Experimental Modelling of Chronic Obstructive Pulmonary Disease

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A method for experimental reproduction of stages of chronic obstructive pulmonary disease formation (from acute inflammation to bronchopulmonary tissue restructuring characteristic of this disease) is presented. Lung injury and inflammation were induced by nitrogen dioxide. Hyperplasia and hypersecretion of goblet cells, squamous cell metaplasia of the ciliary epithelium, emphysema, and focal fibrosis served as the morphological substrate for the formation of bronchial obstruction. The adequacy of the model is confirmed by signs characteristic of chronic obstructive pulmonary disease: hyperexpression of CD3 lymphocytes in the bronchial wall and parenchyma, manifold increased production of TNFα and TGFβ, high concentrations of circulating pathogenic immune complexes. Persistence of the structural and functional shifts throughout 6 months after exposure to nitrogen dioxide indicated a chronic course of the resultant pathological process.

Key Words: *chronic obstructive pulmonary disease; inflammation; nitrogen dioxide*

Pathological inflammatory response of the lungs to inhalation of aggressive gases and particles, specifically, tobacco smoke and anthropogenic oxidant pollutants, underlies the formation of chronic obstructive pulmonary disease (COPD) [4,9]. The difficulties in simulation of COPD are explained by the heterogeneity and polyetiological nature of the disease, when different factors and pathogenetic pathways eventuate in the formation of COPD.

Our aim was to reproduce under experimental conditions structural and functional changes in the lungs corresponding to stages in the formation of COPD: from acute inflammation to pronounced remodeling of bronchopulmonary tissue characteristic of this disease. Nitrogen dioxide (NO_2), a component of tobacco smoke and one of the most aggressive aerogenic oxidants, was selected as the factor initiating lung injury with subsequent development of inflammation and formation of signs characteristic of COPD. Nitrogen dioxide poorly dissolved in water reaches the terminal bronchioles and alveoli, where it affects the pneumocytes highly sensitive to this pneumotoxicant [1,11].

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MATERIALS AND METHODS

The study was carried out on male Wistar rats (180-200 g). Parameters of exposure to NO_2 causing no acute toxic injuries in the lungs and providing 100% survival of animals were selected experimentally. The rats (6-7 animals) were put into a 90-liter box assembled in a ventilation cabinet and connected via a hose to a laboratory device for *ex temporae* production of NO_2. The needed amount of sodium nitrite was put into Wurtz flask and concentrated sulfuric acid was added by droplets through a separating funnel. A mixture of nitrogen oxides formed as a result of chemical reaction:

$2NaNO_2+H_2SO_4=Na_2SO_4+NO+NO_2+H_2O$.

Colorless NO under the effect of air oxygen present in the flask is converted into more stable yellow-brown NO_2 pumped into the box with animals through a tube. The concentration of NO_2 was measured by colorimetrical methods and was 30-40 mg/m^3. The animals were exposed to NO_2 3 times a day, 30 min per session, at 30-min intervals between the sessions, for 15 (n=30), 30 (n=30), 60 (n=40), and 90 (n=30) days. Control rats were put into a similar box with air. Ten animals were observed for 6 months (rehabilitation period) after the end of 60-day exposure to NO_2. The animals were sacrificed by cervical dislocation. Blood specimen was collected by puncture of the left heart ventricle, the lungs were washed with sterile saline. The lungs for histological studies were spread by injecting 10% formaldehyde solution through the trachea. The material was embedded in paraffin, sections (5-7 μ) were stained with hematoxylin and eosin and after van Gieson. Morphometric analysis of lung tissue histological preparations was carried out on a complex consisting of a 3.5 mega pixel camera and computer with Videotest Morpho 3 software. By evaluating color contrasts, the percentage of alveolar septae and the area of fibrous tissue around the lobular bronchus wall, lobular pulmonary artery, and in the interalviolar septae were calculated. Immunohistochemical study was carried out using monoclonal antibodies to CD3 antigens for detection of T-lymphocytes) and CD68 (for detection of macrophages). The count of T-lymphocytes was estimated by CD3 expression in each visual field in the bronchial wall and in the peribronchial zone (up to 20 μ from bronchial wall). The number of cells per mm bronchial wall length was calculated. The levels of TNFα and transforming growth factor β (TGFβ) in the serum and bronchoalveolar lavage fluid (BALF) were evaluated by ELISA using species-specific commercial test systems (DRG). Serum concentrations of circulating immune complexes (CIC) were measured by polyethylene glycol selective precipitation. The results were statistically processed using Statistica 6.0 software.

RESULTS

Changes characteristic of acute reaction to injury were detected after 15 days of NO_2 exposure in the presence of retained plication of the bronchial mucosa. The shifts included submucous edema, sites of lung tissue overstretching, sheets of desquamated epithelium, focal proliferation of bronchial epithelium, hyperplasia of lymphoid formations in the bronchial wall. Signs of moderate degeneration, disorders in secretion production by bronchial glands, cellular infiltration of the interstitium with predominating histiocytes were seen. Focal infiltration of interalveolar septae with swelling of the alveolar epithelium and macrophage accumulation in the lumen were found.

Thirty-day NO_2 exposure resulted in the development, in addition to the above changes, of lymphocytic and leukocytic infiltration of the submucous layer, peribronchial and perivascular fat, degenerative changes in the bronchial gland epithelium. Goblet cell hyperplasia was most pronounced in sites of bronchial ramification. Desquamation of the epithelium with denudation of the basal compartments was seen. The muscle plate of the small bronchi was involved in the pathological process: dilatation of the lumen with thinning of the wall and muscle plate atrophy or spasms were found. Signs of overstretching and lymphocytic infiltration of interalveolar septae progressed in the respiratory part.

After 60-day exposure to NO_2, foci of squamous cell metaplasia and atrophy of the bronchial glands emerged. Focal dilatation of the bronchioles and alveolar passages and moderately pronounced peribronchial and perivascular sclerosis were seen. Signs of emphysema were detected in the respiratory part (Fig. 1). Morphometric analysis showed shrinkage of the specific area of interalveolar septae (14.3±3.1% *vs.* 26.6±3.1% in the control and 28.5±4.6% after 30 days of NO_2 exposure, $p<0.05$), this indicating their thinning and overstretching of lung tissue. The amount of fibrous tissue increased significantly in all the studied structural elements of the lungs (Table 1).

Hence, by histological criteria the changes detected in the lungs after 60 days of NO_2 exposure correspond to manifestations of chronic inflammation leading to the formation of morphological substrate for the development of irreversible bronchial obstruction [8,12]. The changes developing in the bronchi and respiratory compartments during 60-day NO_2 exposure

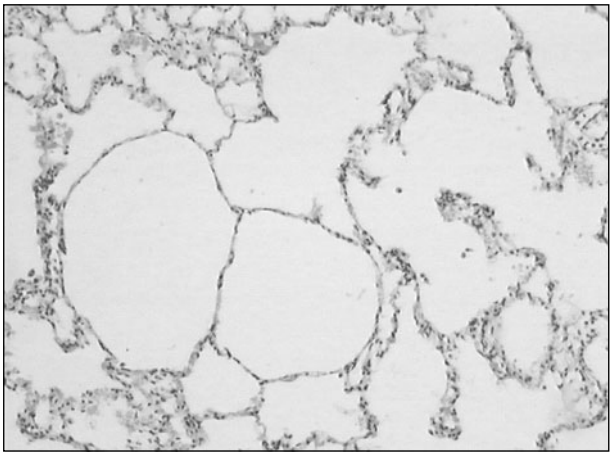

Fig. 1. Rat lung after 60 days of exposure to NO_2. Emphysema foci, thinned interalviolar septae. Hematoxylin and eosin staining, ×120.

TABLE 1. Morphometric Characteristics of Fibrosis Area around Lobular Bronchus, Lobular Pulmonary Artery, and in Interalveolar Septae of Rats after NO_2 Exposure of Different Duration ($M \pm m$)

Experiment conditions		Around lobular bronchus, mm	Around lobular artery, mm	Interalviolar septae, mm
Control		0.970±0.002	1.060±0.002	0.870±0.005
Exposure to NO_2, days	15	1.110±0.034	1.190±0.029	0.990±0.023
	30	1.200±0.026	1.140±0.023	1.060±0.024
	60	1.310±0.008*	1.250±0.005*	1.40±0.01*
	90	2.050±0.029*	2.130±0.024*	1.350±0.023*

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

persisted for 6 months after the end of the pollutant inhalations: foci of dedifferentiated epithelium, accumulations of macrophages with a lymphocyte admixture in the alveoli, goblet cell hyperplasia in the large and small bronchi. Cell accumulations in combination with dystelectasis and hyperplasia of the alveolar epithelium were found around small vessels. Free cells were found in edematous interstitium.

Prolongation of NO_2 exposure to 90 days led to the progress of panacinar emphysema and signs of fibrosis in the lung tissue (Table 1) mainly in the submucous layer of the large bronchi and in the walls of small bronchi. The bronchial glands were characterized by large vacuolar epithelial degeneration with disorders in secretion. The development of panacinar emphysema resulted in a decrease in the number of functioning capillaries in the interalveolar septae. Macrophage accumulations were seen in the alveolar lumen. Morphological changes in the lungs characteristic of different periods of NO_2 exposure are presented in Table 2.

The formation of chronic inflammation in the bronchopulmonary tissue was confirmed by immunohistochemical findings. A significant increase of CD3 expression in the walls of the small and medium-sized bronchi of rats after 60-day NO_2 exposure indicated the presence of numerous T cells (61.60±1.59 *vs.* 28.10±2.36 in the control, $p<0.05$). After 90 days of NO_2 exposure the count of T-lymphocytes in the bronchial wall somewhat reduced in comparison with 60 days, but remained significantly higher than in the control (47.1±5.3, $p<0.05$). A trend to an increase of CD3 expression in the lung interstitium was observed after 90-day NO_2 exposure: 19.1±3.8 (control), 20.9±3.5 (60 days), 26.0±3.6 (90 days; $p<0.05$). The expression of macrophage CD68 in the interstitium decreased after 60-day NO_2 exposure to 2.30±0.43 (4.15±1.65 in the control; $p>0.05$) and increased after 90 days of NO_2 exposure (8.93±2.33; $p<0.05$ vs. 60 days). Macrophages were detected in the bronchial lumen only on day 90 of experiment. Presumably, migration of alveolar macrophages from the parenchyma to the bronchial wall and lumen started after 60 days of NO_2 exposure, while their initial activation took place in the lung parenchyma. Hence, immunohistochemical findings indicated the involvement of not only congenital im-

TABLE 2. Structural Changes in Lung Tissue of Rats after NO_2 Exposure

Duration of NO_2 exposure	Morphological changes in lung tissue		
	bronchial epithelium	bronchial walls	interstitium
15 days	Desquamation of epithelium, focal proliferation, degeneration and disorders of bronchial gland secretion	Submucous edema. Lymphoid hyperplasia	Edema and cell infiltration of interalveolar septae. Accumulation of macrophages in alveoli
30 days	Desquamation. Goblet cell hyperplasia	Bronchial spasm or dilatation, muscle plate atrophy	Lymphocytic and leukocytic infiltration of interalveolar septae
60 days	Metaplasia. Bronchial gland atrophy	Bronchial dilatation, muscle plate atrophy	Leukocytic and lymphocytic infiltration. Signs of emphysema
90 days	Metaplasia, foci of hyperplasia. Increased count of goblet cells	Sclerosing with lymphomonocytic infiltration	Foci of panacinar emphysema, focal fibrosis

TABLE 3. Serum and BALF Levels of TNFα and TGFβ after NO_2 Exposure (*M*±*m*)

Cytokine level	Control	Day after NO_2 exposure		6 months after NO_2 exposure
		30 days	60 days	
Serum, pg/ml				
TNFα	60.93±6.34	120.05±13.47*	125.86±16.21*	61.89±7.87^{+}
TGFβ	N.d.	1.97±0.06	36.72±5.98^{o}	2.28±0.14
BALF, pg/ml				
TNFα	1.50±0.03	178.23±20.57*	204.95±25.76*	234.82±32.46*
TGFβ	N.d.	3.77±0.98	31.27±7.24^{o}	29.69±8.12^{o}

Note. N.d.: not detected. $p<0.05$ in comparison with: *control, $^{+}$60-day exposure, ^{o}30-day exposure.

munity cells (primarily alveolar macrophages) in the inflammatory process, but also of adaptive immunity cells (T-lymphocytes). This confirmed the formation of chronic inflammation in animals on days 60-90 of NO_2 exposure.

An increase of TNFα and TGFβ concentrations in the serum and BALF was detected depending on the length of NO_2 exposure (Table 3). After 30-day exposure to NO_2, the level of TNFα in the blood increased 2-fold, in BALF more than 100-fold. Prolongation of exposure to 60 days caused no appreciable increment in TNFα concentrations in both biological fluids. No TGFβ was detected in the blood or lavage fluid of controls, because normally the representatives of this growth factor family are inert in the extracellular matrix. Reactive oxygen species, including NO_2, can serve as TGFβ activators. A significant level of TGFβ in both media was recorded after 30-day NO_2 exposure. Prolongation of exposure to 60 days was associated with increase of TGFβ concentrations in BALF (8.3 times) and blood (18.6 times). During rehabilitation period serum levels of both cytokines reduced and approached the control values. In BALF, reflecting the focal processes in the lungs, their levels remained high, indicating persistent inflammation. Changes in the levels of TGFβ lagged behind changes in TNFα concentrations: the level of TNFα increased as early as by day 30 of NO_2 inhalations, while a significant increment of TGFβ was recorded only by day 60. This difference can be attributed to chronic degeneration of the inflammatory process and to fibroblast function stimulation and initiation of fibrosing process [3,6]. This is in line with the morphological findings demonstrating the progress of sclerotic changes after 60 days of NO_2 exposure.

Blood levels of medium-molecular-weight CIC increased with prolongation of NO_2 exposure: by 45% *vs.* control after 30 days, by 110 and 137% after 60 and 90 days, respectively ($p<0.05$), and remained 1.9-fold ($p<0.05$) above the normal 6 months after the end of NO_2 inhalations. It is known that medium-molecular-weight immune complexes are characterized by the highest pathogenic activity, as they are adsorbed on lung tissue and promote the release of many cytokines and chemokines [5,7]. High concentration of medium-weight immune complexes can prevent the development of reparation processes in the lungs and promote the persistence of inflammation [10].

Hence, daily exposure to NO_2 in a concentration of 30-40 mg/m^3 for 1.5-2 h leads to the development of a chronic inflammatory process in the bronchopulmonary system by day 60, which can be regarded as a COPD model [2]. The model reproducibility was 90%. The morphological substrate for subsequent formation of bronchial obstruction is goblet cell hyperplasia and hypersecretion, squamous cell metaplasia of the ciliary epithelium, promoting disorders in the tracheobronchial clearance, and progressive changes in the respiratory part of the lungs (emphysema and focal fibrosis). Hypersecretion and impaired discharge of the secretum from the respiratory tract promote the persistence of the inflammatory process and emergence of the vicious circle leading to the progress of morphological remodeling of the lungs, which was most clearly seen after 90 days of NO_2 exposure. The adequacy of COPD model is confirmed by pathophysiological changes characteristic of this condition: hyperexpression of CD3 lymphocytes in the bronchial wall and lung parenchyma, many-fold increased production of proinflammatory cytokine TNFα and growth factor TGFβ promoting the development of fibrosis in the small bronchi, and high concentrations of pathogenic immune complexes circulating in the blood. The persistence of structural and functional changes throughout 6 months after the end of NO_2 exposure confirms chronic course of the resultant pathological process.

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