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Morphological changes in mucous membrane of bronchi in patients with severe chronic obstructive pulmonary disease with frequent exacerbations during long-term maintenance treatment

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Abstract
Chronic obstructive pulmonary disease (COPD) is characterized by irreversible chronic airflow limitation that usually leads to a progressive decline in lung function over time. The development of COPD is associated with both chronic airway and systemic inflammation. Roflumilast – a phosphodiesterase-4 (PDE4) inhibitor –represents a new class of drug in the management of COPD. Through selective inhibition of the PDE4 enzyme, roflumilast prevents the breakdown of cyclic AMP, which plays an important role in regulating inflammatory cell activity. Remodeling of respiratory tracts is a pathologic process observed at chronic inflammatory and obstructive diseases of respiratory tracts, and it is prescribed on the background of a COPD maintenance treatment. For 9 people with severe COPD before treatment and after 30 and 180 days treatment with roflumilast were performed biopsies of the mucous membrane of the bronchi. Verification of the diagnosis and its formulation confirmed with the orders of Ministry of Health of Ukraine № 128 from 19.03.2007. "On approval of clinical protocols of medical care in the specialty pulmonology".

Keywords: Chronic obstructive pulmonary disease, airway remodelling, factors protect mucosal, roflumilast.

1. Introduction
COPD is an inflammatory disease of the airways related mainly to smoking and characterized by airflow limitation, which manifests clinically with dyspnea, cough, and sputum production, symptoms that aggravate disease severity and disease exacerbation [1-3]. COPD is responsible for early mortality, high death rates and significant cost to health systems [15]. Globally, 10%–20% of the population older than 40 years is COPD sufferers, resulting in more than 3 million deaths each year [1-2]. COPD is projected to be the third leading cause of death by the year 2020 [1-2]. Yet, COPD is a complex, multi-component, heterogeneous disease, whose clinical, functional and radiological presentation varies greatly from patient to patient [12-13]. Though tobacco smoking is established as the primary cause of COPD, indoor air pollution from biomass and/or traditional fuels is estimated to be associated with 0.4 million deaths from acute symptoms of COPD. On average near 5 to 15% of adults in industrialized countries have COPD defined by spirometry. COPD is also an important cause of disability, and is linked to comorbid diseases, which adds to the large economic burden associated with this disease.
According to official statistics, in Ukraine, the incidence of COPD is 10 times higher than in bronchial asthma. In percentage terms, COPD (62.4%) is far ahead of other respiratory diseases for the duration of periods of disability in the structure of bronchopulmonary disease (compared to asthma - 21.4%, pneumonia - 7.6%) [1-2, 5, 8].
Consequently, the mortality from COPD increases (41.2 per 100,000 population), 3.2 times higher than the rate for pneumonia (12.8) and 34 times (1.2) in asthma [1, 2]. However, the mortality and disability are quickly growing, especially among men of working age [1, 2, 7]. It is estimated that patients with COPD suffer from one to four or more exacerbations during the year [1, 9]. COPD is associated with a variety of comorbidities and extrapulmonary symptoms. It has been suggested that the association between COPD and these other conditions is due to the inflammatory process extending systemically. Systemic inflammation is associated with, and appears to be a risk factor for, a variety of symptoms and conditions including weight loss, muscle wasting, atherosclerosis, malignancy, osteoporosis, diabetes, and anemia.
The complex of respiratory symptoms (cough, shortness of breath), characteristic of the III

Stage of COPD, signs of chronic pulmonary heart, hypoxia and hypercapnia, rheological disorders, circulatory failure, respiratory muscle fatigue, the most important thing - refractory to bronchodilator drugs. Refractory to bronchodilator means - an important sign of loss of reversible component of airflow obstruction and emphysema progression. Roflumilast and its main metabolite roflumilast N-oxide are selective PDE4 inhibitors which act to decrease immune and inflammatory cell activation. Roflumilast targets the PDE4A, 4B and 4D splicing variants with similar potency in the nanomolar range. The affinity to the PDE4C splicing variants is 5 to 10-fold lower. This mechanism of action and the selectivity also apply to roflumilast Noxide, which is the major active metabolite of roflumilast. Phosphodiesterase-4 (PDE4) inhibitors are a new class of anti-inflammatory drugs that have shown efficacy and acceptable tolerability in preclinical and clinical studies in patients with COPD [3, 14].

The first large clinical trial involved 1411 patients with moderately severe disease (mean post-bronchodilator FEV1 1.5 L, 54% predicted) with a lack of reversibility to 400 μg albuterol and compared the effect of daily treatment with roflumilast 250 μg or 500 μg for 24 weeks with placebo62 [3, 9]. The only other respiratory medications allowed during the study were short-acting β2-agonists (SABAs) and short-acting anticholinergics (SAACs). Approximately a quarter of the patients were treated with xanthines, 20% with ICSs, and 15% with long-acting β2 agonists (LABAs) prior to study entry.

At the end of the study, roflumilast-treated patients experienced greater improvements in post bronchodilator FEV1 (74 mL and 97 mL for the 250 μg and 500 μg dose, respectively) and health-related quality of life, although the difference from baseline did not reach the clinically significant threshold of −4 units. In addition, exacerbations, primarily of mild intensity, were decreased but adverse events were similar in the two groups [9].

2. Material and Methods

For 9 people with severe COPD before treatment and after 30 and 180 days treatment with roflumilast was performed fibrobronchoscopy with biopsy of bronchial mucosa of the bronchi. Verification of the diagnosis and its formulation confirmed with the orders of Ministry of Health of Ukraine № 128 from 19.03.2007. "On approval of clinical protocols of medical care in the specialty pulmonology" [4].

The material of the research was bronchoalveolar lavage and bronchial biopsy materials received on the level of bifurcation of proximal bronchi to segmental bronchi during fiberoptic bronchoscopy.

3. Results

Before treatment we have observed, that in the pseudostratified epithelium of the mucous membrane of a bronchus it was observed a thickening of the basement membrane.

The thickness of epithelium on the perimeter was not the same. The areas with low epithelium alternated with pavement epithelium and a complete absence of epithelial cells and denudation of the basement membrane. In the epithelial layer it was difficult to separate basal and intercalary cells. Goblet cells were barely distinguished. On the surface of epithelium in the preserved areas the cilium were not found and the clots of mucus were localized.

In lamina propria of mucous membrane of a bronchus it was observed a great number of mighty bundles of collagen fibers.

Cell population was presented by a great number of myofibroblasts that were defined by an irregular shape with pointed processes, basophilic cytoplasm, and a big rounded nucleus with hyperchromic chromatin. Myofibroblasts are inclined to torsion in deep areas of lamina propria. Between them there are a lot of macrophages (often with granules in cytoplasm) and lymphocytes. There are few fibroblasts. Inflammatory polymorphic cellular infiltration was not identical in the visual field. The lumen of blood vessels is dilated and often contained blood corpuscles, their wall is thinned. In the vessels of microcirculation bed there are stases. Often the cellular infiltration dominates in perivascular areas. It should be especially noted that in pathogenesis of COPD myofibroblasts have a significant meaning – they express α-actin of smooth muscles and components of territorial matrix [16]. These cells represent clinical steps in the development of irreversible pulmonary fibrosis [17]. They are elementary “effector” cells in the tissue remodeling and fibrosis [18]. This cytokine is secreted by macrophages [19]. Molecular mechanism of pulmonary fibrosis through myofibroblastic differentiation is complex and is performed by TGF-β/Smad [20]. There is information that dis-regulation of myofibroblasts is connected with pro-oxidant shifts of oxidation-reduction homeostasis [21]. Myofibroblasts create microenvironment - territorial matrix that plays an important role in physiological conditions and in pathological process of fibrosis development [22]. Content of territorial matrix plays an important role in defining a structure of respiratory tracts wall at COPD. Myofibroblasts are characterized by the presence of a well-developed contractile apparatus and formation of active fibers. These are mechanically active cells that remodel territorial matrix at fibrous damage [23]. In one case there was discovered a proliferation of epithelium with formation of microfolds of superficial epithelium with subordinate lamina propria.

The electronic microscopic picture was characterized by the signs of chronic inflammatory process. In epithelial cells that were found in the section, a picture of necrosis was often observed. Plasmolemma of an epithelial cell did not contour and its content conjugated with amorphous substance of the subordinate connective tissue. In cytoplasm there are numerous transparent vacuoles, remains of organelles, amorphous structures.

On the transverse section it was observed dilatation and weakening of intercellular contacts with dilatation of intercellular spaces. In one viewing field it was observed a section of cells in their nuclear and anucleate parts. Separate nuclei contained nucleoli. Cytoplasm is homogeneous, organelles are hard to identify. Goblet cell is in the necrobiotic condition with kariopicnoti nucleus. In its enclosing epithelial cells are deeply damaged.

In fibroblasts of the subordinate connective tissue the nucleus is big, chromatin is moderately and evenly condensed throughout the whole nucleus. In cytoplasm there are identified mitochondria, endoplasmic reticulum, and beyond the cells–the products of fibrillogenesis–pro-and microfibrils, elements of amorphous substance. Such fibroblasts occur not often.

More widespread in the connective tissue are myofibroblasts. These cells are localized in the shape of groups of a few cells. The cells bodies are of lengthened fusiform form, the nuclei are lengthened with peripheral condensation of chromatin. In cytoplasm there are identified numerous microfilaments, and separate mitochondria. In the environment of the cells it is observed thick collagen fiber bundles oriented in different directions.

In the inflammatory infiltration there are quite a lot of macrophages. In the nucleus there is a peripheral condensation of chromatin. It contains two fragments of a nucleus. Plasmolemma does not have contours clarity. Numerous twisted processes are coming from the body and that is why there are a lot of their fragments near the cells. In cytoplasm there are big phagosomes one of which is a giant one. Their content is fragments of membrane organelles and thickened osmiophil material. In other areas the cytoplasm is homogeneous, single organelles are barely seen (cisterns of endoplasmic reticulum, mitochondria with shortened cristae). Around the macrophage there is the basic (amorphous) substance of the soft connective tissue in the state of edema.

What attracts attention is that among the cells of inflammatory infiltration in the connective tissue there are few plasmocytes. Plasmocytes show considerable destructive changes. In a nucleus it can be often observed an edema with eccentric displacement of chromatin. In cytoplasm degranulation of rough endoplasmic reticulum, single mitochondria. Primary lysosomes are identified seldom, more often secondary – phagosomes and tertiary - residual corpuscles. Plasmolemma is contoured not clearly.

In many blood capillaries in dilated lumen there is a stasis of blood corpuscles mostly erythrocytes. The basement membrane is thickened and damaged in some places. In the nucleus of endotheliocyte there is a peripheral condensation of chromatin. In the peripheral area of the endotheliocytes cytoplasm is difficult to identify the organelles, but mitochondria and phagosomes are distinguished.

Around the capillaries there are considerable layers of collagen fibers. In the surrounding of the capillary there are observed myofibroblasts and their processes, bundles of collagen fibers that have different directions and maturity.

Sometimes the thickening of the basement membrane and surrounding of the capillary by collagen fibers is considerably expressive. The capillaries have fissural lumen and are “embedded” into the bundles of connective tissue that have circular localization. Endotheliocyte of the capillary wall has pyknotic nucleus and thinned electronically thickened cytoplasm. In lamina propria of mucous membrane there is an evident pulmonary fibrosis, there are myofibroblasts and mighty bundles of collagen fibers. Myofibroblasts in the wall in allergic bronchial irritation have been described by W.R. Roche *et al* [15].

In some capillaries the lumen is free from blood corpuscle but endotheliocytes are in the state of destruction (necrosis). Their cytoplasm is electronically thickened and has vacuoles. The basement membrane is identified only in separate areas. The capillary is surrounded by collagen fibers situated as tight laminae. From the outside of them there are collagen fibers on the transverse section, i.e. collagen fibrous structures have two layers – circular and longitudinal. Near the nucleus-containing area of endotheliocyte the collagen fibers have reticular form and are less dense and thick.

So, at the end of 30-days treatment with roflumilast on the background of COPD mainance therapy, we have identified some good results in comparison with the results before treatment. The main signs of improvement in the morpho-functional state of epithelial cells can be considered: moderate hypertrophy of the nucleus, cytoplasm vacuolization reduce, the appearance of cilia on the apical plasmolemma. In the lamina propria's cells the destruction and dystrophic processes are decreased. In the fibroblasts structure components of endoplasmic reticulum are identified, they are synthesizing of collagen fibers. The number of myofibroblasts are reduced, some of them showed signs of degradation. In some capillaries the changes that were found before treatment are remained - endothelial dystrophy, thickening of their basement membrane.

A six-month therapy with the use of roflumilast gave its positive results. In the pathohistological picture of a bronchus mucous membrane there were outlined positive changes in the epithelial tissue as well as in the connective tissue of lamina propria of mucous membrane.

Microscopically there was observed the renewal of the epithelium on rather long areas of a bronchus surface. On these areas the epithelium normalized and in it there could be distinguished basal, ciliated, high inserted cells covered with cilia. That is, in some places the epithelium acquired the form of a renewed pseudostratified ciliated epithelium. Ciliated cells had mostly cubic form and were intimately adjacent to the basement membrane. The latter had different thickness. Nuclei of epithelial cells were coloring actively basophilic; they were characterized by euchromatin. Anyway, the goblet cells seemed to be absent.

In the connective tissue of lamina propria there was also outlined positive progress. Polymorphocellular infiltration decreased and in some places disappeared. Bundles of collagen fibers are thin; between them there are cells of fibroblastic and macrophage rows.

Fibroblasts looked like elongated cells with elongated nuclei. Micro-fibroblasts were localized one by one or by small groups. They had little processes with basophilic cytoplasm. Sometimes there occurred mast cells with granules in small number. Considerable attention was paid to mast cells by Gh. Nini *et al*. [20, 12]. Authors established that mast cells are present in lamina propria of mucous membrane of a bronchus before and after the treatment of COPD, but before the treatment 90% of mast cells displayed the signs of degranulation. Mast cells in the state of degranulation after the treatment were observed in the center of inflammatory nidus of infiltration and this explains their participation in the cellular immune response. After the treatment the number of mast cells decreased. Among them occurred granulated and degranulated cells. Their granules were immature. Macrophages were mostly related mature macrophages and rarely to monocitoide ones. Blood vessels of microhemocircular bed had an ordinary picture of the build of their wall without blood corpuscles in the lumen with all normalk definitions of the wall of microhemovessels.

In the electronically microscopic picture of a bronchus wall there happened a lot of changes. First of all, this concerns the cells of superficial epithelium. Epithelial cells in a larger amount than in the previous term and before the treatment had cilia on their apical surface. The cilia were not of great thickness but they had all signs of a normal build. In their basis there were observed basal corpuscles that transferred to free protrusions with an axoneme inside and covered with plasmolemma. Cytoplasm of the ciliated epithelial cell contains mitochondria. The mitochondria have different build – from small dark to larger ones with determined cristae and somewhere cleared matrix. Vacuoles are practically absent.

On the transverse section through basal pole of epithelial cells there were observed a few cells with centrally situated nucleus. The nuclei had invaginations, which confirms the active state of the cells. Chromatin of the cells is moderately condensed. Karyolemma is contoured clearly. In cytoplasm there are mitochondria, cisterns of granular and vesicles of agranular

endoplasmic reticulum, small vacuoles, canalicular apparatus, single lysosomes and (in some) phagosomes. Intercellular unions were getting stronger and the cells were situated close one to another connected by simple unions and with invagination of the "lock" type. Among epithelial cells there is identified a cell with rounded granules with osmiophil content - endocrine cell that belongs to dissociated endocrine system. In it there appear mitochondria, cisterns of granular endoplasmic reticulum, Golgi complex with vesicular component.

Fibroblasts of normal build often are determined in connective tissue of lamina propria of mucous membrane. The form of these cells is elongated. A nucleus is rounded with not deep invagination. In a nucleus equally is represented euchromatin and moderately condensed heterochromatin. Karyolemma is contoured clearly. In cytoplasm there are mitochondria in which there are clearly identified cristae and matrix. Cisterns of granular endoplasmic reticulum are localized near mitochondria. Granular endoplasmic reticulum is represented by numeral flat cisterns. Around the fibroblast there is the amorphous substance in which elements of newly created, young collagen fibers are distinguished.

4. Conclusions

1. In severe COPD damaged bronchial mucosa with a significant growth of connective tissue in the lamina propria of clearly identifiable basement membrane alteration, the presence of fibroblasts, activation of fibroblasts/myofibroblasts and mucous glands are determine.
2. After a 180-day treatment by roflumilast on the background of a basic therapy of patients with COPD of III stage there was detected the most positive dymanics of pathohistological picture of a bronchus mucous membrane in the epithelial tissue as well as in the connective tissue of lamina propria of mucous membrane, in comparison with a 30-day treatment.

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