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Research Article

Influence of Basic Treatment of Patients with Stage II Chronic Obstructive Pulmonary Disease with Tiotropium Bromide on the Morpho-Functional State of the Bronchial Mucosa and the Level of Type IV Collagen in Bronchoalveolar Lavage Fluid

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Abstract
The objective of the research was to evaluate the influence of basic treatment of patients with chronic obstructive pulmonary disease with tiotropium bromide on the processes of morphological rearrangement and local barrier defence mechanisms in the bronchial mucosa.
Stage II chronic obstructive pulmonary disease is associated with the damage to the bronchi with proliferation of the connective tissue in its proper plate, clear identification of the basal membrane alteration, the presence of fibroblasts, the activation of fibroblasts/myofibroblasts and mucous glands, which is accompanied by the significant increase of type IV collagen levels by 6.19 times ($p < 0.05$) in bronchoalveolar lavage fluid as compared to the control group indices.
The elimination of stage II chronic obstructive pulmonary disease exacerbation and the use of tiotropium bromide within a month was accompanied only by partial improvement of morpho-functional state in relation to both cells of bronchial epithelial lining and adjacent connective tissue of mucosal plate. The prolongation of tiotropium bromide administration from 2 to 6 months, provided positive dynamics of structural morphological changes of the bronchial mucosa (the restoration of the ciliary apparatus of epithelial cells, the normalization of the secretory function of goblet cells, the inactivation of fibroblasts, the initial degeneration of myofibroblasts), thus leading to complete absence of morphological signs of edema or epithelial cell dystrophy.
Conclusions. In patients with stage II chronic obstructive pulmonary disease, complete absence of morphological signs of edema or dystrophy of epithelial cells, against the background of collagenolysis in the connective tissue of the proper mucous plate of the bronchi and the highest possible decrease in the number of myofibroblasts, with near-complete levels of type IV collagen normalization in the bronchoalveolar lavage fluid, were identified only within a 6-month treatment with tiotropium bromide.

Keywords
chronic obstructive pulmonary disease; morphological rearrangement of the bronchial mucosa; tiotropium bromide

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Problem statement and analysis of the latest research

Chronic obstructive pulmonary disease (COPD) is one of the most severe diseases in terms of disability and economic costs and the second most widespread infectious disease in the world. It ranks fourth in the structure of mortality and is characterized by steady tendency to the increase in its prevalence in future due to the incidence of bad habits on the one hand and extended longevity on the other hand [1, 2, 12]. In the "European Lung White Book", Ukraine is represented as one of the countries with the highest mortality rate due to respiratory pathology among men [5]. In Ukraine, the incidence of chronic bronchitis has increased by 0.51% over the period of 2015-2016, that suggests it to be the strategic issue of national medicine [3].

The course of stage II COPD is the most aggressive with the onset of morphological reorganization of the respiratory tract (sclerosis, fibrosis, bronchospasm, edema, hypercrinia, dyscrinia), leading to bronchial remodeling with subsequent galloping progression of its systemic manifestations. The remodeling of the respiratory tract is a pathological process that is observed in chronic inflammatory and obstructive diseases of the respiratory organs. Fibrotic changes are critical in the re-

arrangement of airways [14, 16, 17]. Recently, scientists have studied the expression and function of muscarinic receptors in pulmonary fibroblasts, which have a stimulating effect on the collagen synthesis [13, 18]. We think that prolonged blockade of muscarinic-induced collagen synthesis may be effective and may contribute to reported beneficial long-term effects of anticholinergics in COPD. Anticholinergic medications are the key aspect in the treatment of broncho-obstructive syndrome, particularly COPD treatment, due to their bronchodilative effects [7, 8, 9]. Based on the UPLIFT (Understanding Potential Long-term Impacts on Function with Tiotropium) research findings, the administration of M_3 selective anticholinergic agent tiotropium to COPD patients within the 4-year period showed lasting improvement of pulmonary function [10]. The following results were recorded in the group of patients treated with tiotropium agents: the rate of mortality risk decreased by 16% ($p = 0.016$), the treatment had positive influence on the survival rate ($p = 0.034$), it also proved to prevent exacerbations of the disease ($p < 0.001$) and considerably decreased the risk of destabilization of COPD course which required further hospitalization (risk ratio 0.86; $p < 0.002$), as compared to the control group of patients [5, 10]. Furthermore, broad-scale clinical studies pointed to the fact that the use of long-acting muscarinic antagonist tiotropium for COPD patients promotes delayed deterioration of the functioning of respiratory tract in dynamics up to 4 years, which would probably contribute to the involution of morphological changes in the bronchi [7, 10, 21].

In our opinion, the effectiveness of treatment of any pathology depends on the deep knowledge of all the components of its pathogenesis. For this reason, the study of complex and multi-system processes, originating in the body during COPD, as well as the peculiarities of local regenerative-restorative potential formation, against the background of the use of tiotropium bromide with its specific effect is important and will contribute to the development of modern science and improve understanding of the need for long-term basic treatment by both patients and healthcare workers.

The objective of the research was to evaluate the influence of basic treatment of COPD patients with tiotropium bromide on the processes of morphological rearrangement and local barrier defence mechanisms in the bronchial mucosa.

1. Materials and Methods

The study involved 61 patients suffering from stage II COPD (GOLD 2 - moderate bronchial obstruction: $FEV_1 > 50\%$ and $> 80\%$ of the predicted value). The diagnosis was verified and formulated in accordance with the Order of the Ministry of Health of Ukraine No 555 of June 27, 2013 – "On Approval and Implementation of Medical-Technological Documents on the Standardization of Medical Care in Chronic Obstructive Pulmonary Disease" and Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017 [1, 13].

All subjects were divided into groups depending on the duration of continuous use of selective M_3 cholinolytics of prolonged action of tiotropium bromide at a dose of 18 μg - 1 inhalation 1 time per day.

Group I included 12 patients who did not receive selective M_3 cholinolytics of prolonged duration in the integrated therapy;

Group II comprised 23 patients, who were prescribed tiotropium bromide preparation on the background of complex treatment of exacerbation phase, for 30 days.

Group III included 15 patients, who received a tiotropium bromide preparation on the background of complex treatment of exacerbation phase, for 60 days.

Group IV comprised 11 patients, who received the preparation of tiotropium bromide on the background of complex treatment of exacerbation phase, for 180 days.

The study was conducted prior to therapy and in the dynamics of treatment using conventional therapy regimens (Ministry of Health of Ukraine No 555 of June 27, 2013 – "On Approval and Implementation of Medical-Technological Documents on the Standardization of Medical Care in Chronic Obstructive Pulmonary Disease"). Tiotropium bromide was administered in an inhalation form at the dose of 18 mcg once a day [1]. Bronchial biopsy specimens were obtained from 9 patients with stage II COPD at admission and in the dynamics of treatment within 1, 2, 3, 4 and 6 months in order to study the effectiveness of treatment programs suggested by us and their influence on the processes of morphological rearrangement of the bronchial mucosa. The research material involved broncho-alveolar lavage fluid and bronchial biopsy materials, which were obtained at the level of bifurcation of the upper lobar bronchi into segmental ones during fiber-optic bronchoscopy. Biopsy specimens of the bronchial tree were taken in accordance with the generally accepted rules, maintaining the cutting speed and their non-invasive cutting into pieces. Bronchial biopsy samples were fixed in the solution of 1% osmium tetroxide in 0.1M phosphate buffer with pH 7.4. The biopsy material was diced (1 mm^2) and fixed in osmium solution which was changed three times within 1.5-2 hours. After fixation, the tissue blocks were washed from the fixator with 0.1 M phosphate buffer followed by dewatering in ethyl alcohol of increasing strength (30°, 50°, 70°, 90°, 100°) for 10 minutes changing it for three times in each of the portions. At the stage of dewatering in 70°alcohol, the tissue blocks were contrasted in 2% solution of uranyl acetate prepared with 70°alcohol use. After the dehydration was completed, the material was passed through absolute alcohol with absolute acetone (10 min). Subsequently, the material was passed through the dilution of acetone with epoxy resins (3: 1 – 30 min, 1: 1 – 1 h); then the material was kept in pure resin for 1 hour. Subsequently, pieces of material were placed in gelatin capsules and poured with resin followed by polymerization at +60°C for 24 hours. Semifine sections, 1 μm thick, were colored with 1% methylene blue solution for light-optical study and orientation of the selected cut for subsequent electron microscopic examination. The cross sections, obtained with

the help of Tesla BS-490A ultramicrotome, were mounted on copper blends 1 mm in diameter with Formvar-coated grid (for surveys) or on a support grid. To increase the contrast, the cross sections were de-contrasted with 2% uranyl acetate solution on 70°alcohol and Reynolds mixture. The material was studied with the help of electron microscope TEM-125 K at an accelerating potential of 75 kV with subsequent photographic recording with the increase in power field from 2000 to 25000 times. Type IV collagen was investigated in bronchoalveolar lavage fluid of 43 patients with stage II COPD before and within the 1st, 2nd and 6th months of tiotropium bromide administration with the help of immunoassay analysis on the StatFax 303 Plus analyzer using "Biotrin Collagen IV EIA" reagents. The control group involved 15 apparently healthy individuals (AHI) without obvious signs of respiratory tract disorders or any other pathology of internal organs.

2. Results and Discussion

Substantial damage to the components of mucosa (epithelial lining and connective tissue of its proper plate) was identified in bronchial biopsy materials of stage II COPD patients hospitalized in the exacerbation phase of the disease prior to treatmet. Ultramicroscopic analysis revealed that epithelial cells of the mucous membrane showed alternative and dystrophic changes. Epithelial cells of varied functional status were identified on the cross sections of mucous membrane of the bronchus, including "dark", "intermediate" and "light" epithelial cells. The nuclei of "dark" epithelial cells were rounded with moderate chromatin condensation. The cytoplasm of "dark" cells included numerous cisternae of smooth endoplasmic reticulum against the dark-coloured cytoplasm with large osmiophilic inclusions of varied shapes and single phagolysosomes. Sparce cilia of varying length were present on the surface of epithelial cells. These cells were often polygonal in shape and their processes immersed between the "light" epithelial cells. The cells were connected by simple intercellular contacts and desmosomes, which are the zones of adhesion and keep the cells together. In the visual field, the intercellular spaces between cell membranes were dilated and their membranes differed. Such changes pointed to catarrhal-sclerotic inflammation.

The nucleus of "light" epithelial cells had slight invaginations and euchromatin, while the perinuclear cistern was not dilated. The cytoplasm involved numerous ribosomes and polysomes, small vacuoles and mitochondria in the state of partial destruction. The Golgi complex was regular. Osmiophilic granules and rod-shaped formations were present in the apical pole.

"Intermediate" (by their intensity of cytoplasm colouring) cells had rounded nuclei and euchromatin. Their cytoplasm looked lighter as compared to the cytoplasm of "dark" cells. It also included small number of vacuoles and osmiophilic granules, small mitochondria, numerous ribosomes and polysomes.

The signs of partial destruction of the cilia apparatus were observed at the apical pole of epithelial cells, namely the typical numerous cilia of varying length and regions of apical surface denudation. The parts of basal cells, located at the base of cilia, were immersed in the epitheliocyte cytoplasm. Membrane-enclosed vesicles (with a rim) were also observed.

Goblet cells were observed in small amounts and in different phases of secretory cycle. Some of them contained a large number of mucus granules accumulated in the cytoplasm without the signs of their excretion outside the cell. The others were at the beginning of the secretory cycle of mucus synthesis; the granules of secret were observed only in the basal part of the cell.

Basal cells had an ordinary structure. Euchromatin prevailed in their nuclei. Some of them had double nuclei. Basal epithelial cells were somewhere identified at various phases of mitosis.

Dystrophic and sclerotic changes with blood supply disruption were observed in the connective tissue of the bronchus proper mucous plate. Sclerotic changes were most pronounced in the subepithelial zone, where thick bundles of collagen fibers were observed.

Numerous macrophages with significant ultrastructural alterations were observed in the cellular component. We identified elongated pseudopodia and microvilli of plasmalemma along the perimeter of macrophage.

The nuclei of macrophages were predominantly round or bean-shaped with condensed patches of heterochromatin. Smooth invaginations of caryolemma and sub-membrane condensation of heterochromatin were also noticed. The perinuclear space was somewhat extended. The elements of granular endoplasmic reticulum and mitochondria were observed around the nucleus. Moderate quantity of small mitochondria, vacuoles, lysosomes and phagolysosomes with vesicular or electron-dense osmiophilic granular material were noticed in peripheral portions of the cytoplasm. Among the mitochondria, organelles of round and elongated shape were determined. Some of them had swollen and clear matrix. The contours of outer mitochondrial membrane were indistinct in some places. The cristae of the internal mitochondrial membrane were unclear. Lysosomes in these areas were predominantly secondary. Tertiary lysosomes (residual corpuscles) occurred in a moderate amount. Vacuolar component of the cytoplasm was represented by a large number of hydrophobic vacuoles. The presence of residual corpuscles and vacuoles in macrophages characterized the development of their dystrophic processes, on the one hand, and the predominace of final stages of phagocytosis on the other one. It is the presence of macrophages in chronic inflammation of COPD patients that indicates specific morphological changes which characterize COPD, as compared to the inflammatory manifestations in asthma [15, 19].

The fibroblastic range of cells in the subordinate to the epithelial lining of the connective tissue in the bronchial mucosa was represented by fibroblasts, fibrocytes and myofibroblasts. The fibroblasts were elongated, and surrounded by numerous

collagen fibers in different projections and of various degrees of maturity, separated by spaces filled with the main amorphous substance. The nucleus corresponded to the external shape of fibroblasts. The nucleus was sometimes in the shape of an "hour glass". It revealed the peripheral condensation of chromatin. The caryolemma was closely related to the surrounding elements of the endoplasmic reticulum. The cytoplasm of active fibroblasts in the connective tissue of the bronchus proper plate was represented by a large number of vacuoles, numerous cisternae of the granular endoplasmic reticulum and free polyribosomes, a small number of vacuoles, hypertrophy of the Golgi complex, multivesicular corpuscles. The Golgi complex was located not only near the nucleus of fibroblasts but in other parts of the cell that characterized its high activity and the ability to secrete products throughout the cell surface [4].

The fibroblasts were distinguished by the degree of colouration of the cytoplasm: there were cells with the "dark" cytoplasm, elongated nuclei and long cytoplasmic projections, and "light" ones with vacuoles and mitochondria.

We also observed a greater number of fibrocytes surrounded by numerous collagen fibers, as compared to AHI. In general, fibrocytes were rarely observed in healhy individuals due to their small number. The presence of fibrocytes, as a definitive form of fibroblasts, indicated the appearance of old cells with reduced secretory activity, but retaining the function of regulation of metabolism and mechanical stability of the connective tissue.

Thin microfilaments were detected in a significant number of fibroblasts in the cytoplasm, which were arranged without a certain order between the organelles, that gave reason to attribute them to myofibroblasts. According to traditional points of view, microfilaments are part of the contractile apparatus of many cells and form the basis of a cell cytoskeleton. The revealed fibroblasts/myofibroblasts had a nucleus with numerous invaginations of the caryolemma (in the state of hyperfunction) and unusual "deposits" of granular material in the cytoplasm of the cell (myofibrils in the cross section). The bundles of microfilaments occupied more than a half of the cytoplasm area. Such elongated microfilament bundles were identical to the contractile apparatus present in smooth myocytes of the bronchial wall. Collagen fibers and thin threads of protofibrillar material occurred around the cells [14, 17, 19]. These authors categorized them as fibroblasts/myofibroblasts. Further research studies suggest that such transformations of fibroblasts occured due to the differentiation of smooth myocytes to myocytes of "secretory" phenotype [11] and their movement into their own plate of the mucous membrane to the subepithelial layer, but they did not find proper confirmation in the scientific literature.

We revealed fibroblasts/myofibroblasts in the subepithelial layer of the connective tissue of the bronchus proper plate. Structural features of fibroblastic cells predominated in their structure, which might play a key role in the surplus production of collagen and the processes of fibrillation of the airway walls in COPD [19, 23]. The increase in type IV collagen level by 6.19 times in bronchoalveolar lavage fluid of stage II COPD patients prior to treatment, as compared to the control group of patients ($p < 0.05$), confirmed the above-described processes (Table 1). In our opinion, the increase in type IV collagen level in bronchoalveolar lavage fluid in COPD indicates the increase of fibroblasts/myofibroblasts activity (on the background of microcirculation disorders, activation of lipid peroxidation and hypoxia), as well as marked thickening of the basal membranes, and thus resulting in dysfunction of both metabolism of their own biological systems, and dissociation of a number of dosage forms, particularly inhalations. Medium and large lymphocytes (plasma cells) were detected in the subepithelial compartment of the bronchus proper plate. Medium lymphocytes had round nuclei. The nucleus was round, with peripheral chromatin condensation and some patches of heterochromatin in its centre. The contours of plasmolemma of lymphocytes were smooth and it was possible to identify some cytoplasmic projections. Moderate quantity of mitochondria and elements of endoplasmic reticulum were observed in the narrow rim of the cytoplasm. According to immunocytochemical methods, they were predominantly attributed to CD8 + T-lymphocytes and confirmed their significant role in the remodeling of the respiratory tract wall in the pathogenesis of COPD [20, 22].

In COPD, the prolonged overload of phagocytic cells with antigenic stimuli and products of the inflammatory process is known to lead to the disorders in the system of alveolar macrophages, lung surfactant and immune defense [5, 8]. The above-mentioned reasons actually provoke a breakdown of synthesis by polymorphonuclear and mononuclear phagocytes of lysozyme [8].

At the same time, we revealed isolated fibroblasts/fibroclasts on cross sections of the bronchial mucosa in the connective tissue of its proper plate. These were cells which had the characteristics of fibroblasts as collagen producers and fibroclasts, which reabsorb collagen. Their characteristics as fibroblasts involved spindle shape, large and light nucleus with the nucleolus. Developed granular endoplasmic reticulum with flat inactive cisternae was observed in their cytoplasm. Their characteristics as fibroclasts involved large vacuoles in the cytoplasm, often with lysosomes inside. The cell surface was invaginated. The cells were surrounded by a fine flakes-like material.

The findings of our research showed that satge II COPD was characterized by damage to the bronchial mucosa with significant sclerosis of its proper plate. It is considered that chronic damage to the epithelium of the mucous membrane and its reparative regeneration occurs due to the activation and permanent secretion of proinflammatory cytokines that affect the epithelium and growth factors that lead to subsequent chronic inflammation and remodeling reactions in the subepithelial compartments. These factors include epithelial growth factor and granulocyte- macrophage stimulating factor that subsequently induce damage to the adjacent basal

Table 1. Dynamics of type IV collagen levels (ng/ml) in bronchoalveolar lavage fluid during treatment of patients with stage II COPD of bronchial obstruction, (M±m)

Indices	AHI, n=15	Prior to treatment, n=61	After treatment				p1	p2	p3
			Group I, n=12	Group II, n=23	Group III, n=15	Group IV, n=11			
Type IV collagen (ng/ml)	9.87±0.52	61.14±1.28	55.21±1.12	43.27±1.17	24.72±1.15	14.07±0.83	<0.05	<0.05	<0.05

Notes.

p1 – significance of differences of parameters between the indices recorded prior to and after the administered treatment;

p2 – significance of differences of parameters between groups of patients;

p3 – significance of differences of parameters between groups of patients and the control indice

membrane, by activating adjacent fibroblasts/myofibroblasts, and deeper structures, including bronchial smooth myocytes, mucous glands and walls of the blood vessels [14].

In the dynamics of treatment, short thin cilia were noticed on the apical pole of mucosal epitheliocytes due to the use of tiotropium bromide for a month as a part of complex treatment of stage II COPD in its exacerbation phase. Basal cells were observed in some epitheliocytes. The cytoplasm was packed, and contained transparent vacuoles, electron-dense inclusions not limited by the membrane, the Golgi complex and granular endoplasmic reticulum. The organelles were difficult to identify due to the densification of the cytoplasm. The nucleus had smooth invaginations and depressions; euchromatin was also present. Granules of mucous were revealed in goblet cells that corresponded to different phases of the secretory cycle. The excretion of mucous granules from the cells was clearly evidenced.

The most pronounced changes occurred in the proper mucous plate of the bronchus.Morphological manifestations of macrophage activity decreased. Macrophages retained villi on the surface, which is one of the permanent signs of these cells. Moderate number of organelles was observed in the macrophage cytoplasm; however, they did not contain phagocytic vacuoles. The nucleus was elongated with peripheral chromatin condensation. Such macrophages may be considered transient, ranging between types I and II [4]. Morphologically, type I macrophages were monocytic, rounded, with bean-shaped nucleus, small volume of the cytoplasm and mild bulging of plasmalemma. The cytoplasm included free ribosomes and polysomes, that provided its density. The elements of both granular and agranular endoplasmic reticulum were also identified. Type II macrophages belonged to mature macrophages, and their activity was ensured by the presence of numerous organelles in the cytoplasm, including a large number of free ribosomes and polysomes, primary, secondary and tertiary lysosomes, sufficiently developed granular endoplasmic reticulum and the Golgi complex. Mature fibroblasts were located within the bundles of collagen fibers and amorphous matter, and their nucleus was elongated with moderate invaginations and a small number of elements of granular endoplasmic reticulum. The cisternae of this organelle showed moderately expanded profiles and involved soft-fiber content. Fine structures, corresponding to synthetic collagen products, were also revealed in the saccules of the Golgi complex. By their morphological characteristics, fibroblasts mostly belonged to cells with moderately pronounced signs of synthetic processes – in this case, the synthesis of collagen. In addition to fibroblasts of the usual structure, there were myofibroblasts of the irregular shape, with the small amount of the thickened cytoplasm. Their nucleus was irregular in shape with significant peripheral condensation (margination) of chromatin. The cytoplasm included vacuoles, heterogenic mitochondria and osmiophilic inclusions. Myofibroblasts differed by their structure from the same cells before treatment. Changes in their structure characterized the processes of shrinkage of myofibroblasts and decrease in their function, which might be caused by the use of selective M_3 anticholinergic agent tiotropium bromide, which is mediated by blocking the muscarinic receptors. At the same time, we identified that the levels of type IV collagen in bronchoalveolar lavage fluid reduced by 1.41 times ($p < 0.05$) (Table 1), as compared to the indices before treatment with the use of tiotropium bromide for 1 month. And it is an evidence of physiological course for repair processes, *neocollagenogenesis* inhibition and thereupon the implementation of a cascade of inhibition effects of remodelling.

At the same time, active plasma cells (plasmocytes) were observed in proper mucous plate of the bronchus in the loose connective tissue, that was evidenced by the presence of dilated cisternae of granular endoplasmic reticulum with moderate electron density in their cytoplasm. The nucleus occupied a significant portion of the cytoplasm. Chromatin of the nucleus was partially condensed proportionally throughout the karyoplasm.

Thus, at the end of the first month of treatment, the histological picture of the bronchial mucosa showed the signs of improvement of the morphofunctional state of both cells of the epithelial lining of the bronchus and the adjacent connective tissue of the proper mucous plate of the bronchus. The signs of ciliary apparatus improvement were observed on the apical pole of epithelial cells. Vacuolization in the cytoplasm decreased, while the cytoplasmic condensation still remained. Macrophages were inherent in a structure that corresponded to moderate activity manifestations. The fibroblasts contained small amount of protein-synthesizing organelles. Plasmocytes retained sufficiently clear outlines of the granular endoplasmic reticulum; however, its content reduced as compared to its state before treatment.

Electron-microscopic examination of the biopsy samples of the bronchial mucosa showed further dynamics of positive structural changes in its components after a two-month treatment with tiotropium bromide. Numerous cilia were observed on the apical surface of epithelial cells. The cytoplasm included a large number of free ribosomes and polysomes, mitochondria and small transparent vacuoles at the apical pole. The granules of secretion in goblet cells showed no differences as compared to those of the normal ones.

Most fibroblasts in the connective tissue of the proper mucous plate had an elongated and irregular nucleus with peripheral condensation of chromatin. The cytoplasm involved a small number of vacuoles and mitochondria; the number of other organelles was scanty, i.e. these fibroblasts morphologically exhibited the signs of inactive state. The bundles of collagen fibers were determined around fibroblasts. Groups of vacuolated myofibroblasts, the nuclei of which had numerous vacuoles of various size and peripheral chromatin condensation (karyopyknosis), were also often identified. The cell cytoplasm included numerous vacuoles and homogenized hyaloplasm. This testified to the degeneration of myofibroblasts under the influence of the use of tiotropium bromide, which resulted in the decrease in collagen synthesis.

We observed subsequent positive tendencies – the inhibition of the processes of collagenogenesis (type IV collagen level decreased by 79.4% ($p < 0.05$)) with the use of tiotropium bromide medication within two moths (Table 1).

By the end of the third month, our attention was attracted by myofibroblasts in the state of degeneration, which often had a pyknotic nucleus, and densely packed, homogeneous, and sometimes vacuolated cytoplasm.

At the end of the fourth month of treatment, epithelial cells of the mucous membrane did not differ in structure as compared to the previous treatment period. Morphological picture was characterized by the presence of fibroblasts of the typical form (elongated with two poles) in its own plate. Their nuclei were predominantly elongated. The distribution of chromatin did not particularly differ. The perinuclear cistern was moderately expanded. Moderate quantity of mitochondria and cisternae of granular endoplasmic reticulum were observed on the outside of the nucleus and closely adhered to it. The cytoplasm of fibroblasts included free ribosomes and polysomes, mitochondria and cisternae of granular endoplasmic reticulum. The surface of plasmalemma of fibroblasts had small appendages, namely, these were fibroblasts which were close to fibrocytes by their structure. It is considered that the synthesis of collagen and glycosaminoglycans of amorphous connective tissue substance in these cells drastically reduced [4].

After a six-month treatment, epithelial cells closely adhered to each other. Their nuclei were round on the cross section. The nucleoli were clearly identified. Chromatin of the nuclei was predominantly euchromatin. The cytoplasm of epithelial cells included mitochondria of the usual structure, elements of granular and agranular endoplasmic reticulum, thin cytophilaments, free ribosomes and polysomes, i.e. morphological signs of edema or dystrophic changes in epithelial cells were not detected. The correlation of cells in the connective tissue of the proper mucous plate was normal and typical for this tissue. Collagen fibers were observed near the fibroblasts. They showed some signs of disintegration along various regions, namely the disarrangement from parallel thick bundles of collagen fibers to small reticulum and disintegration to granular masses. We consider the presence of the bundles of collagen fibers of different thickness and orientation to be the manifestation of collagenolysis.

The issue of collagen degeneration under the conditions of COPD treatment is of primary importance, since its development is associated with the intensification of the processes of fibrotization of the connective tissue of the proper plate of the bronchial mucosa. Different cells are known to have significant differences in collagenolytic function. In particular, macrophages and fibroblasts can perform this function in the bronchial wall in case of inflammation. Macrophages cause extracellular destruction of collagen fibers by secretion of lysosomal enzymes and their release beyond the macrophage. Thereafter, macrophage phagocyte destroy collagen and complete the process by the breakdown of collagen molecules. Therefore, the role of macrophages, under these conditions, is to purify the connective tissue of the btonchus proper mucous plate. The phagocytic function of fibroblasts differs from the macrophagal one in that it is immune-dependent due to the absence of receptors to immunoglobulins and complements on their surface. At the same time, specific collagen receptors were found on the surface of fibroblasts. This allows us to suppose that the occurrence of excess collagen in the intercellular substance is recognized by the fibroblasts and serves as a signal to its phagocytosis. Fibroblasts that contain vacuoles and numerous lysosomes, carry out collagen resorption and those in which the processes of fibroclasia predominate, become fibroclasts. We observed fibroclasts in the connective tissue before treatment, which testified their involvement in the processes of collagen resorption. Tissue basophils and lymphocytes also take part in the processes of collagenolysis, as they produce the corresponding cytokines that enhance the formation of collagenase by other cells. Under normal con-

ditions, there is a dynamic balace between biosynthesis and collagen catabolism, when these two opposite processes are performed by fibroblasts.

High activity of tissue basophils was not revealed in the process of studying the mucose membrane under the influence of basic six-month treatment with tiotropium bromide. Thus, the mechanism of collagen level reduction in the proper mucous plate could be caused not by the increased collagenolysis, but by the decrease in its synthesis. The latter was evidenced by the gradual decrease in the number of myofibroblasts in the connective tissue. Based on the detection of muscarinic receptors on the human pulmonary fibroblasts (M_1, M_2, M_3) and the conclusion about the control of collagenesis with muscarinic mechanisms [13], we can admit the idea of the influence of selective M_3 cholinolytic tiotropium bromide on myofibroblasts with the aim of their recovery. It may be confirmed by a significant decrease in type IV collagen levels in the broncho-alveolar lavage fluid of patients with stage II COPD in the dynamics of 6-month observation under the influence of tiotropium bromide treatment (Table 1). It was also accompanied by the restoration and stabilization of the local protective barrier of the mucous membrane of the bronchial tree.

3. Conclusions

Stage II COPD was associated with the damage to the bronchi with proliferation of the connective tissue in its proper plate, clear identification of the basal membrane alteration, the presence of fibroblasts, the activation of fibroblasts/myofibroblasts and mucous glands, which was accompanied by the significant increase of type IV collagen levels by 6.19 times ($p < 0.05$) in bronchoalveolar lavage fluid as compared to the control group indices. The elimination of stage II COPD exacerbation and the use of tiotropium bromide within a month was accompanied only by partial improvement of morpho-functional state in relation to both cells of bronchial epithelial lining and adjacent connective tissue of the mucosal plate, on the background of moderate decrease in type IV collagen level in bronchoalveolar lavage fluid.

The administration of tiotropium bromide for 2 months, as a part of basic therapy for stage II COPD, provided further positive dynamics of structural morphological changes in the bronchial mucosa (the restoration of the ciliary apparatus of epithelial cells, the normalization of the secretory function of goblet cells, the inactivation of fibroblasts, the initial degeneration of myofibroblasts) associated with progressive depression of neocollagenogenesis in bronchoalveolar lavage fluid.

Complete absence of morphological signs of edema or dystrophy of epithelial cells on the background of collagenolysis in the connective tissue of the proper mucous plate of the bronchi and the highest possible decrease in the number of myofibroblasts, with near-complete levels of type IV collagen normalization in the bronchoalveolar lavage fluid, were identified only within a 6-month treatment with tiotropium bromide in patients with COPD stage II.

Conflict of Interest

The authors stated no conflict of interest.

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