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Ultrastructural Changes in the Air—Blood Barrier of Rats in Acute Intoxication with Furoplast Pyrolysis Products

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Rats were exposed to fluoroplast-4 pyrolysis products (sample weight 2.6 g, pyrolysis temperature 440-750°C, pyrolysis duration 4 min) containing perfluoroisobutylene over 15 min. Lung tissue samples for histological and electron microscopic examination were isolated in 3 and 30 min after intoxication and processed routinely. Histological examination revealed no structural changes in the lungs. In ultrathin sections of rat lungs, some changes in the structure of type I pneumocytes were detected in 3 min after the exposure: detachment of cytoplasmic processes and the appearance of transcytosis pores. These changes attested to impaired cell—cell interactions and their adhesion to the basement membrane, where structural disorganization and edema of the collagen matrix were observed. In 30 min following exposure, the signs of damage to type I pneumocytes became more pronounced. The increase in the equivalents of transcellular and paracellular permeability in the alveolar lining profile was observed. No changes in the pulmonary capillary endotheliocytes were detected, which suggest that type I pneumocytes are the primary target of the toxic effect of perfluoroisobutylene. The vulnerability of a particular cell population, in view of specific metabolism of these cells, can be the key to deciphering of the mechanisms of the toxic effect of pyrolysis products of fluorinated polymer materials.

Key Words: *fluorine-containing polymers; perfluoroisobutylene; pneumocytes; toxic mechanism of action; electron microscopy*

Acute respiratory distress syndrome (ARDS) is viewed as a complication of serious diseases, intoxications, and injuries and manifests by nonspecific lung lesion and clinical picture of rapidly increasing respiratory failure. Inhalation of various toxic substances is among the factors that directly affect the lungs and lead to ARDS development [1]. In clinical practice ARDS caused by direct lung damage (inhalation of chemicals, gases, and fumes) is defined as acute pulmonary edema (ICD-10 code J68.1). In experimental studies on laboratory animals, the term toxic pulmonary edema (TPE) is used. Among different toxicants, perfluorizobutylene [$(F_3C)_2C=CF_2$] (PFIB) is the substance that can induce TPE upon inhalation [5]. PFIB inhalation poisoning can occur at fire [5,8] during pyrolysis of various fluorine-containing polymeric materials (polytetrafluoroethylene), in case of emergency at fluoropolymer production facilities or at their processing facilities (by method of thermal decomposition) [7].

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To date, there are no effective therapies for PFIB-mediated lung damage [7,8], and the mechanism of TPE caused by exposure to PFIB remains unclear. There is published data on the acylating effect of PFIB on all components of the air—blood barrier (surfactant, pneumocytes, endotheliocytes), which leads to the release of proinflammatory agents and triggers a cascade of inflammatory reactions accompanied by an increase in the permeability of the air—blood barrier [4,7].

An important way to elucidate the mechanism of the toxic effect of PFIB is the search for its primary target. In our previous studies, an assumption was made that the presence of surfactant, even in case of acylation of its molecules, does not unambiguously determine the likelihood of TPE development after exposure to PFIB [2]. The mechanism of its direct toxic effect is probably realized through the cellular components of the air—blood barrier (pneumocytes or endotheliocytes) [2].

In view of compartmentalization and specificity of metabolic processes at the early stages following exposure, it is important to establish the primary cell target among the components of the air—blood barrier subjected to toxic effects of PFIB, in particular, via detection of ultrastructural changes.

Here we studied ultrastructural changes in cellular components of the air—blood barrier at the early stages following inhalation exposure to fluoroplast-4 pyrolysis products.

MATERIALS AND METHODS

The study was performed on male Wistar rats (body weight 180-220 g) according to the requirements of regulatory legal acts on experimental work using animals and on humane animal care. The animals were divided into experimental and control groups (6 rats each).

Fluoroplast-4 pyrolysis products (weight 2.6 g) were obtained at a temperature of 440-750°C within 4 min. Static inhalation effect of pyrolysis products on animals of the experimental group was modeled in a sealed chamber over 15 min (chamber volume 0.1 m^3), after which the animals were removed from the chamber and breathed atmospheric air. Animals of the control group were placed in an inhalation chamber for 15 min, where they breathed atmospheric air.

The animals were sacrificed by overdose of Zoletil (Valdefar) in 3 and 30 min after the end of the exposure and lung tissue samples were isolated for histological examination and electron microscopy. The material for histological examination was placed in 10% neutral formalin; after standard processing, the samples were stained with hematoxylin and eosin and examined under a MIKMED-6 light microscope (Analit-Neva) with photographic recording.

For electron microscopy, the lung parenchyma samples (2-3 mm^3) immediately after isolation were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h at room temperature, washed in three changes of phosphate buffer, postfixied in 1% OsO_4, dehydrated in ethanol solutions of increasing concentration (30, 50, 70, and 96%) and in absolute alcohol, impregnated with acetone, and embedded in epon. For electron microscopy, ultrathin sections (50-70 nm) were sliced on a Leica UC7 microtome (Leica Microsystem), transferred to copper grids, and contrasted in an alcohol solution of uranyl acetate and in aqueous solution of lead citrate. Electron microscopy was performed using a JEM 1011 microscope (Jeol). Digital micrographs were taken using a Morada camera (Digital Imaging Solutions Inc.).

Qualitative changes in the ultrastructure of type I and type II pneumocytes and endotheliocytes were evaluated on electron diffraction patterns. The length of the profile of the alveolar lining was measured and the number of fused pinocytotic vesicles determining the possibility of transepithelial transport, as well as the number of denuded basement membrane sites were evaluated. Thus, we evaluated transcellular (transcytosis and aggregates of pinocytotic vesicles) and paracellular (expansion of intercellular contacts and denudation of the basement membrane) equivalents of permeability of the alveolar epithelium.

Statistical analysis of the results of experimental studies was performed using the program Statistica 10.0 (StatSoft, Inc.). In case of data distribution different from normal law, non-parametric Kruskal—Wallis and Newman—Keuls tests were used for multiple pairwise comparisons. The data are presented as the Me (Q1; Q3). The differences between the groups were significant at $p<0.05$.

RESULTS

Toxicological experiments showed that inhalation of the pyrolysis products of 2.68±0.6 g fluoroplast-4 in a 0.1-m^3 chamber caused death of 50% laboratory animals on the first day after intoxication. Products of fluoroplast-4 pyrolysis were delivered to the inhalation chamber by means of natural convection. No irritating effect of the smoke was observed in animals. The condition of animals removed from the inhalation chamber did not differ from that of control animals. Histological examination of micropreparations (×200 and ×400) isolated from animals in 3 and 30 min after the exposure revealed normal histoarchitectonics of the lung tissue, no pathological changes were revealed in comparison with the control group.

Electron microscopy of lung preparations from control group rats demonstrated components of the air—blood barrier (Fig. 1). Type I pneumocytes (respiratory) were presented by flattened cells of irregular shape of endodermal origin. They had extensive nuclear part and thinned cytoplasmic processes containing numerous pinocytotic vesicles. The processes of type I pneumocytes were usually overlapped. In some cases, the processes formed flat terminal surface that contacted in the "end-to-end" manner. Adjacent surfaces in this case formed tight junctions creating a dense barrier. Type II pneumocytes (granular type) were presented by highly differentiated cubic cells; their cytoplasm contained secretory granules, osmiophilic lamellar bodies, and numerous of mitochondria. Type III pneumocytes (brush type) were prismatic cells with well-recognizable microvilli. All types of pneumocytes were located on the basement membrane formed by regular bundles of collagen fibers. In many areas of the air—blood barrier, pneumocytes and endotheliocytes had common basement membrane. Endotheliocytes lined the alveolar capillaries. Their processes contained different number of transcytotic vesicles; somewhere they became thinner and formed fenestra. Alveolar macrophages were somewhere seen in the lumen of the alveoli or in the epithelial lining.

In 3 min after exposure, the formation of contacts of pinocytotic vesicles with the luminal and abluminal membranes of the processes (transcytosis) was observed in respiratory pneumocytes. Sometimes, fusion of pinocytotic vesicles into polyform lacunae contacting with the process membranes was noted. Destruction of the membrane contacts in end-to-end contacting processes led to the formation of intercellular gaps up to basement membrane structures as soon as in 3 min after intoxication. Thus, the structural equivalents of both transcellular and paracellular pathological permeability were revealed at early stages after intoxication, which indicated impairment of the air—blood barrier (Fig. 2). In 30 min after inhalation of the toxicant, outgrowths and exfoliated fragments created a picture of "serpentine structures" in the lumen of the alveoli. The formation of cytoplasmic outgrowths of pneumocyte processes was accompanied by destruction of occluding junctions between the overlapping processes. Widening of the contact slit was irregular in length and was often seen starting from the abluminal part. At the same time, signs of destruction of the collagen matrix of the basement membrane were found only in 30 min after intoxication (Fig. 2).

Aside from the processes, significant pathological changes were detected in bodies of respiratory pneumocytes (type I) with condensation of heterochromatin on the karyolemma and deformation of the nucleus. In some cases, these changes can be classified as apoptotic, however, pronounced destruction of mitochondrial cristae limits the possibility of completing this type of cell death and suggests further lytic fusion according to the type of necrosis. In other cells, karyorrhexis was initially detected with a marked expansion of the perinuclear space and destruction of mitochondria. In both cases, desquamation of cells surrounded by a halo of "serpentoid processes" into the lumen of the alveoli was observed (Fig. 3).

Changes in granular pneumocytes (type II) with karyorrhexis, destruction of the mitochondrial matrix and osmiophil lamellar bodies, and dilatation of endoplasmic reticulum cisterns were observed only in 30 min after inhalation exposure. Despite critical changes in the cells throughout the observation period, their desquamation and exit from the epithelial lining were not revealed. In brush pneumocytes (type III), a decrease in the number of outgrowths was observed.

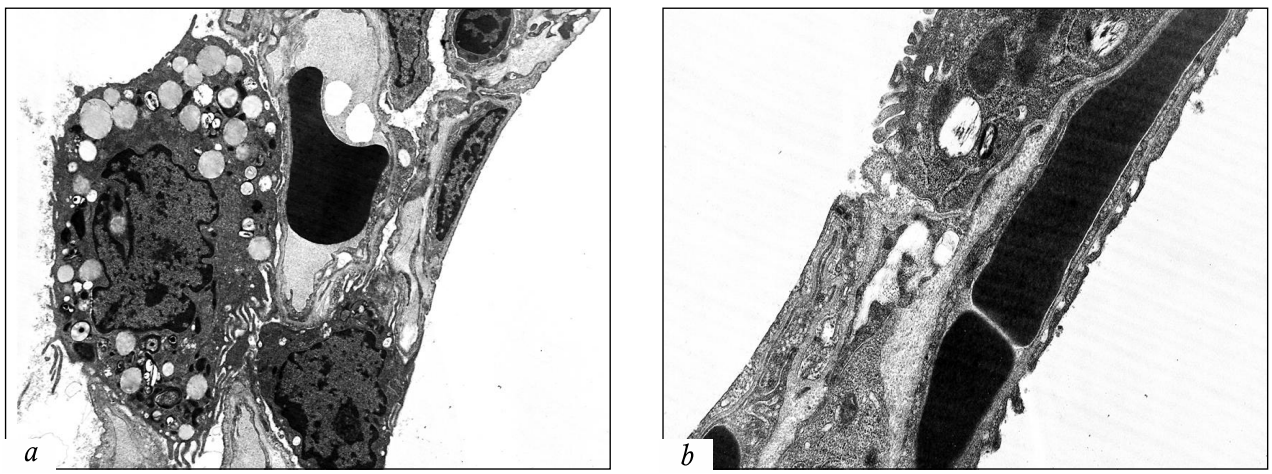

Fig. 1. Normal structure of the air—blood barrier in rats. The thickness of the barrier is determined by the presence of nucleated bodies of type I and type II pneumocytes located around the vessel (*a*) or on one side of it (*b*). Electron diffraction pattern, ×5000.

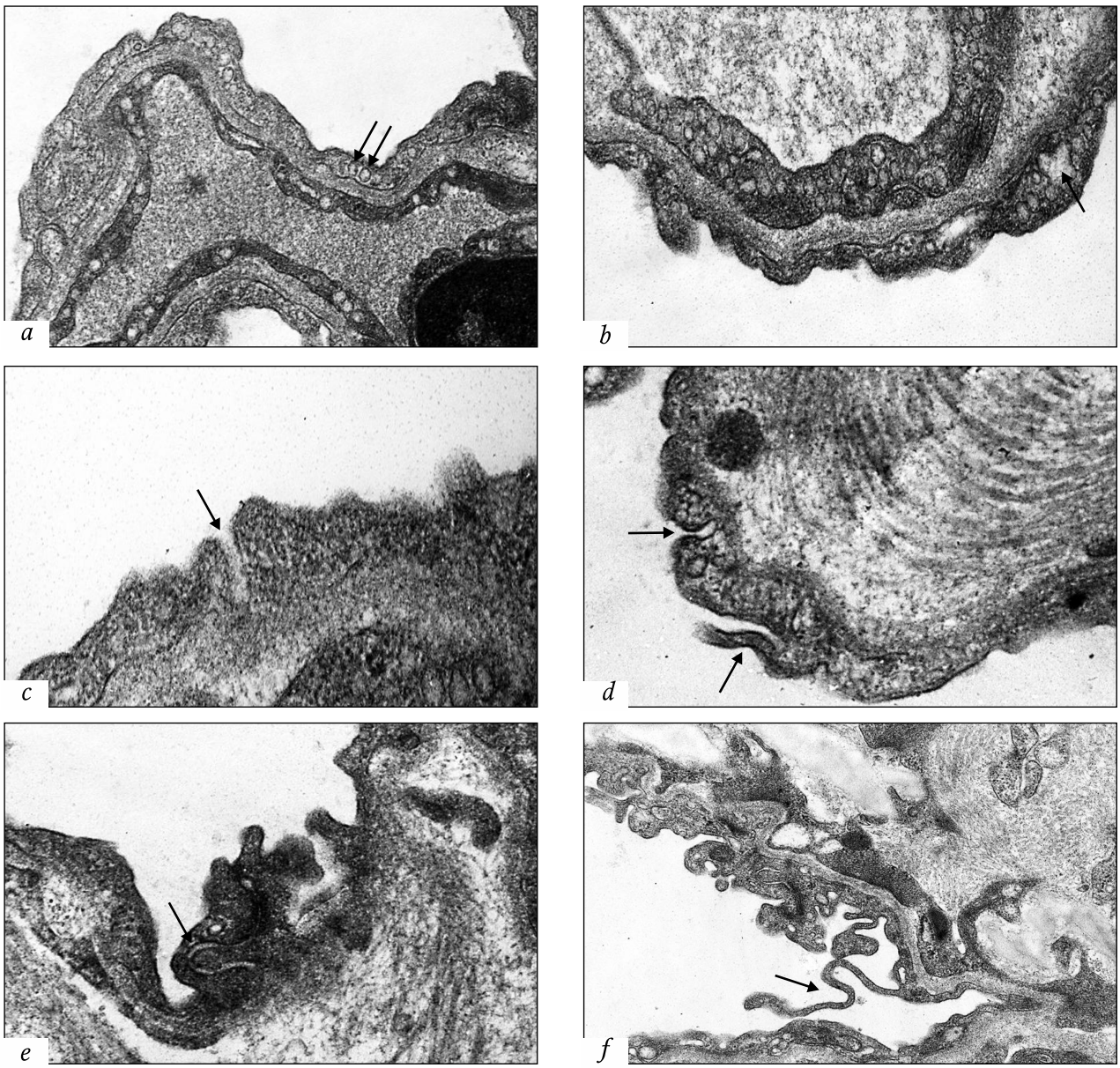

Fig. 2. Variants of early changes in the processes of type I pneumocytes. Electron diffraction pattern, ×6000. The formation of contacts of pinocytotic vesicles with the luminal and abluminal membranes of the process (transcytosis) in the respiratory pneumocyte (*a*), the fusion of pinocytotic vesicles into lacunae also contacting with the membranes (*b*) in 3 min after PFIB intoxication. Destruction of membrane contacts of end-to-end contacting processes leads to the formation of intercellular clefts up to basement membrane structures in 3 (*c*) and 30 min (*d*) after intoxication. Formation of cytoplasmic outgrowths (*d*) and thickening of the lining is accompanied by destruction of the occluding contacts between overlapping processes (*e*); desquamation of these processes also led to the formation of "serpentine structures" (*f*) in 30 min after intoxication.

Changes in their cytoplasm were similar to type II pneumocytes (Fig. 3).

No pathological changes in endotheliocytes were detected at the specified terms.

Analysis of the number of equivalents of transcellular and paracellular permeability revealed an increase in the number of transcytotic vesicles and aggregates of pinocytosis vesicles, as well as an increase ($p<0.05$) in the number of dilated cell—cell contacts and denuded basement membrane sites in 3 and 30 min following intoxication (Table 1). The number of cytoplasmic intraalveolar outgrowths significantly increased in 3 min. By the 30th minute after exposure, this parameter decreased, however, the length of the processes of pneumocytes exfoliated into the lumen of the alveoli increased.

The toxicity of the fluoropolymer pyrolysis products for humans is primarily determined by perfluoroisobutylene [6]. According to published data, PFIB entering via inhalation route produces an acylating

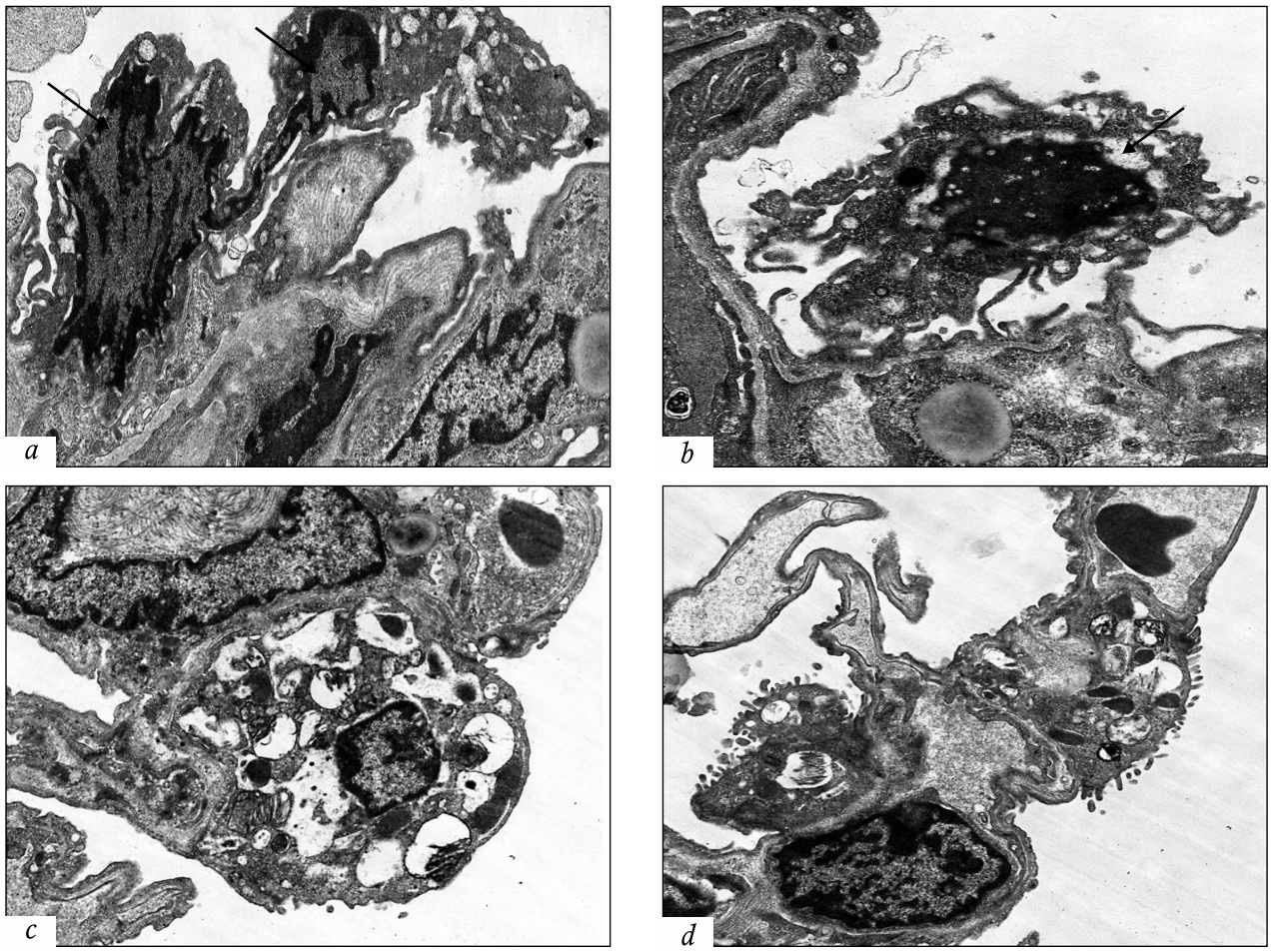

Fig. 3. Changes in the bodies of respiratory pneumocytes (type I) with condensation of heterochromatin on the karyolemma and deformation of the nucleus (*a*) or karyorrhexis with a marked widening of the perinuclear space (*b*), destruction of mitochondria and desquamation of cells that lost contact with the basement membrane into the lumen of the alveoli. Changes in granular pneumocytes (type II), accompanied by karyorrhexis, destruction of the mitochondrial matrix and osmiophilic lamellar bodies with widening of endoplasmic reticulum cisterns (*c*). Brush border pneumocytes (type III) with degenerative changes in organelles (*d*). Electron diffraction pattern, ×6000 (*a*, *b*), ×5000 (*c*, *d*).

effect on the macromolecules of the air—blood barrier components, which leads to activation of the proinflammatory cascade, increase in air—blood barrier permeability, and the development of TPE [4,5,7]. However, to date, the primary cellular target of PFIB has not been determined [7,8], which makes it difficult to identify molecular targets of the poison, considering the differences in the organization of metabolic processes in cells of different populations.

Previous electron microscopy studies [3] revealed ultrastructural changes in both pneumocytes and capillary endotheliocytes in rats with PFIB as soon as in 5 min after intoxication. However, in this study, chemically pure PFIB was used (LCt_{50}=117 ppm/min); intoxication with this agent in its pure form in such high concentrations is unlikely. At the same time, the effect of the pyrolysis products of fluorine-containing polymeric materials containing PFIB is of current interest nowadays [5,7,8].

Histological examination of the lung preparations obtained in 3 and 30 min after the exposure revealed no disturbances in histoarchitectonics and cytopathological changes in the elements of the air—blood barrier. Nevertheless, at the ultrastructural level, changes in pneumocytes (predominantly type I) were observed as soon as in 3 min after the exposure. We observed enhanced vacuolization of cell processes, pronounced transcytosis, and destruction of tight junctions in end-to-end contacting processes with the formation of funnel-shaped defects of the epithelial lining (often to its entire depth) by the 3rd minute. Overlapping processes also dissociated at different lengths and freely hung in the lumen of the alveoli. By the 30th min, desquamation of bodies of type I epithelial cells into the alveoli was observed with the appearance of many bizarre convoluted tape structures formed by processes that retained connection with edematous basement membrane. Changes in types II and III pneumocytes were

TABLE 1. Changes in the Number of Equivalents of Transcellular and Paracellular Permeability as well as the Number of Cytoplasmic Outgrowths per 1 μ of the Transverse Profile of the Processes of Type I Pneumocytes in the Dynamics of Early Post-Intoxication Period (Me (Q1; Q3); arb. units)

Experimental conditions	Equivalents of transcellular penetrability	Equivalents of paracellular penetrability	Cytoplasmic inraalveolar outgrowths
Control (*n*=6)	0.054 (0.030; 0.095)	0.012 (0.005; 0.022)	0.007 (0.001; 0.016)
3 min after exposure (*n*=6)	0.102 (0.074; 0.163)	0.051* (0.032; 0.071)	0.102* (0.07; 0.17)
30 min after exposure (*n*=6)	0.083 (0.070; 0.123)	0.054* (0.048; 0.070)	0.018 (0.001; 0.036)

Note. *$p<0.05$ in comparison with the control.

rare and consisted of destruction of secretory granules, appearance of cytoplasmic vacuoles, often with activation of lysosomes, and decrease in the number of processes; however, the cells always retained their contours and were located within the epithelium.

The detected increase in the number of equivalents of transcellular and paracellular permeability in the profile of the alveolar lining indicates the early development of a pathological cascade in type I pneumocytes leading to impairment of the integrity of the alveolar barrier and an increase in the possibility of vascular leakage.

The results of our experimental study indicate that cytopathological changes in the structure of the air—blood barrier after PFIB poisoning are mainly associated with damage to type I pneumocytes, which can be used for further studies of the mechanisms of toxic effects of the pyrolysis products of fluorinated polymer materials, as well as for the search for correction tools for PFIB-based lung lesions.

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