clinical stage and is easily overlooked.
Fig 4. Representative electronmicroscope photomicrographs of AT-II from rats sacrificed at different times pre- and post- PFIB (140 mg/m³ × 5 min) exposure. The results from left to right are control, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h and 72 h post PFIB exposure. Nuclear chromatin compaction, segregation and localization on the nuclear envelope (▲), swollen mitochondrion (▲), destroyed double membranes (▼), swollen or broken cristae (●) and fallen off microvilli (△) were observed in temporal order of description post PFIB exposure. The most serious injuries were observed at 24 h, and were followed by gradual recovery.
Consequently, the best time for treatment is often missed, the reason being that injuries of AT-I, AT-II and PMVEC in this period are, in fact, in progress according to the ultrastructure results. Other research teams also observed the infiltration of inflammatory cells under an electron microscope in this period\(^2\). Therefore we suggest that the term “the latency period” should be substituted by “the early damage period” in order to describe early ALI more objectively and remind doctors to adopt proper therapeutic measures during this period.

The changes of ultrastructural injuries of the blood-air barrier, which were observed under a transmission electron microscope as early as 30 min post PFIB exposure in the present study, were consistent with those of the lung wet-to-dry weight ratio, the total protein concentration of BALF and the HE stained lung slices from 8 h post PFIB exposure. Accumulated data concerning this early damage of the blood-air barrier probably indicate the direct effect of PFIB: the concentration of fluorion in blood significantly increased.
Fig. 5. Representative electronmicroscope photomicrographs of PMVEC from rats sacrificed at different time pre- and post- PFIB (140 mg/m\(^3\) × 5 min) exposure. The results from left to right are control, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h and 72 h post PFIB exposure. Nuclear chromatin compaction, segregation and localization on the nuclear envelope (▲), swollen mitochondrion (▲), destroyed double membranes (▲), swollen or broken cristae (▲) and swollen or broken basal lamina (▲) were observed in temporal order of description post PFIB exposure. The most serious injuries were observed at 24 h, with gradual recovery thereafter.

Fig. 6-1. Light photomicrographs of paraffin-embedded and in situ apoptosis detection sections of representative lungs from rats sacrificed at different times pre- and post- PFIB (140 mg/m\(^3\) × 5 min) exposure. The white arrows point to apoptotic cells (the nuclei are stained brown). The results from left to right are control, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, 72 h post PFIB exposure.

Fig. 6-2. Apoptosis of rat lung tissue at different times pre- and post- PFIB (140 mg/m\(^3\) × 5 min) exposure. The results from left to right are control, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, 72 h post PFIB exposure. Mean ± SD, N=8, **: p<0.001, versus control group, ANOVA.
at 0 h post PFIB exposure, and was still higher than normal at 1 h post PFIB exposure25); the aggregation and sequestration of a great number of PMNs were observed at 1 h post PFIB exposure17); and the level of interleukin-1 (IL-1β) in sera of PFIB-exposed mice dramatically increased at 30 min and reached its maximum at 1 h post PFIB exposure. The level of IL-8 in sera of PFIB-exposed mice climbed to a peak at 1 h, followed by a plateau till 4 h post PFIB exposure18). The direct injuries probably arise from PFIB itself. The halogen and p-π electron conjugation in the molecular structure of PFIB makes it very electrophilic, allowing it to easily attack the thiol (-SH), amino (-NH2) and hydroxy (-OH), nucleophilic groups, which are widely distributed in the alveolar membrane and are crucial to the maintenance of the cellular structure and function30). Moreover, as a micromolecular gas, PFIB can pass through the blood-air barrier easily to attack the epithelial barrier and endothelial barrier together. However, later injuries in ALI due to PFIB exposure are probably attributable to the inflammatory cascade or networking by inflammatory cells and their secreted substances26): the concentration of fluorion in blood returned to normal levels at 1.5 h post PFIB exposure25); and multiple mediators, especially PMN, probably became a key factor in the period. Elastase and collagenase, which are important damage factors, were delivered to attack the blood-air barrier27, 28); and macrophages also probably participated in the process of injury in the later period27, but the details are still unknown.

Apoptosis is now known to key to the maintenance of normal cell turnover within structural cells in the parenchyma of virtually every organ7). In the current experiment, the number of apoptotic cells was abnormally large at 30 min post PFIB exposure and kept increasing until 24 h, after which they gradually declined. A great deal of apoptotic evidence was also observed under the transmission electronic microscope. Apoptosis in the early period might be beneficial to the blood-air barrier in that it can prevent the leakage of entocytes. Thus, necrosis, which is a serious injury in ALI, could be partially prevented. It follows that slight apoptosis in the early period might be responsible for the formation of the latency period. Of course, excessive apoptosis in the later period is deleterious29, 30). Studies of mechanisms of apoptosis now pay more attention to the Fas/FasL system. Activation of Fas activates intracellular caspases and culminates in apoptosis31, 32). In the process, a functioning rennin-angiotensin system in the target cell is necessary30). However whether the Fas/FasL system is involved in apoptosis induced by PFIB requires further investigation.

Western blot analysis revealed that the level of actin in the blood-air barrier showed no significant changes within the first 4 h post PFIB exposure. However, it decreased at 8 h, and a minimum appeared at 24 h, which was followed by gradual recovery. The changes of actin were consistent with the formation of pulmonary edema and increased permeability of the blood-air barrier post PFIB exposure. Actin is a main ingredient of cytoskeleton. So cytoskeleton injury was one of the mechanisms of the lung injury induced by PFIB. Besides the changes of the cellular structure of the blood-air barrier, other factors also probably contribute to the increase of permeability. In ALI induced by LPS, thrombin, mechanical ventilation, changes cell junctions and cystoskeleton play critical roles in the increase of permeability, in which the myosin light chain kinase is a very important factor1, 3, 33). All results indicate the complicated mechanisms of ALI.

In conclusion, no significant pulmonary edema or increased permeability was found within the first 4 h post PFIB exposure. They were observed at 8 h, peaked at 24 h, and then recovered. However the pathological process develops persistently from the onset of PFIB exposure and we suggest that the term “the latency period” be substituted by “the earlier damage period”. Due to the characteristics of PFIB, the injuries induced by PFIB probably include direct effects and indirect effects through the inflammatory cascade or networking by inflammatory cells and their secreted substances. Western blot analysis revealed that cytoskeleton injury was one of the mechanisms of lung injury induced by PFIB. Given the complicated mechanisms, the role of PFIB exposure...
induced ALI calls for much more detailed clarification.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (30800904) and The National Key Technologies R&D Program for New Drugs (2008ZX09305-003).

References


