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EFFECTS OF U-74389G IN EXPERIMENTAL MODELS OF PULMONARY TOXICITY AND COLITIS IN RATS

ABSTRACT
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CONTENTS

ABBREVIATIONS

AM	Amiodarone
AIBT	Amiodarone -induced pulmonary toxicity
BALT	Bronchoalveolar lavage fluid
BC	Crohn's disease
VChZ	Inflammatory bowel diseases
IBB	Interstitial lung disease
CT	Computed tomography
NMC	Unsaturated fatty acids
PNMK	Polyunsaturated fatty acids
UK	Ulcerative colitis
5-ASA	5-aminosalicylic acid
AEC	Alveolar epithelial cells
AGOP	Granulomatous and organizing pneumonia
AFOP	Acute fibrinous and organizing pneumonia
AIPT	Amiodarone - induced pulmonary toxicity
AM s	Alveolar macrophages
AMP	Antimicrobial peptides
AMPK	Adenosine monophosphate activated protein kinase
ARDS	Acute respiratory distress syndrome
BALF	Bronchoalveolar lavage fluid
Breg	In regulatory cells
CAT	Catalase
CL	Cardiolipin
CTD	Connective tissue disease
DAD	Diffuse alveolar damage
DAH	Diffuse alveolar hemorrhage
DC	Dendritic cells

DEA	Desethylamiodarone
DI - ILD	Drug-induced interstitial lung disease
DIP	Desquamative interstitial pneumonia
DLCO	Diffusion capacity of the lungs
DMARDs	Disease-modifying antirheumatic drugs
ELISA	Enzyme-linked immunosorbent assay
ENS	Brain nervous system
FFA	Free fatty acids
FVC	Forced vital capacity
GCS	Glucocorticosteroids
GSH	Reduced glutathione
GPX	Glutathione peroxidase
HLA	Human leukocyte antigen
HP	Hypersensitivity pneumonitis
IBD	Inflammatory bowel diseases
ICI	Immune checkpoint inhibitors (immune checkpoint inhibitors)
IL	Interleukin
ILC	Innate lymphoid cells
ILD	Interstitial lung disease
IL- 1α	Interleukin -1 alpha (interleukin-1 alpha)
IL- 1β	Interleukin -1 beta (interleukin-1 beta)
IMID	Immune- mediated inflammatory changes
iNOS	Inducible nitric oxide synthase
ip .	Intraperitoneal
IPF	Idiopathic lung fibrosis
it	Intratracheal
LH	Lung homogenate
LP	Lipid peroxidation
MDA	Malonov dialdehyde
MDP	Muramil dipeptide
MMR	Matrix metalloproteinases
MPO	Myeloperoxidase

MSC	Mesenchymal stromal cells
NF - κB	Nuclear transcription factor- κB
NOD	Nucleotide-binding oligomerization domain
NSIP	Nonspecific interstitial pneumonia
NLR	NOD-like receptors
OP	Organizing pneumonia
PAMPs	Pathogen-associated molecular patterns
Pr	Prednisolone
PF - ILD	Progressively fibrosing interstitial lung disease
PMN	Polymorphonuclear cells
PPF	Progressive pulmonary fibrosis
PPFE	Pleuroperienchyma fibroelastosis
PRRs	Pattern recognition receptors
RA	Rheumatoid arthritis
RNS	Reactive nitrogen species (reactive nitrogen species)
ROS	Reactive oxygen species (reactive oxygen species)
SOD	Superoxide dismutase
SS	Sulfasalazine
TBARS	Reactive substances of thiobarbituric acid
TBI	Traumatic brain injury
TGF-β	Transforming growth factor β
TLR	Toll -like receptors
TNF - α	Tumor necrosis factor alpha (tumor necrosis factor alpha)
TZ	Tirilazad mesylate (U -74006 F)
WGS	Whole genome sequencing

INTRODUCTION

The oxidative stress and chronic tissue inflammation represent two of the most fundamental and interconnected pathophysiological mechanisms involved in the development and progression of a wide range of diseases. The disturbed balance between the production of reactive oxygen species and the oxygen types and capacity of antioxidant protective systems of the body leads to damage to lipids, proteins and nucleic acids heartburn, with subsequent activation of pro-inflammatory signal cascades.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS): superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (• OH) and peroxynitrite ($ONOO^-$) are physiologically generated during cellular metabolism , but under pathological conditions their overproduction exceeds antioxidant capacity , caused by enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), as well as non-enzymatic molecules as reduced glutathione (GSH) and vitamins E and C. A consequence of oxidative stress is the peroxidation of membrane phospholipids , process whose The end product malondialdehyde (MDA) is routinely used as reliable biochemical marker. In parallel with oxidative damage, activation of the nuclear transcription factor NF - κB trigger cascade production of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF - α), interleukin-1β (IL - 1β), and interleukin-6 (IL -6) and inducible nitric oxide synthase (iNOS). These mediators support chronic inflammatory reaction and contribute to tissue destruction in a number of clinically significant nosologies. The interaction between oxidative stress and inflammation forms a pathologically reinforcing vicious circle: Reactive oxygen and nitrogen types activate NF - κB, and pro-inflammatory cytokines in turn stimulate additionally generation of reactive radicals.

Amiodarone is iodinated benzofuran derivative with wide application in cardiology practice due to its effective control over life-threatening ventricular and supraventricular arrhythmias. Amiodarone-induced pulmonary toxicity (AIBT) develops in 5 - 10% of patients on long-term therapy. Pathomorphologically, AIBT manifests itself as organizing pneumonia, diffuse alveolar disability or chronic interstitial pneumonia with progressive Pulmonary fibrosis. At the root of the damage stands the direct cytotoxicity of amiodarone and its main active metabolite desethylamiodarone (DEA), whose plasma concentration during prolonged use exceeds that of the output compound. At the cellular level, amiodarone disrupts mitochondrial respiration, inhibits lysosomal phospholipases and induces oxidative stress in alveolar macrophages and type II pneumocytes — key effector cells in lung defense. Increased levels of MDA , decreased SOD and GPX activity , and characteristic histological changes (“foamy” macrophages , fibrosing alveolitis) have been verified in multiple in in vivo studies, including in the rat model by intratracheal or systemic administration of amiodarone . As effective specific treatment of AIBT remains unsatisfactory, discontinuation of amiodarone and corticosteroids are the main therapeutic options. The search for new protective molecules represents a current and clinically relevant research question.

Inflammatory bowel diseases (IBD) are chronic, relapsing immune-mediated nosologies with increasing global incidence . Despite advances in biological treatment, a significant proportion of patients do not achieve durable remission, justifying the continued interest in new therapeutic molecules. Intrarectal administration of 2,4-dinitrobenzenesulfonic acid (DNBS) in rats is an established and widely validated animal model that resembles the pathomorphological and pathochemical features of Crohn 's disease . DNBS -induced colitis is characterized by transmural neutrophil and macrophage infiltration, crypt abscesses, mucosal ulcerations and significant elevation of proinflammatory biomarkers, including myeloperoxidase (MPO) - a specific enzymatic marker for neutrophil activation, TNF - α , IL - 1β and IL -6. Simultaneously with the intense inflammatory response, pronounced oxidative stress in the intestinal wall with increased MDA, reduced levels of SOD, CAT and GSH was documented. The severity of colitis was assessed by macroscopic scoring , histological analysis using standardized scales (e.g. Ameho scale) and quantitative determination of the above-mentioned

biochemical markers. The model is sensitive for registering the therapeutic effect of antioxidant and anti-inflammatory agents, making it a suitable tool for studying the potential of U - 74389 G.

U -74389 G is a synthetic 21-aminosteroid from the group of " lazaroids ", a class of compounds developed by Upjohn in the late 1980s to reduce oxidative damage in ischemic -reperfusion injuries and various forms of acute organ damage. The name " lazaroid " is a metaphor referring to the "revival" of cells threatened by oxidative death. Structurally, U -74389 G possesses a steroid skeletal base devoid of glucocorticoid activity, but containing an amine side chain at position C-21, which provides additional electron donor capacity. The main molecular mechanism of action is the inhibition of iron-dependent and iron-independent peroxidation of membrane lipids by scavenging peroxyl and hydroxyl radicals. Unlike methylprednisolone , U -74389 G does not bind to glucocorticoid receptors and does not exhibit immunosuppressive or metabolic side effects typical of classical corticosteroids. Preclinical studies published to date demonstrate that U -74389 G reduces MDA levels , normalizes antioxidant enzyme activity , and suppresses proinflammatory cytokines. cytokines in a variety of pathological models; ischemia- reperfusion injury of the liver, kidneys and brain, sepsis, acute pancreatitis and X-ray radiation. Data on its effect on pulmonary toxicity with amiodarone and DNBS -induced colitis remain scarce, which justifies the present study. The present study was aimed at testing the hypothesis that U -74389 G can exert a significant protective effect in two pathologically different, but related to oxidative stress and chronic inflammation models: amiodarone -induced pulmonary toxicity and DNBS -induced colitis in rats. The choice of these two models was dictated by their clinical relevance, their good characterization in the literature and the fact that both combine intense oxidative stress with a pro-inflammatory response, precisely the spectrum of action for which U -74389 G is a theoretically justified protector. The results of the present work would contribute to clarifying the therapeutic potential of U -74389 G in diseases in the pathogenesis of which oxidative stress and inflammation play a central role.

PURPOSE, OBJECTIVES, MATERIALS AND METHODOLOGICAL APPROACHES

1. **Objective:** To investigate the effects of the lazaroid U-74389G in experimental models of pulmonary toxicity and inflammatory bowel disease.

2. **Tasks**

2.1. Experimental model of amiodarone -induced pulmonary toxicity (AIPT)

2.1. 1. To select an appropriate model of amiodarone -induced pulmonary toxicity.
2.1.2. To investigate the effects of U-74389G on markers of inflammation and cytotoxicity in BALT (broncho -alveolar lavage fluid) in amiodarone -induced pulmonary toxicity
2.1.3. To investigate the effects of U-74389G on basic units of antioxidant defense system, lipid markers peroxidation and pulmonary fibrosis in pulmonary homogenate (LH)
2.1.4. To investigate the effects of U-74389G by histopathological methods

2.2. Experimental model of IBD (inflammatory bowel disease)

2.2.1. Selection of an appropriate experimental model of IBD (inflammatory bowel disease)
2.2.2. To investigate the effects of U-74389G on integral parameters and macroscopic changes in 2,4-Dinitrobenzenesulfonic acid hydrate (DNBS) – induced colitis
2.2.3. To investigate the effects of U-74389G on markers of inflammation and lipid peroxidation
2.2.4. To investigate the effects of U-74389G using histopathological methods

3. Materials and methodological approaches:

The experiments were conducted in the Laboratory of Experimental Physiology and Pharmacology of the Department of Pharmacology and Toxicology at MU-Pleven. The study was conducted in accordance with international standards for care and use on experienced animals (European Directive 2010/63/EU), as well as with the national legislation and policies and has been approved from The Bulgarian agency by safety on Food – Permit for the use of animals in experiments No. 120/18.06.2015.

3.1. Experimental animals

A total of 206 white male Wistar rats weighing 200-250 grams (age 8-10 weeks), supplied by the Experimental Breeding Base for Experimental Animals Slivnitsa, were used in the experimental models. The animals were raised in the vivarium of the Medical University – Pleven for one month at a temperature of 22.0±2.0°C, a 12:12 hour light-dark cycle, air humidity of 50 ± 10% and free access to pelleted food and water ad ad libitum .

3.2. Methodological approaches to studying the effect of the lazaroid U-74389G in an experimental model of a miodarone-induced pulmonary toxicity (AIPT)

Two AIBT models were tested. In the first model (M1) AM was applied intratracheally , once at a dose of 6.25 mg / kg as water solution with a concentration of 7.8 mg / ml . In the second model (M2) AM was introduced intratracheally, twice on days 0 and 2, at a dose of 6.25 mg / kg as water solution with a concentration of 3.125 mg / ml. Based on the results obtained results, described in detail in the "Results" section for appropriate model the one with double application of AM was chosen.

- **Experimental groups**

Animals were randomly divided into five groups of 6 animals in each:

Group 1 (control group) received two intratracheal (it) instillations of **sterile distilled water** on days 0 and 2 [Taylor MD et al., 2000].

Group 2 received two i.t. instillations of **amiodarone (AM)** (6.25 mg / kg, in 3.125 mg / mL aquatic solution) on days 0 and 2.

Group 3 was treated with **AM** it on days 0 and 2 and **U-74389G** intraperitoneally (i.p.) three times – before and twice after AM administration with 5 mg / kg body weight [Dancheva, 2014].

Group 4 was treated with **U-74389G** at the same dose and route.

Group 5 was treated with **AM** it on days 0 and 2 and **prednisolone (Pr**) 10 mg / kg body weight intraperitoneally (ip) three times – before and twice after AM. In this group, the effects on markers of inflammation and pulmonary fibrosis were studied.

For intratracheal administration of the drugs, AM is dissolved in distilled water at 60°C and allowed to cool to room temperature before intratracheal infusion, as described above [Card et al ., 1999]. The first infusion was performed on a day marked as day 0; the second application was done on day 2.

U-74389G is dissolved in CS-4 (aqueous 20 mM solution citric acid monohydrate, 3.2 mM sodium citrate dihydrate, 77 mM NaCl, pH=3) with a concentration of 2 mg / mL.

Prednisolone is dissolved in DMSO (dimethyl sulfoxide), then diluted in PBS (phosphate buffered saline). buffered saline) and a dose of 10 mg / kg body weight is administered intraperitoneally (i.p.).

After treatment the animals are coming back to their respective diets and water ad libitum for the remainder of the study period. In all applications, used individual sterile needles.

Under thiopental anesthesia (50 mg / kg), the animals are killed by exsanguination on days 3, 7, 14 and 28.

- **Integral characteristics**

Measured are bodily mass in grams; weight lung coefficient (lung mass in mg per 100 grams bodily table) on days 3, 7 and 28.

- Obtaining bronchoalveolar **lavage lavage liquid (BALT, BALF)**. To obtain bronchoalveolar lavage fluid lavage liquid, six rats from each group were euthanized on days 3, 7 and 28 under thiopental

anesthesia (50 mg / kg) by exsanguination through incision of the renal vein. The thorax was opened and the lungs were perfused in situation through the right heart chamber with saline (30 mL). The volume of the recovered liquid varies from 80 to 90% of the entered fluid. Bronchoalveolar lavage was obtained as in the trachea of the severed left white The fraction is placed plastic cannula. With a syringe in the lung are scored successively 5 ml of saline solution, divided into 3 portions (2, 2, 1 ml). Each portion of solution is retained for about 1 minute in the organ before being aspirated back. The aspirated amount of liquid is placed in plastic tubes stored in a container with ice cubes. The bronchoalveolar lavage The liquid was centrifuged at 300xg for 10 minutes. The supernatant was decanted and used for biochemical research. To 1 ml of 0.15 M sodium chloride solution was added to the sediment to enumerate the total number of cells in a Burker chamber. For differential BALT cell counts are 500 microliters were taken from the remaining part of the sediment.

- **Methods for studying bronchoalveolar lavage liquid:**

Total number of cells in BALT (x105/ml) - by counting in the Burker chamber;
Differential cell counting in BALT (x105 /ml) – by counting in the Burker chamber Wright -Giemsa staining;
Lactate dehydrogenase enzyme activity in IU/ml – by the method of *Bergmeyer* (1974) [Bergmeyer et al.,1974];
Enzyme activity alkaline phosphatase in IU/ml – by the Bergmeyer method (1974) [Bergmeyer et al.,1974];
Enzyme activity acid phosphatase in IU/ml – by the method of Bergmeyer (1974) [Bergmeyer et al.,1974];
Total protein content in mg/ml – according to the Bradford (1976) method [Bradford,1976].
The enzyme activity is expressed in IU/ml (one unit is defined as as 1 nmol of substrate converted in 1 minute).
Cytological BALT research. An aliquot of BALF is used for a total cell count of x105 / ml. Then The cells were removed by centrifugation at 300xg for 10 minutes. The cell The pellet is resuspended in 1 ml of saline. The cytological preparation is stained using the Wright-Giemsa method and is differential cell counting.
Alveolar macrophages were analyzed using a modification of the Danos and Keebler procedure [Saltini C et al., 1984]. The cells are collected on nitrocellulose 25 mm diameter filters with 5 μm pores (SWP-025-00; Millipore Corp.) and stained with hematoxylin-eosin.
Biochemical BALF assays. After centrifugation of BALF, the supernatant is used to measure enzyme activity such as lactate dehydrogenase (LDH), acid phosphatase (AcPh), alkaline phosphatase (AlPh). The activities are measured by the Bergmeyer method [Bergmeyer HU et al., 1974], and the general protein content was measured by the Lowry method [Lowry OH et al., 1951]. The measured Enzyme activities are presented in U/L, and total protein in mg / mL BALF.

- **Getting a lung homogenate.**

homogenate was prepared from the right lung of rats from each group , and lungs from 3-4 were used for histopathological studies. The chest was opened and the lungs were perfused through the right ventricle with 30 ml of saline (3 x 10 ml). The right white liver near the hilus and was cut for weight measurement and homogenization. The right white The liver was removed and homogenized in ice-cold 0.25 M sucrose in a buffer containing 50 mM Tris-HCl buffer, pH = 7.4 (in a ratio of 1:10). The pulmonary The tissue was cut into small pieces, then homogenized in a homogenizer with a Teflon pestle in the indicated solution. Homogenization was performed at 2000 rpm for 18 tractions. The resulting homogenate was centrifuged in a refrigerator centrifuge K-24 at 4°C at 9000xg for 30 minutes and the supernatant was stored on ice.

- **Methods for examining pulmonary homogenate**

Superoxide dismutase enzyme activity in IU/g tissue – according to the method of Maral (1977) [Maral and al.,1977]. One unit of superoxide dismutase enzyme activity is defined as 50% inhibition of the reduction of tetranitro blue tetrazolium to formazan.
Catalase enzyme activity in mkat /g tissue – by the method of Koroljuk (1988) [Koroljuk and al ., 1988]. One unit of catalase activity is expressed as millikatals /g;
Glutathione enzyme activity peroxidase in ME/g tissue – by the method of Bershneider , modified by Pereslegina (1989) [Pereslegina , 1989]. One unit of activity of the enzymes glutathione peroxidase is defined as nanomole of product formed per minute;

Malon content Dialdehyde (MDA) in nmol /g tissue – for measurement his pulmonary tissue was homogenized with 1.15% potassium chloride solution in a ratio of 1:10 – according to the Ohkawa method and al . [Ohkawa H and al ., 1979].
Plasma concentrations of hydroperoxide in nmol /ml (ROOH) are measured by the Yagy method [Yagi K and al., 1987].
Total *protein* content in *mg* /ml – by the method of Lowry (1951) [Lowry and al., 1974];
L - hydroxyproline content in microg /g tissue – by the method of *Bergman* (1963) [Bergman et Loxley, 1963];
With collagen content in micrograms /gram of tissue – by the Komsa method and al . 1996).

- **Histopathological research**

Researched are white materials liver, fixed in 10% formaldehyde solution for 24 hours and embedded in paraffin blocks. The fabrics materials are taken at the relevant temporal intervals (days) after treatment with a chemical factor. The tissues Sections 3 micrometers thick with a stained with hematoxylin - eosin (HE) for histological research.

- **Chemicals.**

Amiodarone hydrochloride (C25H29I2NO3.HCl; Cat. No. A8423., Sigma-Aldrich), U-74389G (C37H50N6O2-C4H4O4; Cat. No. U5882, Sigma - Aldrich), all others chemicals and reagents are purchased from Sigma-Aldrich Company.

- **Statistical analysis.**

The data are presented in tables and graphs. Kolmogorov - Smirnov test and Shapiro - Wilk test are used to check the normality of the distributions of the studied variables. The amplitudes of the data are compared by parametric hypothesis testing tests for two independent excerpts – Student t -test, and one-factor ANOVA post yes LSD test (at least significant difference procedure) is used to compare amplitudes between more than two sets of data with normal distributions. The data are presented as medium values and standard error (m ± SEM); a statistically significant difference between groups is considered at p <0,05. The statistical processing of the data was carried out using a computer program STATGRAPHICS Plus 4.1 for Windows, SPSS 14 (Statistical Package for the Social Sciences) and Excel (Office 2007).

3.3. Methodological approaches in studying the effect of U-74389G in an experimental model of colitis

The experiments were approved by the Bulgarian Food Safety Agency and were conducted in accordance with the Animal Welfare Regulations. 3 models of experimental colitis were tested by applying the ulcerogenic agent **2,4-Dinitrobenzenesulfonic acid hydrate (DNBS)** : DNBS 10 – with experimental colitis induced by 10 mg DNBS dissolved in 0.25 ml of 50% alcohol, introduced rectally, with a probe 8 cm into the colon ; DNBS 20 and DNBS 30, with a DNBS dose of 20 and 30 mg , respectively . Based on the results obtained, described in detail in the "Results" section, the one with DNBS at a dose of 30 mg was chosen as a suitable model , inducing reproducible colitis in all experimental animals and is a suitable model for studying the effect of potential drugs, for example U-74389G.

- **Experimental groups**

The experienced animals are divided into 4 groups of 8 animals randomly:
Group 1. Control group - rats are treated intrarectally with **saline** (n=8)**.**
Group 2. DNBS group (n=8) The dose of 30 mg **DNBS**, dissolved in 0.25 ml of 50% ethanol, is inserted into the colon intestine one-time with rubber catheter according to the procedure described by Morris et al. [Morris GP et al., 1989] and Wallace and al. [Wallace JL et al., 1995].
Group 3. U-74389G group (n=8) – the lazaroid **U-74389G** is injected intraperitoneally at a dose of 5 mg / kg for 6 days, starting 1 day before **DNBS -** induced colitis. U-74389G was dissolved in a solution of CS-4 (aqueous 20 mM solution citric acid monohydrate, 3.2 mM sodium citrate dihydrate, 77 mM NaCl, pH=3) at a concentration of 2 mg / mL.
Group 4. Sulfasalazine (SS) group (n=8) – rats with experimental colitis were treated with Sulfasalazine, orally, at a dose of 300 mg / kg, once daily for 6 days, starting 1 day before the induction of **DNBS** colitis.

Sulfasalazine dissolves in phosphate buffered saline, pH 7.2 at a concentration of 0.2 mg / mL [Dik B, Sonmez G and al., 2018].
Published are results from U -74389G alone, which were not statistically different from controls, due to which in the experiment no group with only U-74389G [Stavreva GT et al., 2008].
On day 6, animals were sacrificed with a combination of ketamine 90 mg / kg and xylazine 10 mg / kg , and colonic segments were then collected. The severity of intestinal inflammation was assessed macroscopically and histologically, following the criteria reported by Antonioli [Antonioli L at al ., 2007].

- **Assessment of the severity of experimental colitis. Integral characteristics**

A number of parameters were measured from day 0 to day 6: **body weight, food and water intake, stool consistency** (0=normal, 1=loose stools, 3=diarrhea), **presence of blood in stool** (0=negative, 1=positive, 3= profuse bleeding).

- **Macroscopic evaluation method.**

The criteria for macroscopic assessment of colonic damage were as follows: **presence of adhesions between the colon and other organs** (0 – none, 1 – small and 2 – large adhesions); **presence of ulcer** (0 – none, 1 – hyperemia , 2 – ulcer without inflammatory reaction, 3 – wound with inflammation in one place, 4 – ulcers and inflammation in two places, 5 – large lesions extending 1-2 cm along the length of the colon, 6 – large lesions extending more than 2 cm) and the score was increased by 1 for each millimeter of colonic wall thickness. **The length of the colon was measured** . All parameters were assessed independently by two people.
Length of the colon intestine. After euthanasia of the animals, the colon is removed and cleaned of fecal matter. It is then measured weight and length of the organ. In DNBS- induced model of colitis, no significant collagen deposition was observed in the tissues, but rather the observed shortening is due to contraction of the smooth muscle layer in the colon wall intestine. Rectal instillation of DNBS causes inflammation with subsequent increase in inflammatory mediators with a contracting effect on the smooth muscles of the intestinal wall.
Weight of the colon and the distal 10 cm of the organ. Treatment with DNBS causes weight gain in the obese intestine, correlating with the severity of inflammation in the affected The weight gain is mostly due to tissue Edema. Exudation of fluid into the interstitial space during inflammation is due to increased vascular permeability associated with the expansion of the spaces between the endothelial cells of the vascular wall. This is caused by the inflammatory mediators, inducing biochemical changes in endothelial cells.
Thickening of the intestinal wall. The thickening of the intestinal wall is proportional to the degree of inflammation and is due to the tissue edema, tissue infiltration with inflammatory cells (lymphocytes, monocytes, macrophages, neutrophils granulocytes), as well as the proliferation of mesenchymal cells with deposition of interstitial matrix.
Area of damage. DNBS causes damage visible to the naked eye, expressed in necrosis and ulceration of the epithelial layer, and in more severe cases, of the underlying layers of the intestinal wall. The area of epithelial damage is direct relationship with the severity of inflammation. DNBS is strongly reactive hapten and because this binds covalently to tissue proteins and them modifies. The cells of the monocyte-macrophage system recognize and phagocytize haptenized molecules and them presented to CD4 positive cells. This leads to the induction of cell-mediated delayed-type hypersensitivity immune reaction with the main involvement of Th1 and Th17 lymphocytes.
Adhesions to neighboring organs. In inflammatory processes in the abdomen cavity in the recovery process are often formed adhesions – adhesions from connective tissue fabric to neighboring covered with peritoneum authorities, as well as to The formation of adhesions is caused by the action of the secretions resulting from inflammation cytokines, growth factors and molecules for cell adhesion, which lead to proliferation of fibroblasts, mesothelial cells, as well as neoangiogenesis. The degree of expression of adhesions to neighboring organs is in direct depending on the intensity of the inflammatory reaction.

- **Immunological studies.**

Levels of pro- and anti-inflammatory cytokines TNF - α , IL - 1β , IL -6 and IL -10 were assayed in the culture medium after a 24-hour culture period. Cytokine protein concentrations were determined by enzyme -linked immunosorbent assay (ELISA). The ELISA was performed according to the manufacturer's instructions. Each sample was tested in duplicate. The amount of IL-1, IL-6 and tumor necrosis factor-alpha (TNF-α) in plasma was measured in picogram (pg)/ ml using ELISA. The kits were purchased from R&D Systems, UK. Blood samples were centrifuged at 2000xg for 20 minutes and plasma was separated. It was stored at ≤ 20°C. All reagents were stored at room temperature. 1 ml of distilled water was added to the mouse IL-6 kit control. To prepare a sufficient amount of conjugate for one plate (96 wells) 0.5 ml of conjugate concentrate to 11 ml conjugate diluent. 625 ml of wash buffer was prepared (25 ml of original wash buffer + 600 ml of distilled water). A substrate solution was prepared by mixing color reagents A and B in equal volumes for 15 minutes. 100 µl of the resulting mixture was added to each well. Mouse IL-6 standard was mixed with 5 ml calibrator solvent RD5T to obtain a stock solution of 500 pg / ml . 200 µ l of calibrator solvent RD5T were pipetted into each tube . The stock solution was used to prepare a dilution series (decreasing dilution) and to construct a standard curve. Mouse IL-6 standard was used as a high standard (500 pg / ml). Calibrator solvent RD5T was used as a zero standard (0 pg / ml). 50 µ l of the diluted solution was added to the center of each well. 50 µ l of standard, control or sample were added. The plate was covered and incubated for 2 hours at room temperature. After casting, each well was washed five times with 300 µ l of wash buffer. 100 µ l of conjugate in each well. The plate was re-covered and incubated for 2 hours at room temperature, with each well washed five times. 100 µl of conjugate was added substrate solution in the wells, incubated for 30 minutes at room temperature. Finally, 100 µl of stop solution was added . The samples were read at an optical density of 450 nanometers by ELISA Reader , model Stat Fax – 2100, manufactured by Awareness Technology Inc.

- **Determination of malonate dialdehyde in serum of rats with DNBS -induced colitis.**

The determination of MDA (malonov dialdehyde) on day 6 was performed with competitive ELISA kits from Elabscience ® (cat. No. E - EL -0060) according to the manufacturer's protocol (7th ed., 2023). Samples and standards were analyzed in duplicate . Absorbances were read at 450 nm with a Berthold Technologies Mithras LB 943 multi-function microplate reader .

- **Histopathological research**

Microscopic examination of the inflamed distal colon is performed by light microscopy on slides stained with hematoxylin and eosin . Histological criteria include changes in the architecture of the mucosa, cellular infiltration, the presence of crypt abscess, and depletion of goblet cells. For the purposes of histopathological examination, samples are taken from the altered areas of the organ, fixed in 10% buffered formaldehyde, and then 5-micrometer-thick sections are prepared , and the preparations are stained with hematoxylin and eosin . The changes in the individual areas are described sequentially: mucosal epithelium with lamina propria , crypts, submucosa, muscularis. The individual layers are described according to the presence and severity of the following indicators: edema, polymorphonuclear infiltration, mononuclear infiltration, ulcerations , granulation tissue, dilatations and deformations of crypts, mitotic activity, reduction in the number of goblet cells.

- **Chemicals and reagents.**

2,4-dinitrobenzenesulfonic acid hydrate (DNBS, CAS No. 698999-22-3), U-74389G (CAS No. 153190-29-5) sulfasalazine (CAS No. 599-79-1) and all chemicals were obtained from SigmaAldrich Com Ltd. ELISA kits (Rat IL-1 alfa ELISA kit, Rat 1L-6 ELISA kit, Rat 1L-10 ELISA kit and TNF alpha rat ELISA kit) were purchased from R&D systems.

- **Statistical analysis.**

Results were presented as means ± SEM and were tested by one-way ANOVA followed by Fisher 's least significant difference procedure as a post hoc test. A P-value lower than 0.05 was considered significant. Analyses were performed using STATGRAPHICS® Centurion XV statistical software.

RESEARCH RESULTS AND DISCUSSION

1. Effect of U-74389G in an experimental model of amiodarone -induced pulmonary toxicity (AIBT)

1.1.Selection of an appropriate model of amiodarone- induced pulmonary toxicity

In the first model (M1) AM was applied intratracheally, once at a dose of 6.25 mg / kg as aquatic solution with a concentration of 7.8 mg / ml. In the second model (M 2) AM was introduced intratracheally, twice on days 0 and 2, at a dose of 6.25 mg / kg as aquatic solution with a concentration of 3.125 mg / ml. The control groups were introduced sterile distilled water intratracheally.

cyanosis and bristled fur were observed . In 10 out of 20 treated animals at M1, a picture of respiratory failure and exitus was observed within 36 hours. Massive pulmonary hemorrhages were found in the lungs. In M2, out of 20 treated animals, 2 lethal cases were registered (one on day 0 and one on day 2, after the second introduction of AM).

On the 14th and 28th day it was studied , the content of hydroxyproline and collagen in lung homogenate as pulmonary fibrosis markers [Bergman I and al . 1963; Komsa and al. 1996]. Made were histopathological research on the same points.

On days 14 and 28, a significant increase in **hydroxyproline** content was observed in both the model compared to controls (Fig. 1). The content on day 14 at M1 it was 1,749 mcg / ml, and in M2 – 1,071 mcg / ml. On day 28, the hydroxyproline content in the lung homogenate of the groups with model one was 2,677 mcg / ml, and in model two – 2,083 mcg / ml (without a statistically significant difference between them). The **collagen content** in ml of lung homogenate was increased statistically significantly on day 28 in M1 (Fig. 2).

In both studied models, biochemical and histopathological changes were observed, proving the development of pulmonary fibrosis. The histopathological changes in both models were identical : on day 14 they showed cyanosis and edema , single hemorrhages and siderophages . Vascular endothelium, and also vascular myocytes were with vacuolated cytoplasm. There was were venous microthrombi. Fibrosis was slightly presented perivasal, interstitial and peribronchial, and subpleural and intraalveolar – scarce.

On day 28, persistence of cyanosis and edema, single hemorrhages and small groups of siderophages were observed , vascular endothelium and myocytes had vacuolated cytoplasm. Fibrosis was moderately represented perivascularly and interstitially , mildly represented peribronchially , subpleurally and intraalveolarly.

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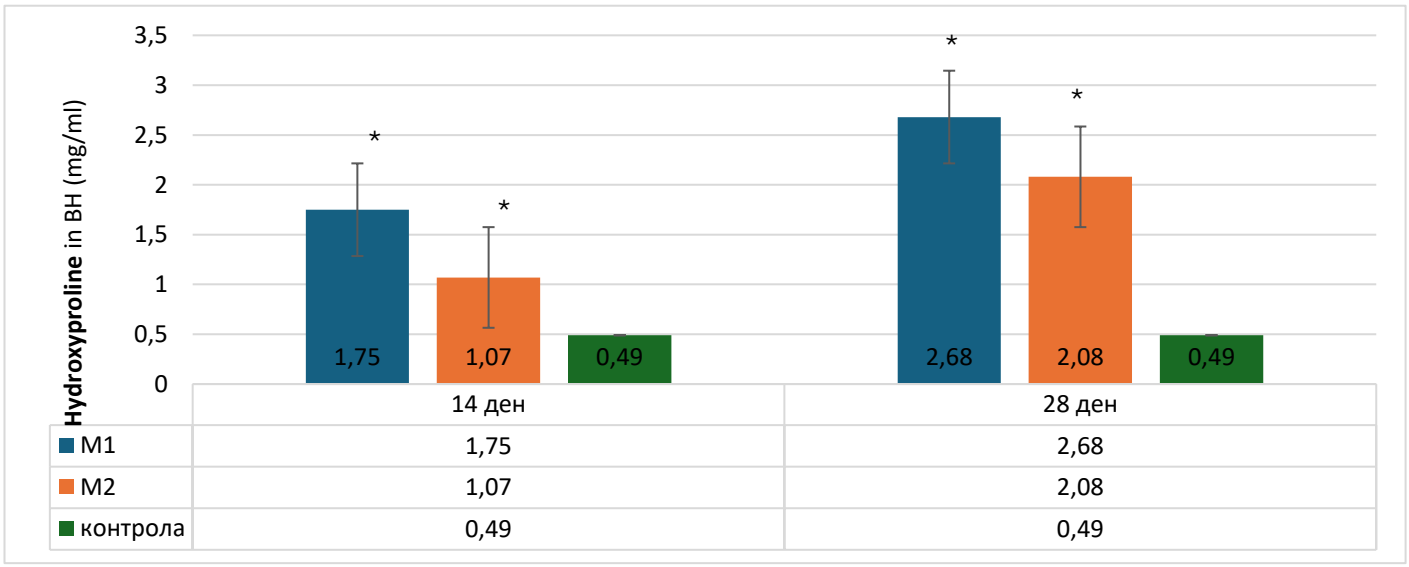


Fig. 1. **Hydroxyproline content** in ml pulmonary homogenate on day 14 and 28 after AM treatment . * denotes statistical credibility compared to the control group, p< 0.05.

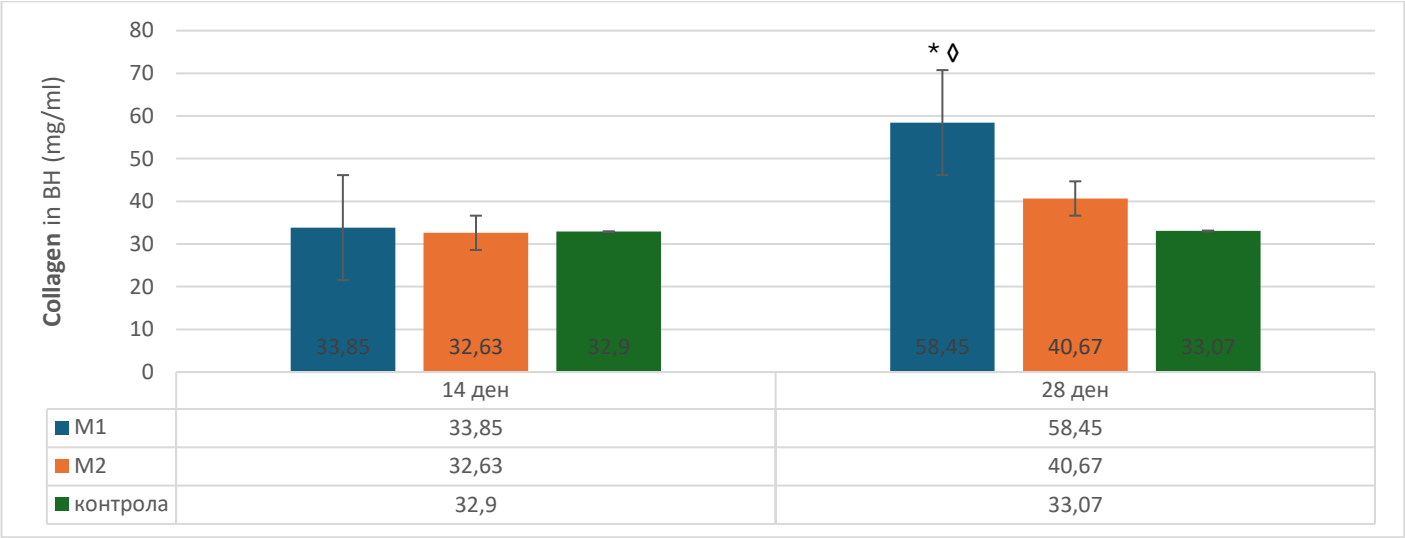


Fig. 2. **Collagen content** in ml pulmonary homogenate on day 14 and 28 after AM treatment . * denotes statistical credibility compared to the control group, ◊ – between M1 and M2; p< 0.05.

One-time intratracheal introduction of AM at a dose of 6.25 mg / kg with a concentration of 7.8 mg / ml (M1) leads to a pronounced pulmonary fibrosis comparable to that induced by the reference model (M2) - double, intratracheal administration on days 0 and 2, at a dose of 6.25 mg / kg with a concentration of 3.125 mg / ml . Although M1 is technically easier feasible, due to the one-time intratracheal treatment and equality fibrogenic with M2, cannot be recommend as a good model for AIBT because the higher one lethality among experienced animals.

1.2.The effects of U-74389G on markers of inflammation and cytotoxicity in BALT (broncho - alveolar lavage fluid) in amiodarone -induced pulmonary toxicity

After intratracheal administration of amiodarone on days 0 and 2, quickly superficially breathing, loss of appetite, cyanosis and goosebumps. These symptoms are weaker expressed in the group treated with AM and U-74389G. **The body The weight** of the rats in the AM group and in the group with the application of a combination of AM and U-74389G decreased slightly on day 7. The right white liver was weighted and relative weight was calculated for each animal. The increase in wet weight of the white Fraction is one of the indicators representing pulmonary edema. As shown in Table 1, the **pulmonary edema coefficient weight** increases approximately twice compared to the control group on day 3 after AM administration and decreased gradually thereafter. The AM- induced increased coefficient decreased after the application of U-74389G, but not significantly (Fig. 3).

The **protein** content in the cell-free BALT is measured as an index of alveolar -capillary permeability barrier (Fig. 4). In accordance with the development of pulmonary inflammation and damage to the alveolar integrity, treatment with AM leads to significant abruptly increased levels of total protein content on day 3 (387%). Combined administration of AM and U-74389G increased protein content, but it is significant lower – two-fold decrease compared to AM on the 3rd day after AM instillations. The total protein content in BALT is not statistically different from that of the control group in later time points.

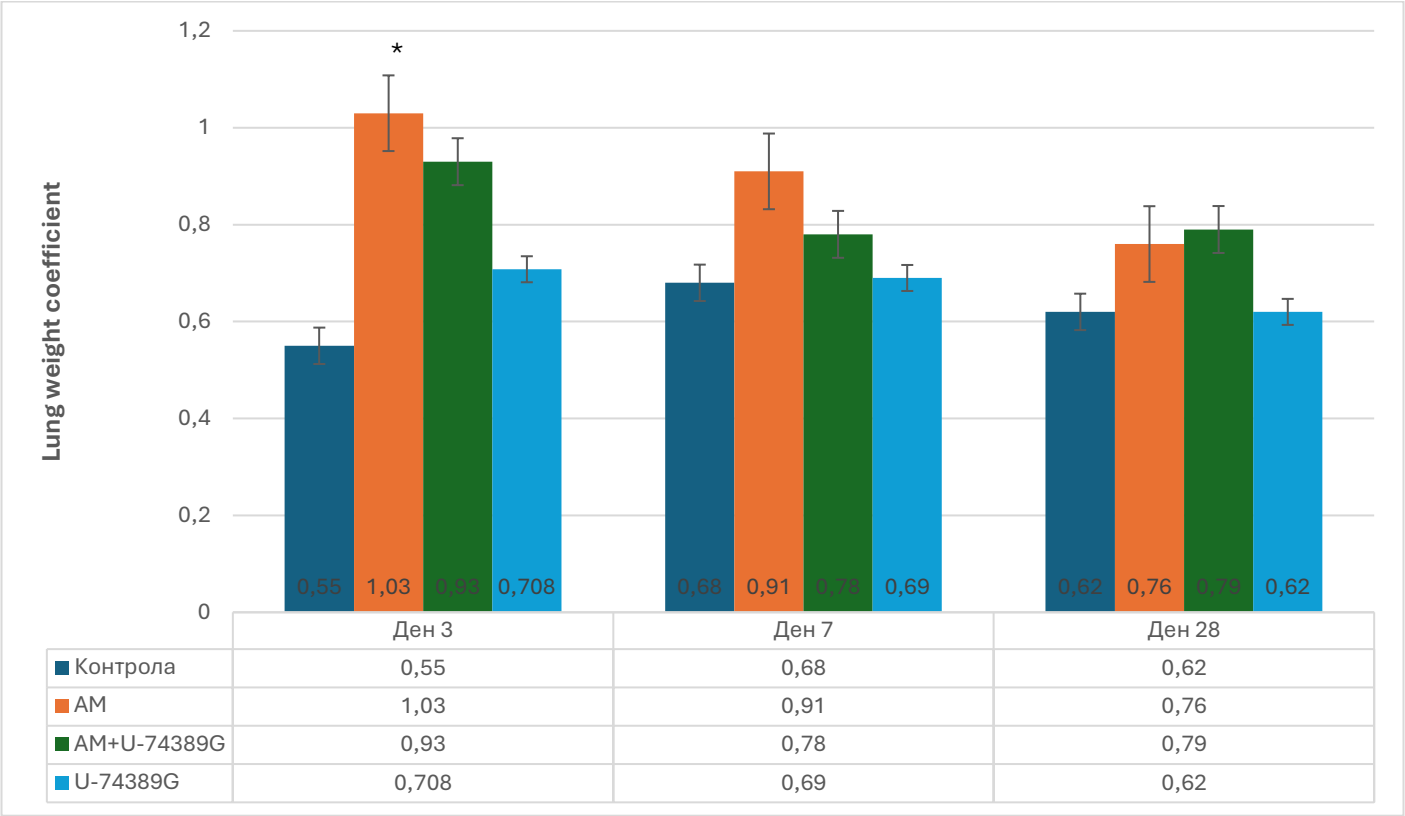
The application of AM was accompanied by an extreme increase in both **total number of cells**, as well as the individual cell types isolated from BALT on day 3. The total number of cells was significantly affected by treatment with AM (425%) and combination of AM and U-74389G (313%) on day 3, compared to controls (Fig. 5). No open significant differences in the total BALT cell count between groups on day 7 and 28.

The effect of lazaroid on alveolar macrophages was assessed by determining their absolute number and their morphology using a modification of the Danos and Keebler procedure [Saltini C et al ., 1984]. The absolute Alveolar **macrophage (AM) counts** in BALT were increased after intratracheal AM on day 3 (Fig. 7).
On the 3rd day of the experiment Alveolar macrophages in BALT of the amiodarone - treated group were present in a wide range of sizes , from small, monocyte- like cells to very large , mature macrophages containing two or more divided nuclei and vacuolated foamy cytoplasm (Figures 8 and 9). The cells are large, irregular to rounded in shape . Abundant, pale violet cytoplasm , with unclear boundaries. A characteristic "smeared" cytoplasmic periphery is observed, typical of macrophages. Abundant cytoplasm with a vacuolated , foamy appearance. The nuclei are eccentrically located. Rounded to kidney-shaped. A nucleolus is visible in some cells - a sign of activation. Apoptotic cells with impaired membrane integrity are often observed , as well as multinucleated giant cells.

Table 1. Effect of U -74389 G on the pulmonary coefficient weight and cytological parameters in the bronchoalveolar lavage fluid after intratracheal AM application

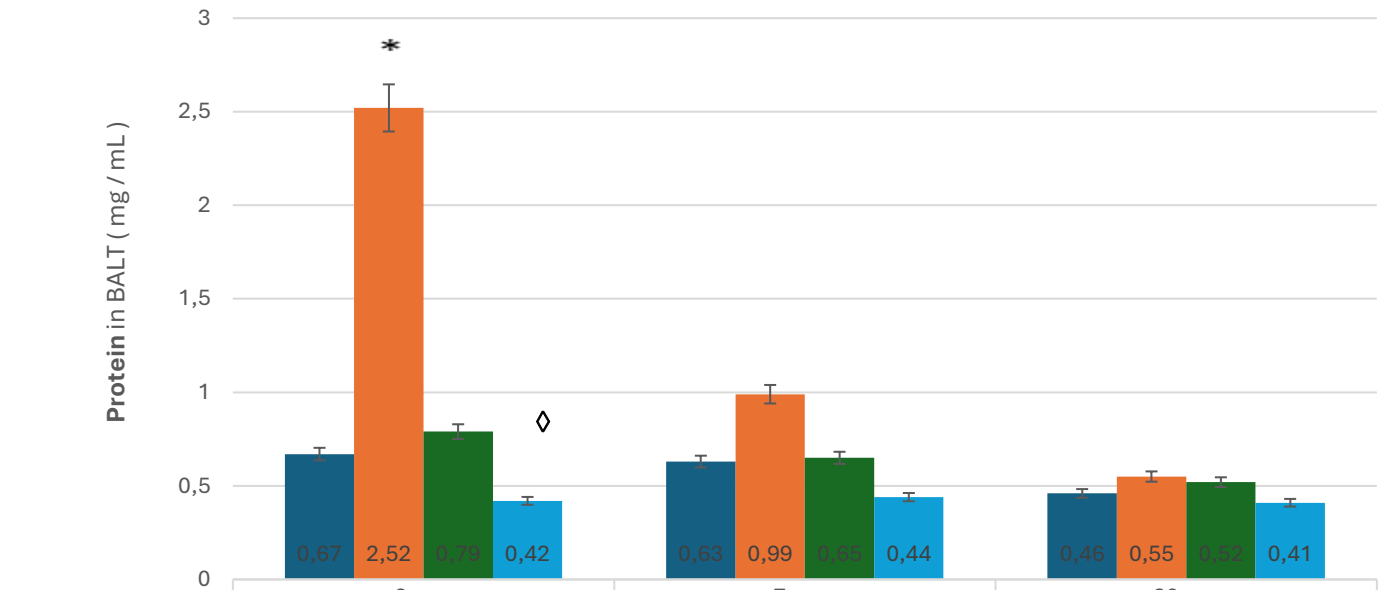
parameters / treatment	Time in days after initial AM administration		
	Day 3	Day 7	Day 28
	Mean ± SEM	Mean ± SEM	Mean ± SEM
Lung weight coefficient			
Control	0.55 ± 0.07	0.68 ± 0.09	0.62 ± 0.63
AM	1.03 ± 0.13 (*p=0.012)	0.91 ± 0.08	0.76 ± 0.04
AM + U -74389 G	0.93 ± 0.08	0.78 ± 0.12	0.79 ± 0.07
U -74389 G	0.708 ± 0.11	0.69 ± 0.09	0.62 ± 0.07
Protein in BALT mg / mL			
Control	0.67 ± 0.03	0.63 ± 0.12	0.46 ± 0.04
AM	2.52 ± 0.09 (*p=0.034; ◊p=0.043)	0.99 ± 0.17	0.55 ± 0.03
AM+U-74389G	0.79 ± 0.02	0.65 ± 0.02	0.52 ± 0.09
U-74389G	0.42 ±0.05 (◊p=0.001)	0.44 ± 0.04	0.41 ± 0.03
Total cell count x 10 ^{5} / mL			
Control	7.43 ± 2. 9 9	7.43 ± 2. 9 9	6.69 ± 0.97
AM	31.62 ± 2.96 (*p=0.001)	1 1.37± 1.58	6.78 ± 0.55
AM+U-74389G	23.26 ± 3.50 (*p=0.016)	10.52 ± 1.59	5.78 ± 0.48
U -74389 G	10.74± 1.76 (◊p=0.018)	8.81 ± 1.16	5.23 ± 0.75
PMNs ^{x105} / mL / %			
Control	0. 32± 0.02/4.3%	0. 32± 0.02/4.3%	0.29 ± 0.01/4.5%
AM	22.92 ± 1.85 (*p=0.008;	3.26± 0.45/28.7% (*p=0.001;	0.85 ± 0.07/12.7% (*p=0.001;
AM+U-74389G	◊p=0.033)/71.0%	◊p=0.041)	◊p=0.002)
U -74389 G	13.02 ± 2.69 (*p=0.024)/52.1%	0.96 ± 0.15/10.1% (*p=0.001)	0.57 ± 0.05/9.8%
	1.62± 0.99 (◊p=0.006)/10.6%	0.48± 0.02/5.3% (◊p=0.012)	0.39 ± 0.05/7.4% (◊p=0.001)
AMs x10 ^{5} / mL / %			
Control	6.74 ± 0.38/92.6%	6.74 ± 0.38/92.7%	6.20 ± 0.29/92.7%
AM	8.07 ± 0.65/24.8%	7.15 ± 0.99/63.9%	5.41 ± 0.47/80.6%
AM+U-74389G	10.32 ± 2.14 (*p=0.044)/44.5%	8.83 ± 1.34/84.2%	4.87 ± 0.40/87.0%
U-74389G	12.38 ± 1.54 (*p=0.026)/84.2%	7.93 ± 1.05/89.9%	4.66 ± 0.63/77.5%
Lyme x10 ^{5} / mL / %			
Control	0.23 ± 0.01/3.1%	0.23 ± 0.01/3.1%	0.21 ± 0.61/3.1%
AM	1.36 ± 0.11% (*p=0.001)/4.2	0.89 ± 0.12 (*p=0.001)/7.9%	0.43 ± 0.04 (*p=0.001; ◊p=0.021)/6.7%
AM+U-74389G	1.09 ± 0.13 (*p=0.001)/4.7%	0.59 ± 0.09 (*p=0.001)/5.7%	0.25 ± 0.02/4.2%
U-74389G	0.76 ± 0.09 (*p=0.012)/5.2%	0.42 ± 0.06/4.8%	0.19 ± 0.03/3.6%

Abbreviations: PMNs - polymorphonuclear leukocytes; A M s - alveolar macrophages; Lym - lymphocytes; BALT - bronchoalveolar lavage liquid;* p < 0.05 versus control group , ◊p <0.05 versus AM + U -74389 G group



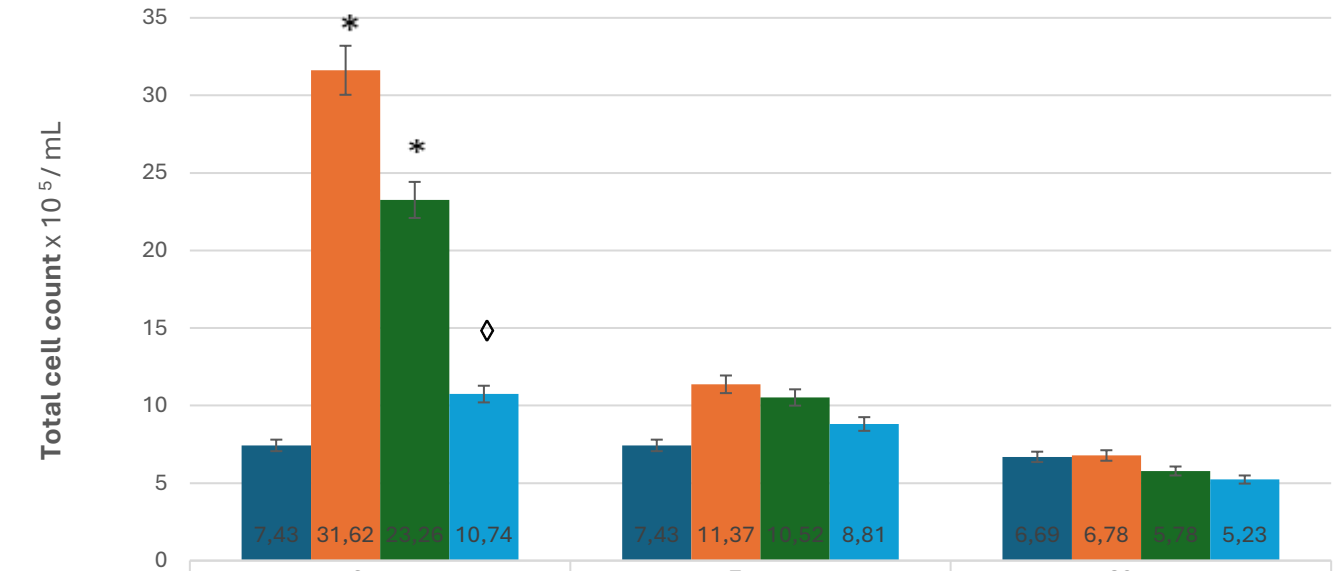
	Ден 3	Ден 7	Ден 28
Контрола	0,55	0,68	0,62
AM	1,03	0,91	0,76
AM+U-74389G	0,93	0,78	0,79
U-74389G	0,708	0,69	0,62

Fig .3. Influence of U -74389 G on **the pulmonary coefficient weight** after AM- induced pulmonary injury; * p < 0.05 vs. the control group



	3 ден	7 ден	28 ден
контрола	0,67	0,63	0,46
AM	2,52	0,99	0,55
AM+U-74389G	0,79	0,65	0,52
U-74389G	0,42	0,44	0,41

Fig. 4. Effect of U -74389 G on **protein content** in BALT after AM-induced lung injury; * p <0.05 versus control group ; ◊p <0.05 versus AM + U -74389 G combination group .



	3 ден	7 ден	28 ден
контрола	7,43	7,43	6,69
AM	31,62	11,37	6,78
AM+U-74389G	23,26	10,52	5,78
U-74389G	10,74	8,81	5,23

Fig. 5. Influence of U -74389 G on **the general number of cells** in BALT after AM - induced lung damage liver

* p < 0.05 vs. the control group; ◊p < 0.05 vs. the group with the combination AM + U -74389 G

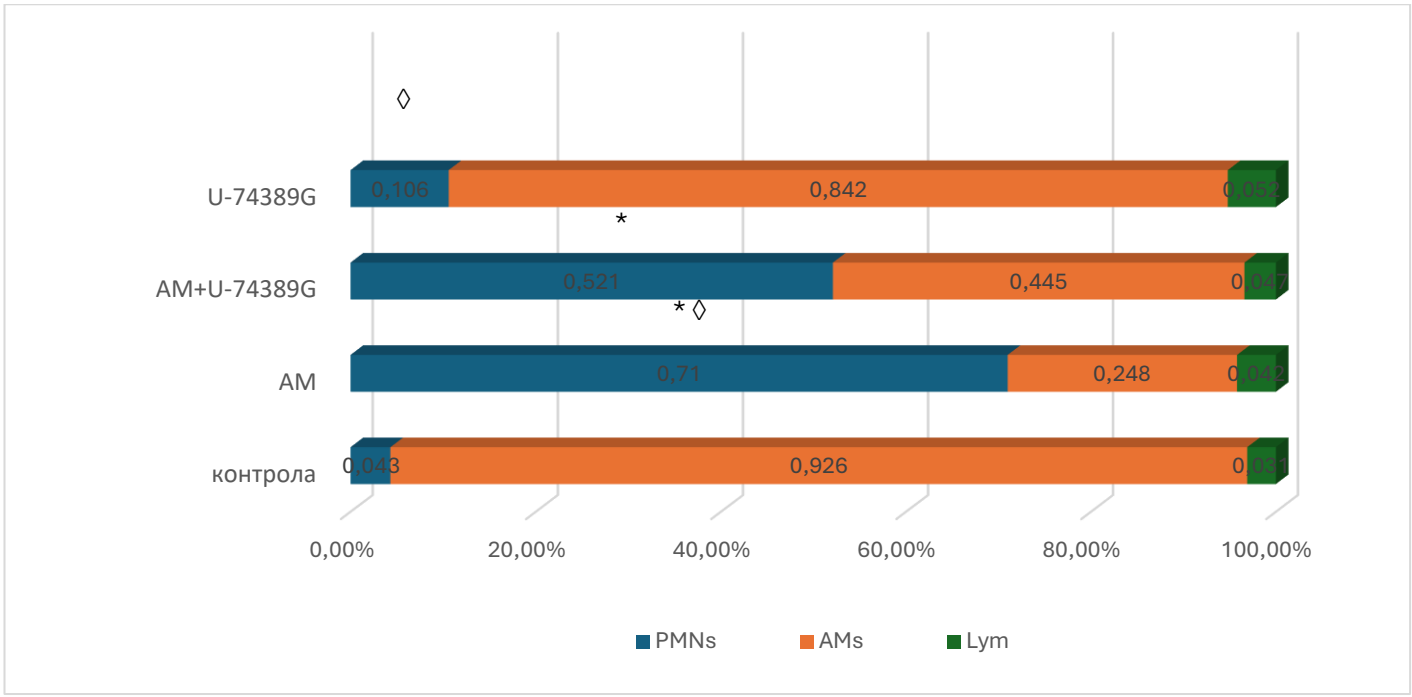
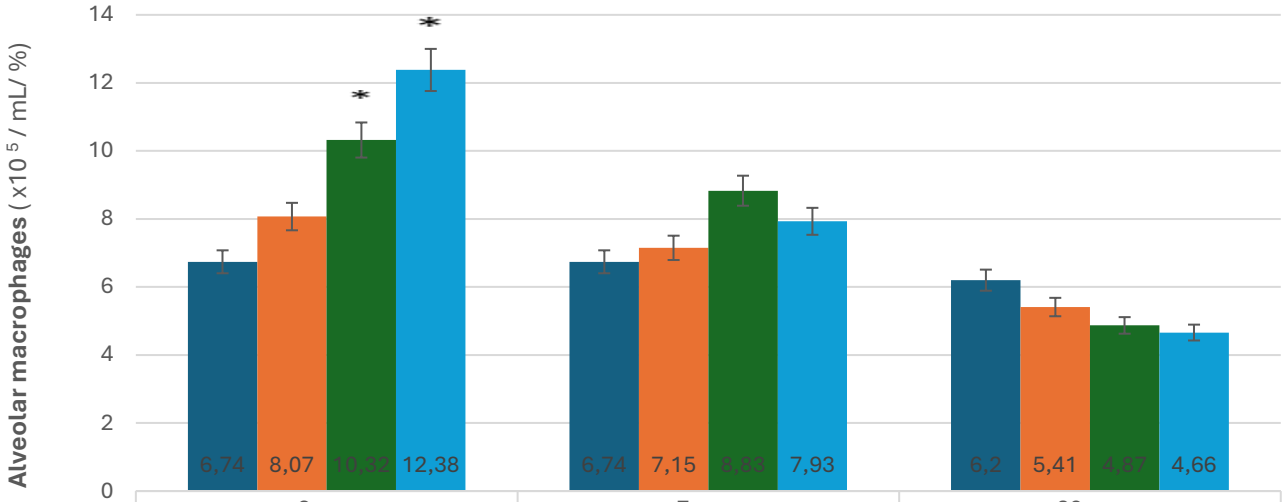


Fig 6. Influence of U -74389 G on **cell types** in BALT after AM - induced lung damage liver on day 3. Abbreviations: PMNs - polymorphonuclear leukocytes; A M s - alveolar macrophages; Lym - lymphocytes

* p < 0.05 vs. the control group; ◊p < 0.05 vs. the group with the combination AM + U -74389 G



	3 ден	7 ден	28 ден
контрола	6,74	6,74	6,2
AM	8,07	7,15	5,41
AM+U-74389G	10,32	8,83	4,87
U-74389G	12,38	7,93	4,66

Fig 7. Effect of U -74389 G on **alveolar macrophages (AMs) in BALT after** AM -induced lung injury; * $p < 0.05$ versus control group

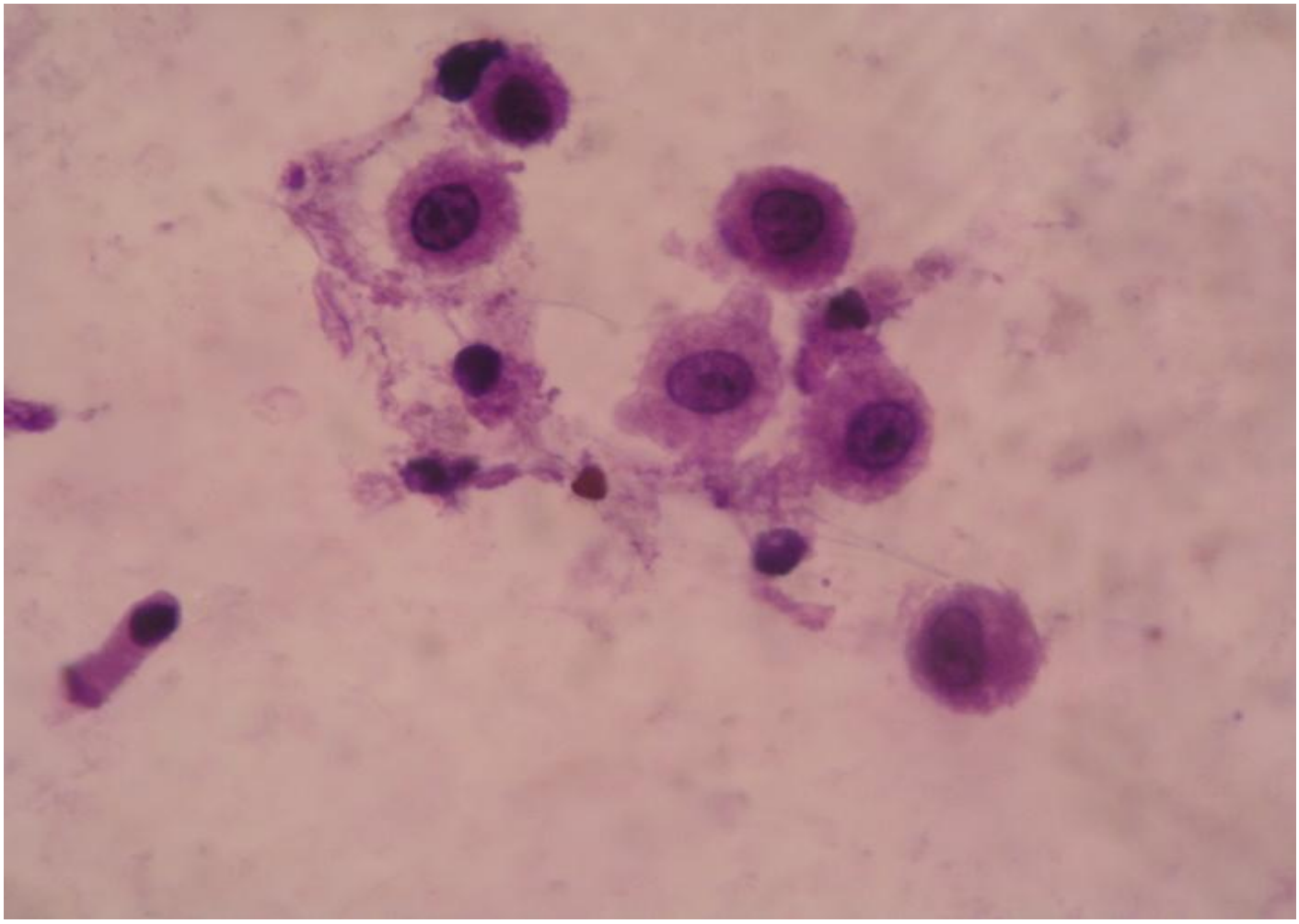


Fig. 8. Morphological hematoxylin-eosin view stained cells present in BALT on day 3 of the experiment after amiodarone administration. Magnification 40x

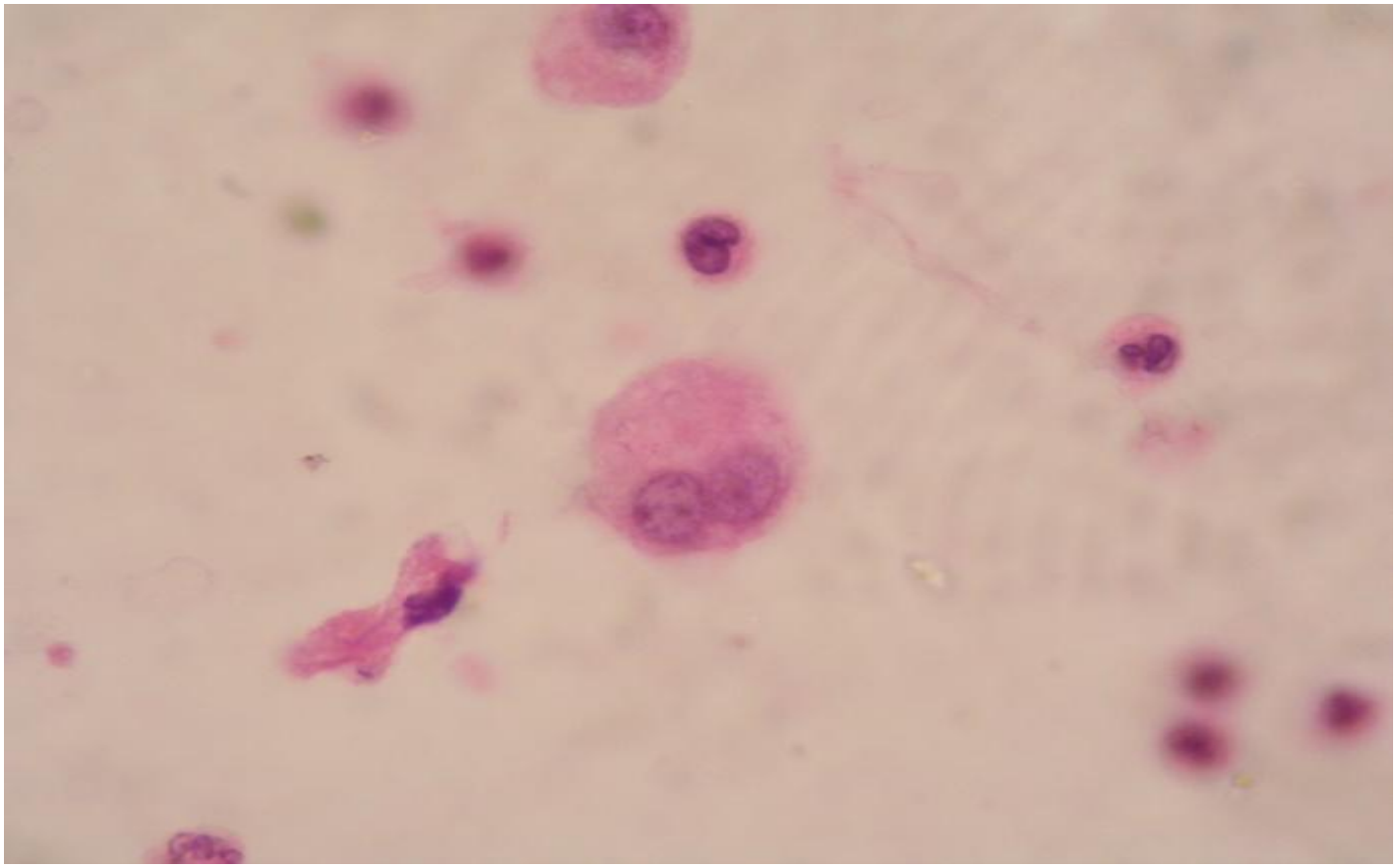


Fig. 9. Morphological hematoxylin-eosin view stained cells present in BALT on day 3 of the experiment after amiodarone administration. Magnification 40x

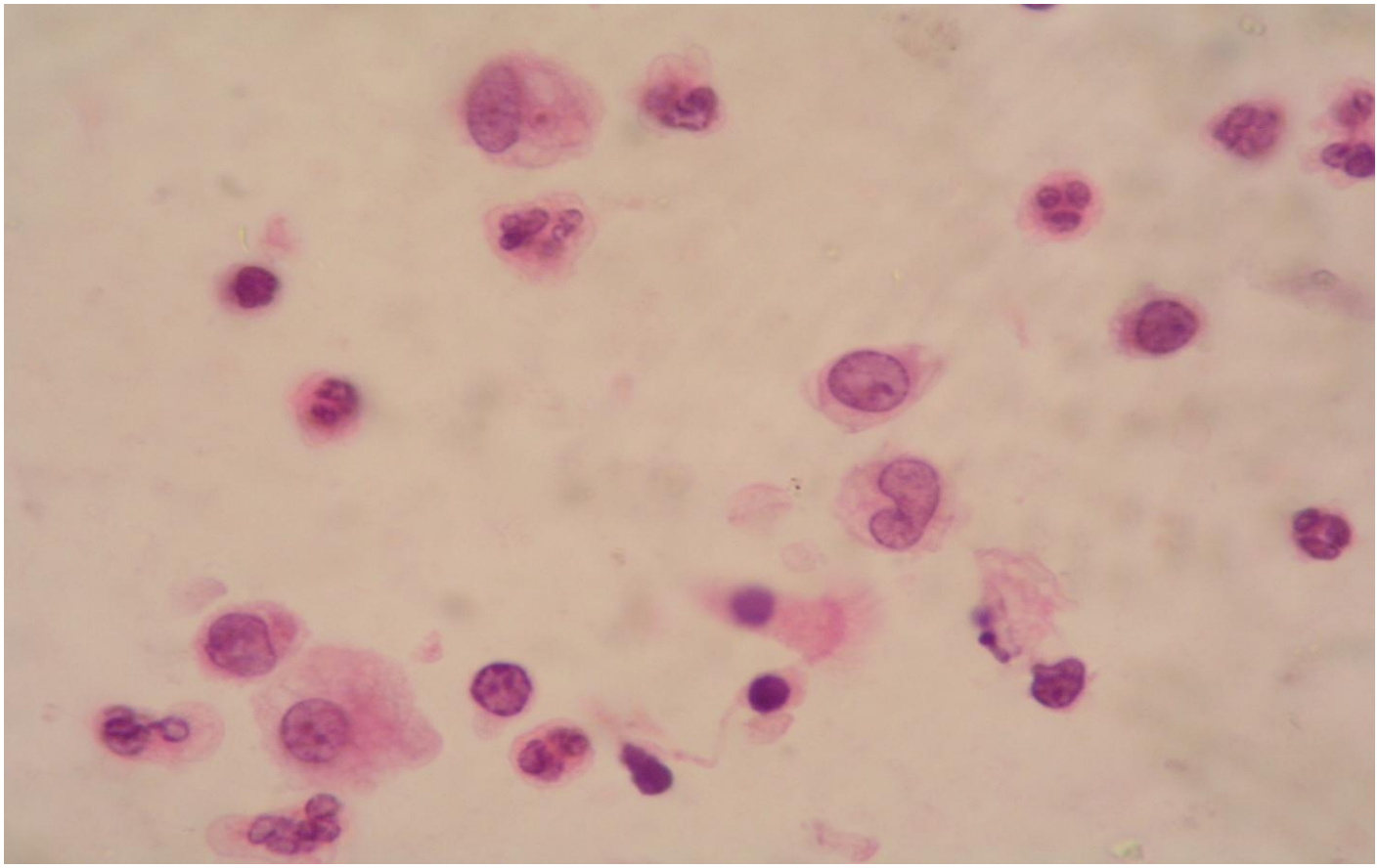
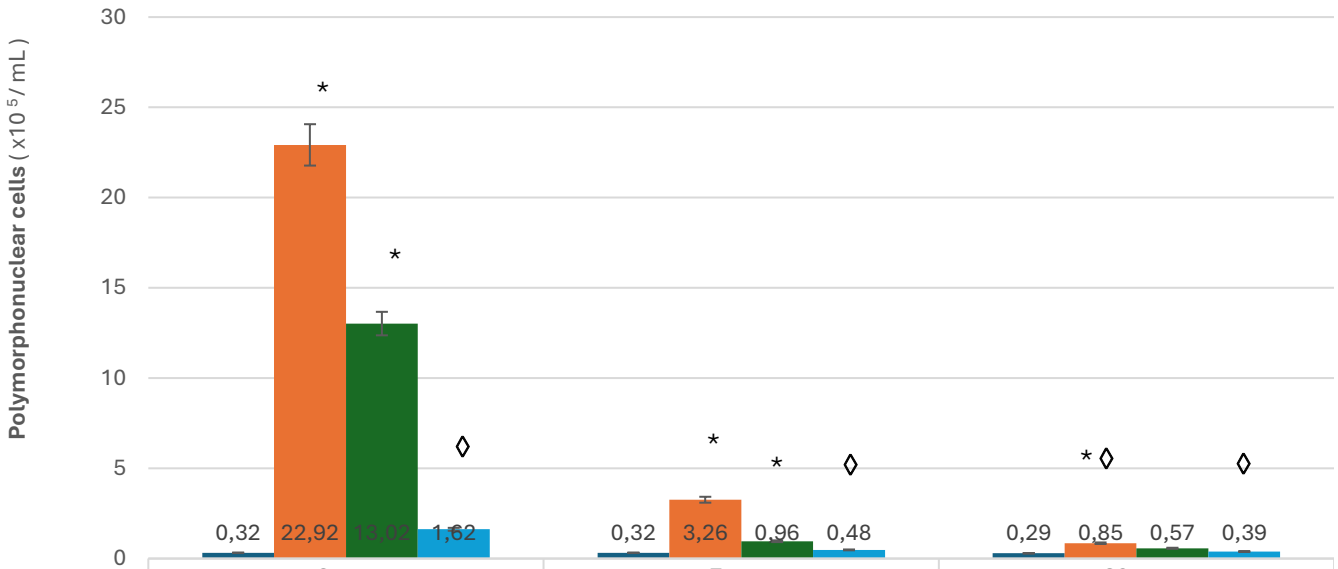


Fig. 10. Morphological hematoxylin-eosin view stained cells present in BALT on day 3 of the experiment after amiodarone administration + U -74389 G. Magnification 40x

On day 3 of the experiment, in the group treated with amiodarone but protected by U -74389 G, there were almost no big activated alveolar macrophages in BALT. The size of the macrophages is closer to normal, they have reduced vacuolization and better reserved membrane integrity. Significantly less are also apoptotic cells, significantly reduced are also the cells multi-core giants (Fig. 10)

The percentage of **polymorphonuclear cells (PMN)** in BALT is increased significantly in the group treated with AM on days 3, 7 and 28, when reaches respectively 71.0%, 28.7% and 12.7% of the total number of cells (Fig. 11). It remains relatively higher, despite the trend for reduction on days 7 and 28. The administration of U-74389G significantly is weakening AM- induced increase in the number of polymorphonuclear cells.



	3 ден	7 ден	28 ден
контрола	0,32	0,32	0,29
AM	22,92	3,26	0,85
AM+U-74389G	13,02	0,96	0,57
U-74389G	1,62	0,48	0,39

Fig. 11. Influence of U -74389 G on **polymorphonuclear cells (PMN)** in BALT after AM - induced lung damage liver on day 3
* $p < 0.05$ vs. the control group; ◊$p < 0.05$ vs. the group with the combination AM + U -74389 G .

Lymphocytes obtained through BALT are significantly increased in the AM group and the AM plus U-74389G group compared to the control. Lymphocyte counts in the combination treatment group were lower on days 3 and 7 and significantly decreases at day 28 compared to group AM (Fig. 12). Sometimes in the samples are observed eosinophils and basophils.

The results of **enzyme activities** in BALT are shown in Table 2. **Lactate dehydrogenase activity (LDH)** in the first BALT faction was used as an indicator of cellular death and disability. LDH leaks from dead or damaged cells when membrane integrity is compromised. After AM infusion, LDH activity in BALT is significantly increased, with a peak increase on day 3 (294%) and normal level was observed on day 28 (Fig. 13). Administration of U-74389G did not attenuate AM- induced increase in LDH activity in BALF on days 3 and 7.
It can be seen that the impact of AM treatment on the activity **of acid phosphatase (AcPh)** follows the same trend as LDH activity (Fig. 14). AcPh activity is slightly reduced by U-74389G in animals treated with AM on day 7. A significant increase in AcPh activity in the BALF of AM rats during the experiment and treatment with U -74389G did not lead to a decrease in the increase in AcPh.

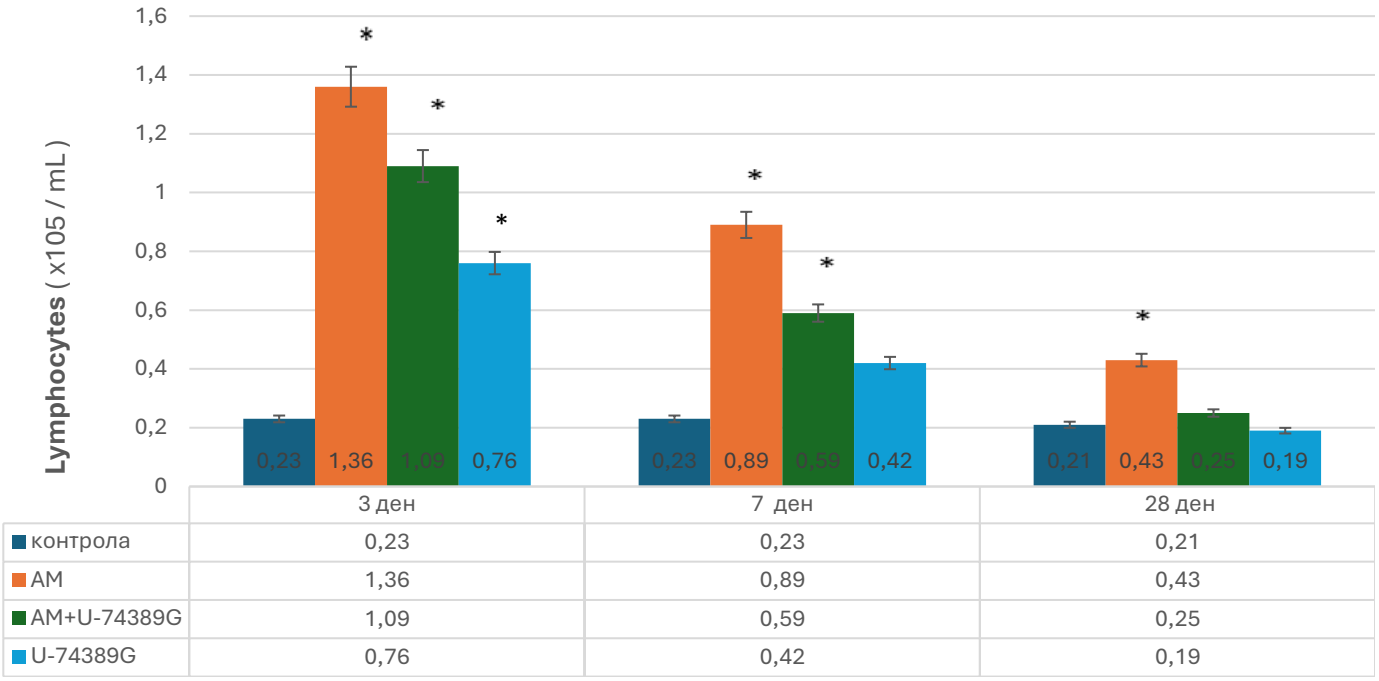
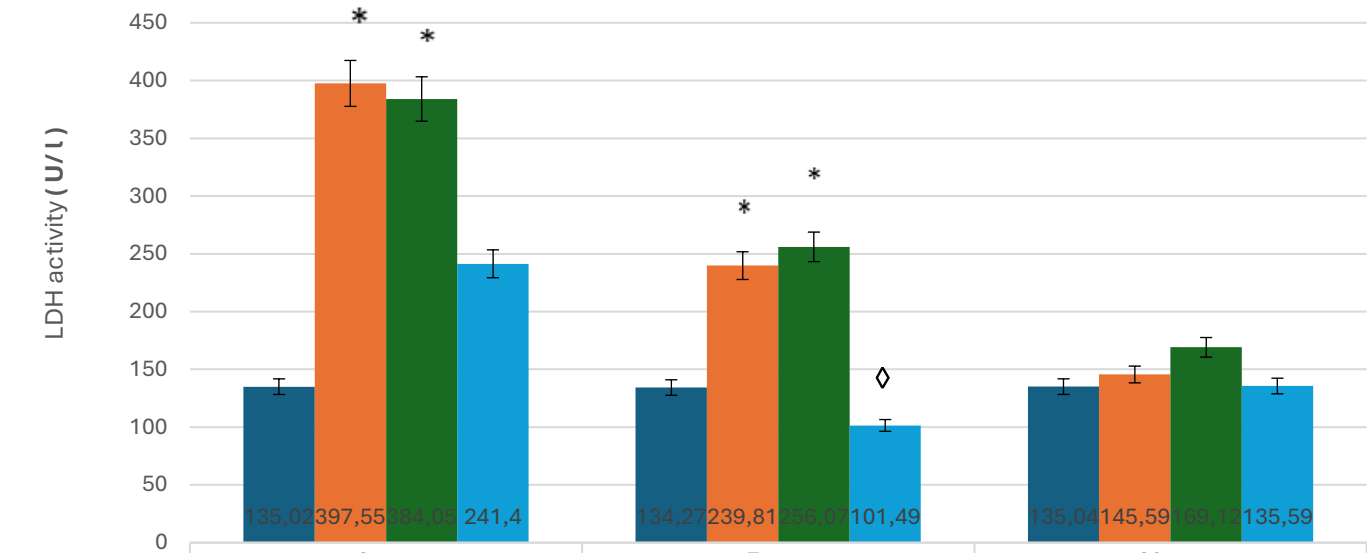


Fig. 12. Influence of U -74389 G on **lymphocytes (Lym)** in BALT after AM - induced lung damage liver on day 3; * p < 0.05 vs. the control group

Table 2. Effect of U -74389 G on biochemical parameters in BALT after intratracheal AM application

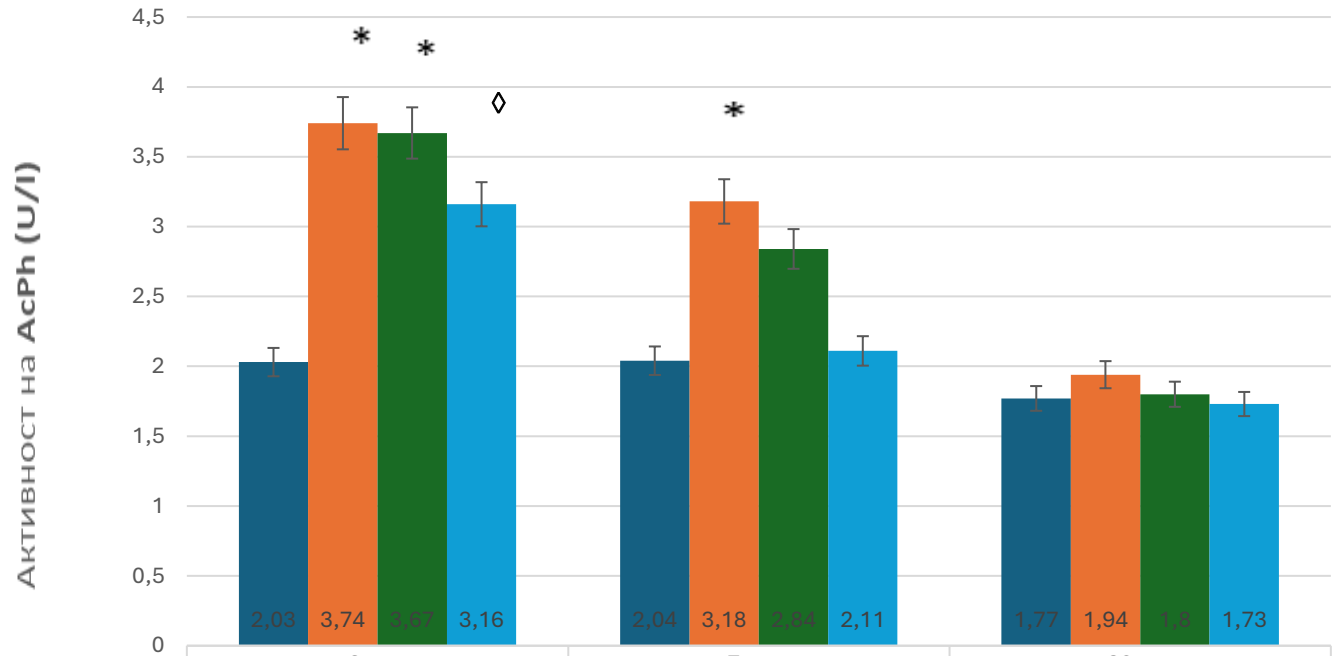
parameters /treatment	Time in days after initially application on the AM		
	Day 3	Day 7	Day 28
	Mean ± SEM	Mean ± SEM	Mean ± SEM
LDH U/l			
Control	135.02 ± 10.04	134.27 ± 28.67	135.04 ± 16.23
AM	397.55 ± 24.36 (*p=0.008)	239.81 ± 47.73 (*p=0.048)	145.59 ± 20.61
AM+U-74389G	384.05 ± 25.76 (*p=0.009)	256.07 ± 4550 (*p=0.047)	169.12 ± 15.56
U-74389G	241.40 ± 17.28	101.49 ± 13.50 (◊p=0.009)	135.59 ± 12.55
AcPh U/l			
Control	2.03 ± 0.09	2.04 ± 0.18	1.77 ± 0.21
AM	3.74 ± 0.19 (*p=0.025)	3.18 ± 0.32 (*p=0.018)	1.94 ± 0.22
AM+U-74389G	3.67 ± 0.23 (*p=0.039)	2.84 ± 0.92	1.8 0± 0.24
U-74389G	3.16 ± 0.26 (◊p=0.097)	2.11 ± 0.19	1.73 ± 0.15
AlPh U/l			
Control	2.02 ± 0.24	2.01 ± 0.35	2.01 ± 0.13
AM	4.46 ± 0.48 (*p=0.025)	4.20 ± 0.72 (*p=0.021)	3.24 ± 0.46(*p=0.027)
AM+U-74389G	4.50 ± 0.61 (*p=0.001)	4.63 ± 0.72 (*p=0.018)	3.12 ± 0.61(*p=0.029)
U-74389G	2.09 ± 0.13 (◊p=0.021)	1.39 ± 0.24 (◊p=0.004)	1.84±0. 30 (◊p =0.021)

Abbreviations: LDH - lactate dehydrogenase ; AcPh - acid phosphatase ; AlPh - alkaline phosphatase
* p < 0.05 versus control group , ◊p <0.05 versus AM + U -74389 G combination group



	3 ден	7 ден	28 ден
контрола	135,02	134,27	135,04
AM	397,55	239,81	145,59
AM+U-74389G	384,05	256,07	169,12
U-74389G	241,4	101,49	135,59

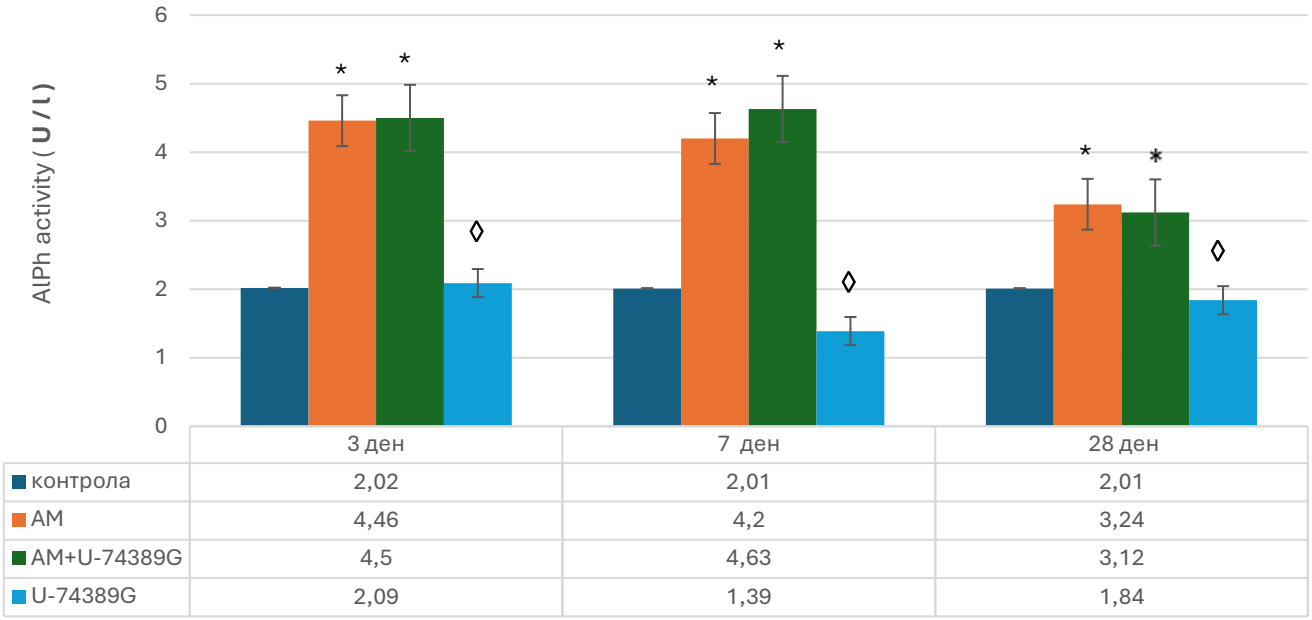
Fig. 13. **LDH** activity in BALT after AM-induced lung injury; * p<0.05 versus control group; ◊p <0.05 versus AM+U-74389G combination group.



	3 ден	7 ден	28 ден
контрола	2,03	2,04	1,77
AM	3,74	3,18	1,94
AM+U-74389G	3,67	2,84	1,8
U-74389G	3,16	2,11	1,73

Fig. 14. **AcPh** activity in BALT after AM-induced lung injury; * p<0.05 versus control group; ◊p <0.05 versus AM+U-74389G combination group.

The activity of another enzyme marker **alkaline phosphatase (AlPh)** in the bronchoalveolar lavage shows a little different concentration dynamics profile in the weather - was increased on day 3, remained higher on day 7 and 28 in both groups – with AM administration, and combined treatment with AM and U-74389G. Interesting, with a statistically significant deviation on day 3, 7 and 28 compared to the group with the AM+U-74389G combination, is the dynamics in the group with only U-74389G administration – very slight increase on day 3, decrease in the concentration of A l Ph even below that measured in the control group on day 7 and onwards increase , but still with a value below the control group on day 28 (Fig. 15).



	3 ден	7 ден	28 ден
контрола	2,02	2,01	2,01
AM	4,46	4,2	3,24
AM+U-74389G	4,5	4,63	3,12
U-74389G	2,09	1,39	1,84

Fig. 15. Influence of U -74389 G on **AlPh** activity in **BALT** after AM - induced pulmonary disability; * p< 0.05 versus the control group; ◊p < 0.05 versus the AM+U-74389G combination group.

Comparison of the effects of U-74389G and prednisolone on markers of inflammation and cytotoxicity in BALT (bronchoalveolar lavage fluid)

We were equal . the effects of **U-74389G** and **prednisolone (Pr)** on the early ones inflammatory changes and the manifestations of cytotoxicity in bronchoalveolar lavage fluid on days 3 and 7 after intratracheal administration of amiodarone (Tables 4 and 5).

LDH activity, AcPh , AlPh , total protein content and cytological analyses of bronchoalveolar lavage fluid were examined on days 3 and 7. Increased **LDH** activity is a marker of increased membrane permeability and cell lysis, **AcPh** for increased phagocytic activity or cytotoxicity , and **AlPh** for toxic injury or proliferation of type 2 pneumocytes .

Fig. 16 shows the influence of prednisolone and U -74389 G on the pulmonary coefficient weight after AM-induced pulmonary disability .

Fig. 17 and Fig. 18 present the effect of U -74389 G and prednisolone on the protein content and total cell count in the bronchoalveolar lavage fluid. lavage on days 3 and 7 after intratracheal administration of AM, as well as statistically significant deviations of the indicators. The significant reduction in the concentration of protein in BALT and the weaker decrease in the total number of cells due to suppression are seen. damage to the alveolar-capillary permeability barrier of prednisolone and U -74389 G.

Table 4. Effect of U -74389 G and prednisolone (10 mg / kg) on the weight coefficient of the white liver and cytological BALT parameters after intratracheal AM application

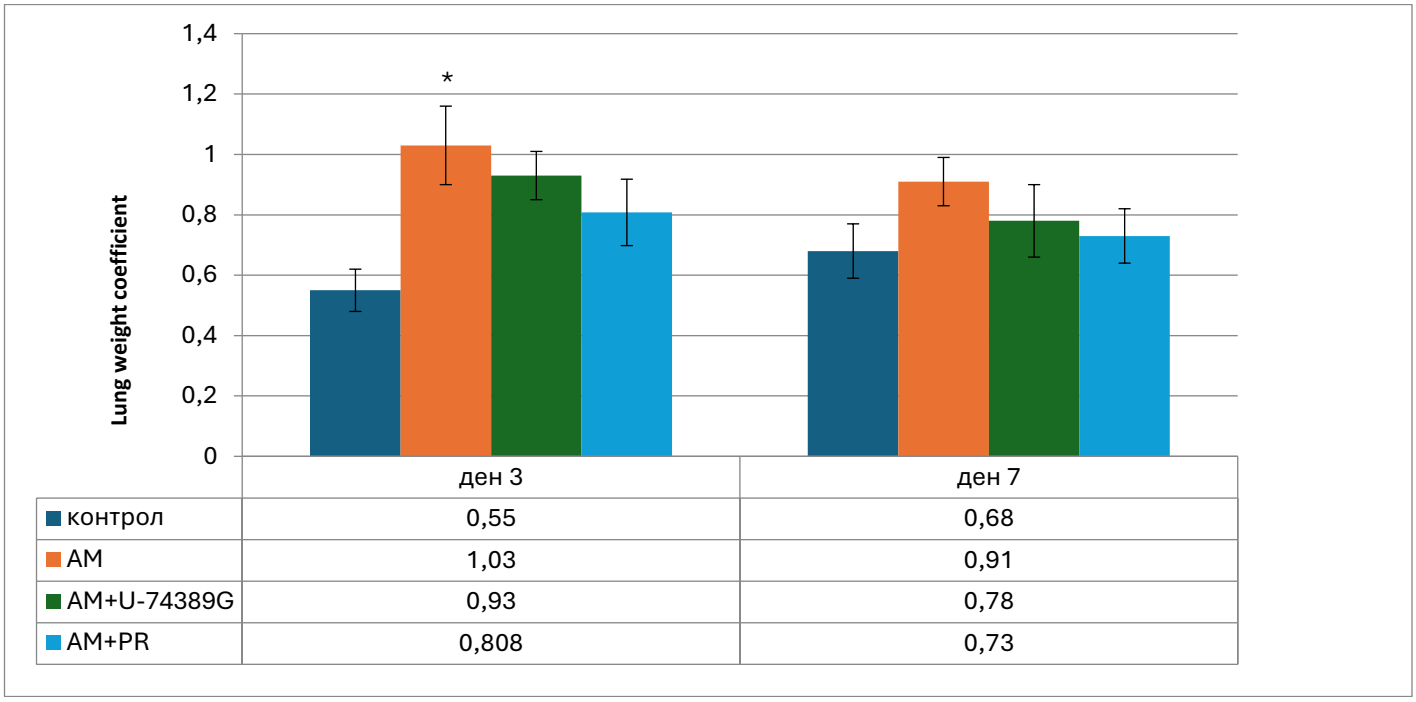
Parameters/treatment	Day 3	Day 7
	Mean±SEM	Mean±SEM
Lung weight coefficient		
Control	0.55±0.07	0.68±0.09
AM	1.03±0.13 (*p=0.012)	0.91±0.08
AM + U -74389 G	0.93±0.08	0.78±0.12
AM + Pr	0.808±0.11	0.73±0.09
Protein in BALT mg / mL		
Control	0.167±0.03	0.201±0.02
AM	0.88±0.09 (*p=0.03)	0.734±0.17 09 (*p=0.036)
AM+U-74389G	0.42±0.02 (*p=0.05; ◊p=0.043)	0.65±0.02 (*p=0.05)
AM + Pr	0.32±0.05; (◊p=0.043)	0.318±0.04 (◊p=0.043)
Total cell count x 10 ^{5} /mL		
Control	7.43±2.99	7.43±2.99
AM	31.62±2.96 (*p=0.001)	11.37±1.58
AM+U-74389G	23.26±3.50 (*p=0.016; ◊p=0.02)	10.52±1.59
AM + Pr	18.74±1.76 (*p=0.046; ◊p=0.018)	8.81±1.16
PMNs ^{x105} / mL / %		
Control	0.32±0.02/4.3%	0.405±0.02/4.3%
AM	22.92±1.85 (*p=0.008; ◊p=0.033)/71.0%	4.98±0.45/28.7% (*p=0.001; ◊p=0.041)
AM+U-74389G	13.02±2.69 (*p=0.024)/52.1%	1.96±0.15/10.1% (*p=0.001; ◊p=0.01)
AM + Pr	6.02±0.99 (◊p=0.006)/10.6%	1.15±0.02/5.3% (*p=0.05; ◊p=0.012)

Abbreviations: PMNs - polymorphonuclear leukocytes, Pr – prednisolone, AM – amiodarone
* p <0.05 versus control group ; ◊p <0.05 versus AM group

Table 5. Effect of U -74389 G and prednisolone (10 mg / kg) on biochemical parameters in the bronchoalveolar lavage fluid after intratracheal AM application

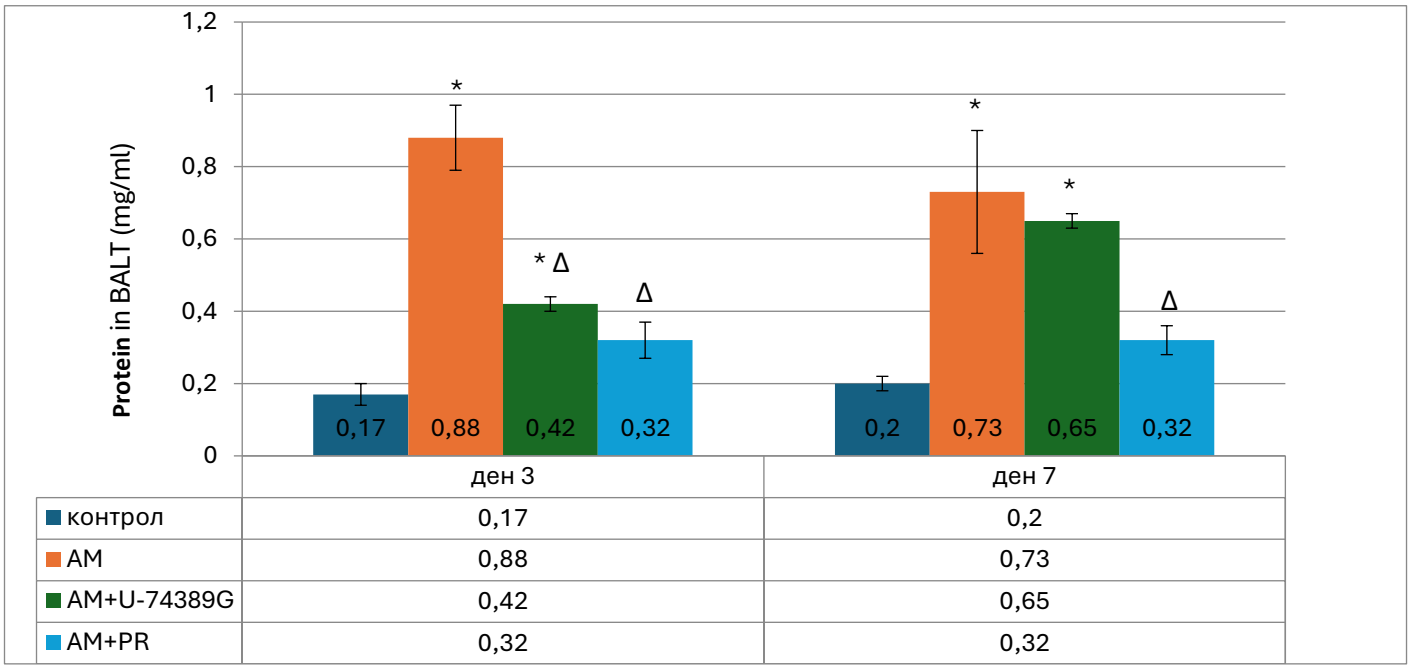
Parameters/treatment	Day 3	Day 7
	Mean±SEM	Mean±SEM
LDH U/l		
Control	140.7±10.04	134.27±28.67
AM	406.55±24.36 (*p=0.008)	300.01±47.73 (*p=0.048)
AM+U-74389G	384.05±25.76 (*p=0.009)	256.07±4550 (*p=0.047)
AM + Pr	206.90±17.28 (◊p=0.021)	206.49±13.50 (◊p=0.009)
AcPh U/l		
Control	2.03±0.09	2.04±0.18
AM	3.74±0.19 (*p=0.025)	3.18 ±0.32 (*p=0.018)
AM+U-74389G	3.67±0.23 (*p=0.039)	2.84±0.92
AM + Pr	3.00±0.26	2.11±0.19
AlPh U/l		
Control	2.02±0.24	2.01±0.35
AM	4.46±0.48 (*p=0.025)	4.20±0.72 (*p=0.021)
AM+U-74389G	4.50±0.61 (*p=0.001)	4.63±0.72 (*p=0.018)
AM + Pr	2.09±0.13 (◊p=0.021)	1.39±0.24 (◊p=0.004)

Abbreviations: LDH – lactate dehydrogenase , AcPh – acid phosphatase , AlPh – alkaline phosphatase , BALT – bronchoalveolar lavage fluid; * p < 0.05 versus control group ; ◊p <0.05 versus AM group



	ден 3	ден 7
контрол	0,55	0,68
AM	1,03	0,91
AM+U-74389G	0,93	0,78
AM+PR	0,808	0,73

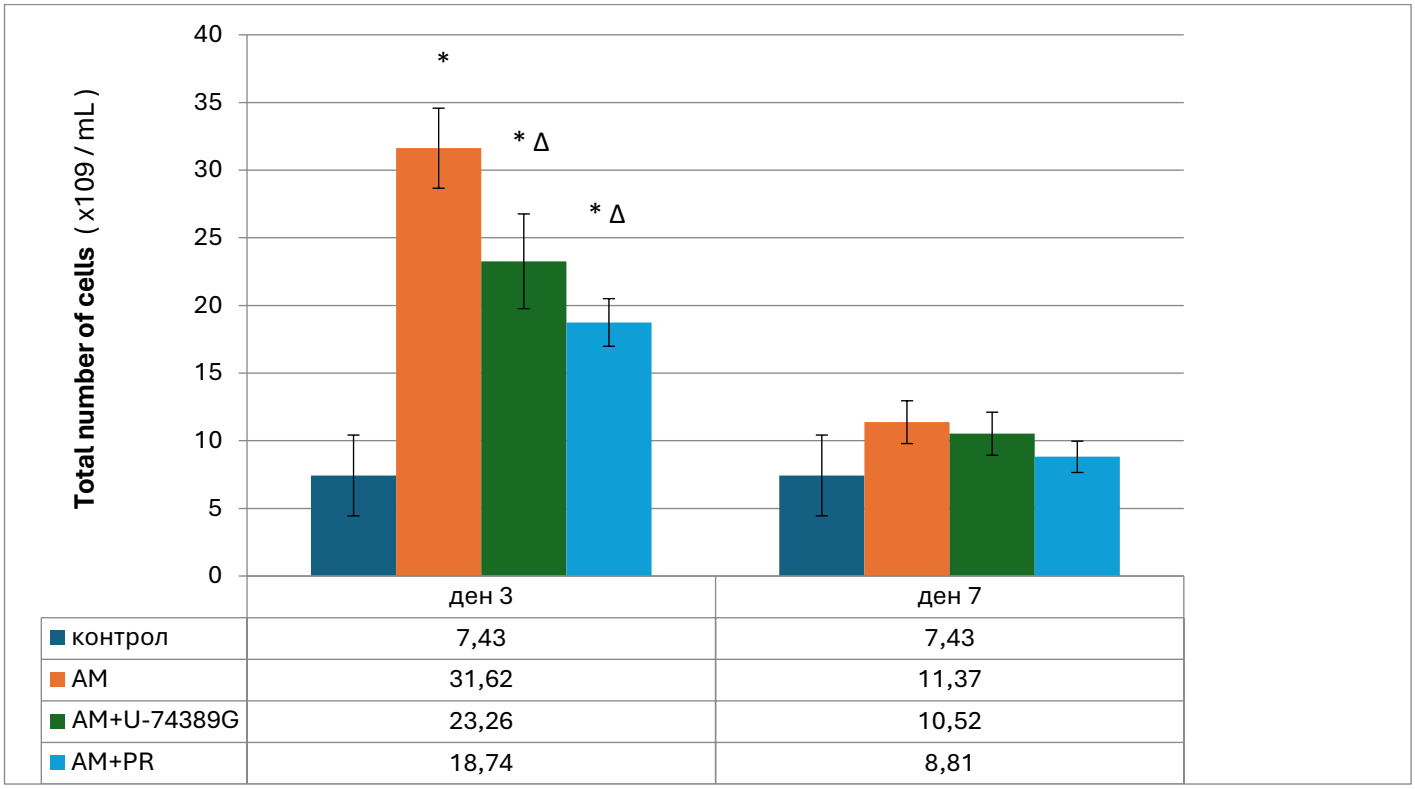
Fig. 16. Effect of prednisolone and U -74389 G on **the pulmonary coefficient weight** after AM- induced pulmonary disability; * p < 0.05 vs. the control group



	ден 3	ден 7
контрол	0,17	0,2
AM	0,88	0,73
AM+U-74389G	0,42	0,65
AM+PR	0,32	0,32

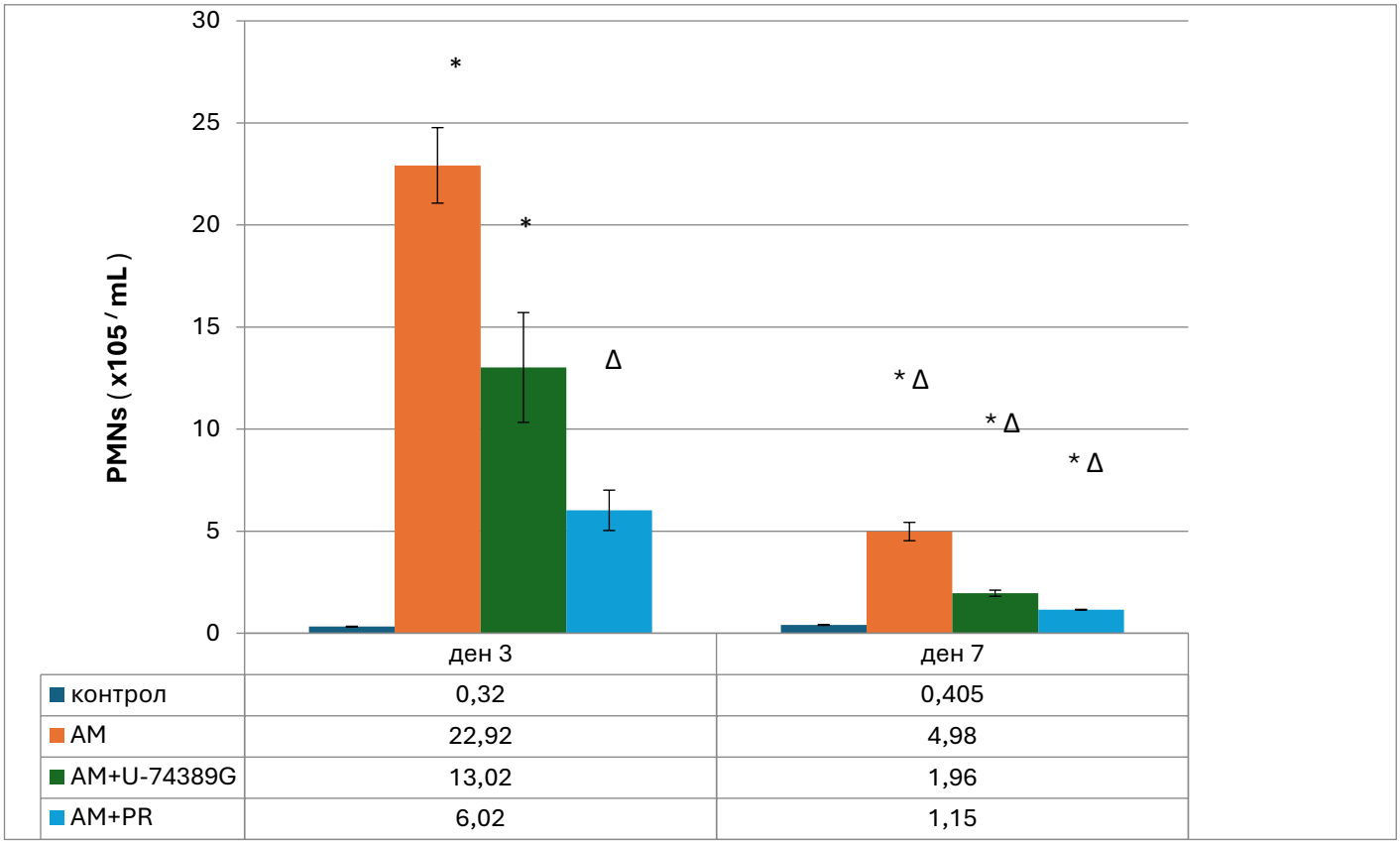
Fig. 17. Effect of U -74389 G and prednisolone (10 mg / kg) on **protein** content in bronchoalveolar lavage after i . t. administration of AM; * p<0.05 versus control; Δ p<0.05 versus AM

Fig. 19 shows the relationship between the administration of U -74389 G and prednisolone on the content of **polymorphonuclear leukocytes (PMNs)** in the bronchoalveolar lavage on days 3 and 7 after intratracheal administration of AM, as well as statistically significant deviations of the indicators. After the initial significant increase on day 3, a decrease in the amounts of polymorphonuclear leukocytes on day 7, approximately The dynamics of the indicator is tied to the trend for a more pronounced protective effect of prednisolone over that of U -74389 G .



	ден 3	ден 7
контрол	7,43	7,43
AM	31,62	11,37
AM+U-74389G	23,26	10,52
AM+PR	18,74	8,81

Fig. 18. Effect of U -74389 G and prednisolone (10 mg / kg) on **total cell count** in the bronchoalveolar lavage after i . t. administration of AM; * p<0.05 versus control; Δ p<0.05 versus AM

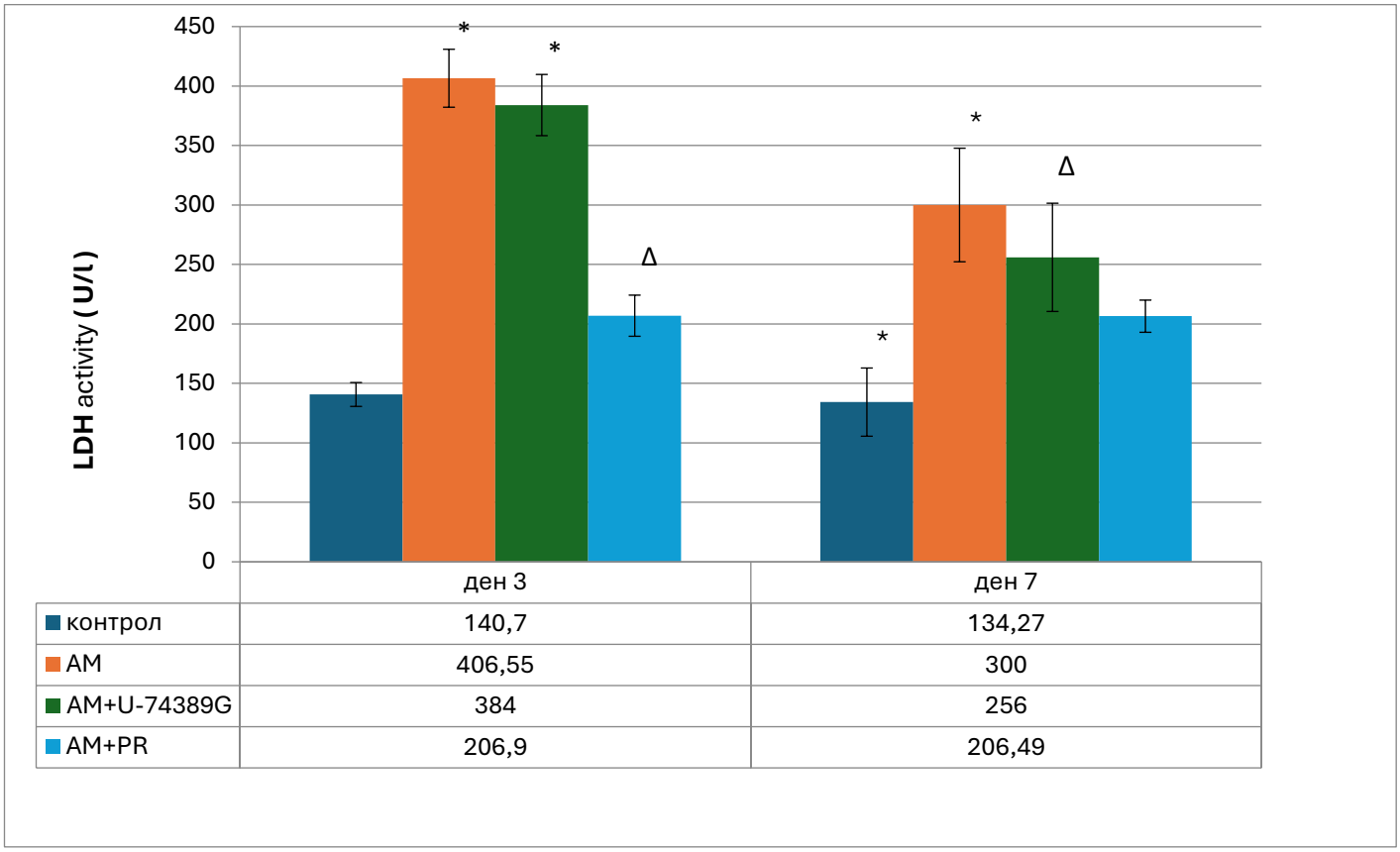


	ден 3	ден 7
контрол	0,32	0,405
AM	22,92	4,98
AM+U-74389G	13,02	1,96
AM+PR	6,02	1,15

Fig. 19. Effect of U -74389 G and prednisolone (10 mg / kg) on **polymorphonuclear leukocytes (PMNs)** in the bronchoalveolar lavage after i . t. administration of AM; * p<0.05 versus control; Δ p<0.05 versus AM

Figures 20, 21 and 22 present the effect of U -74389 G and prednisolone on the content of the enzymes **LDH, AcPh , AlPh** , as well as the statistically significant deviations in their indicators in BALT after intratracheal administration of AM. As expected, on days 3 and 7 there is a reduction in lactate concentrations dehydrogenase and acid phosphatase in the groups with prednisolone and U -74389 G administration , an expression of their suppressive effect on the increased membrane permeability and cell lysis – LDH ; as well as increased phagocytic activity or cell destruction – AcPh [Dusinska M and al ., 1998].

More curious is the case of **alkaline phosphatase (AlPh) concentrations** as an expression of toxic damage or proliferation of type 2 pneumocytes [Dusinska M and al ., 1998], visible in Fig. 22. A slight increase in AlPh is observed in the group with AM+ U -74389 G compared to this one with AM only, possibly due to early toxic effects and proliferation of type 2 pneumocytes, also supported by our histological research also showing the pronounced proliferation of type 2 pneumocytes.

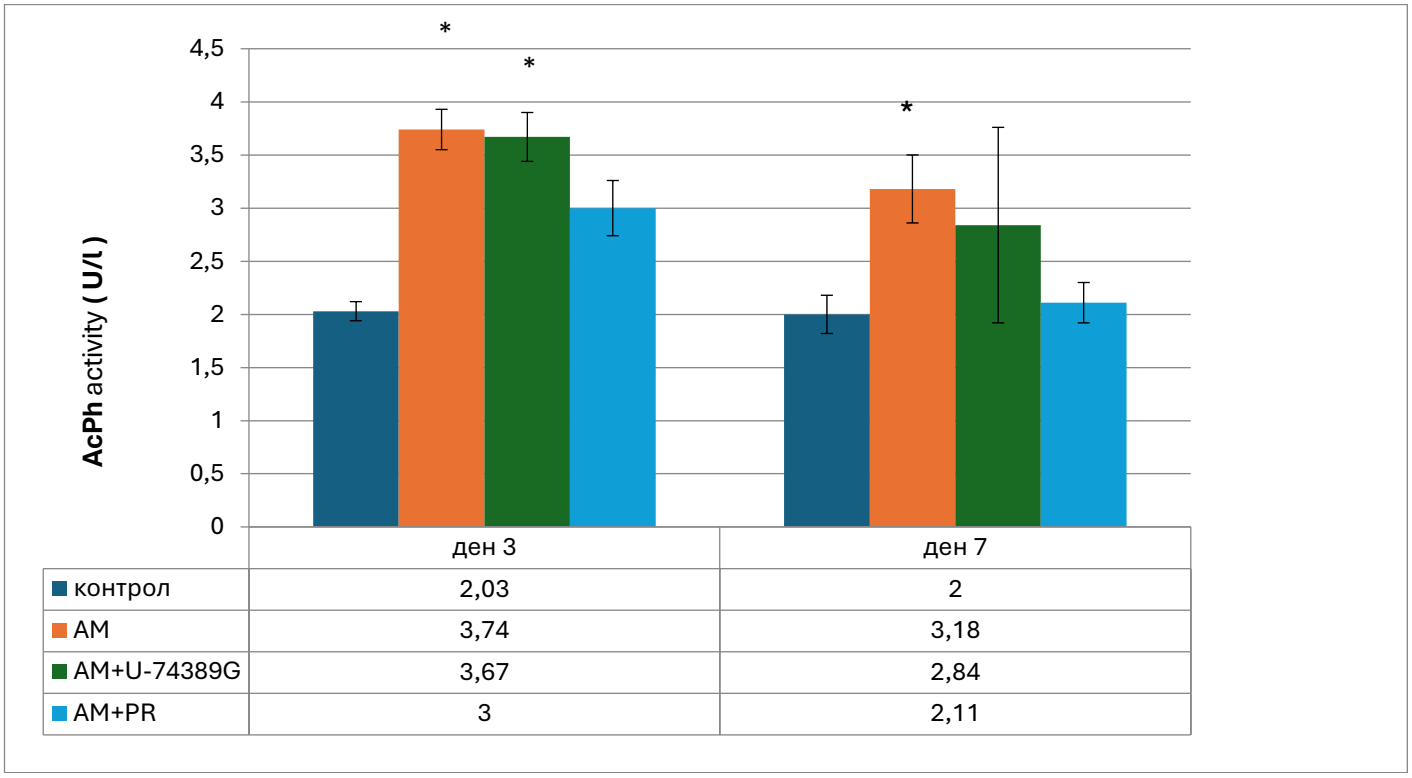


	ден 3	ден 7
контрол	140,7	134,27
AM	406,55	300
AM+U-74389G	384	256
AM+PR	206,9	206,49

Fig. 20. Effect of **U** - 74389 G and prednisolone (10 mg / kg) on **lactate dehydrogenase (LDH)** activity in the bronchoalveolar lavage after i . t. administration of AM; * p<0.05 versus control; Δ p<0.05 versus AM

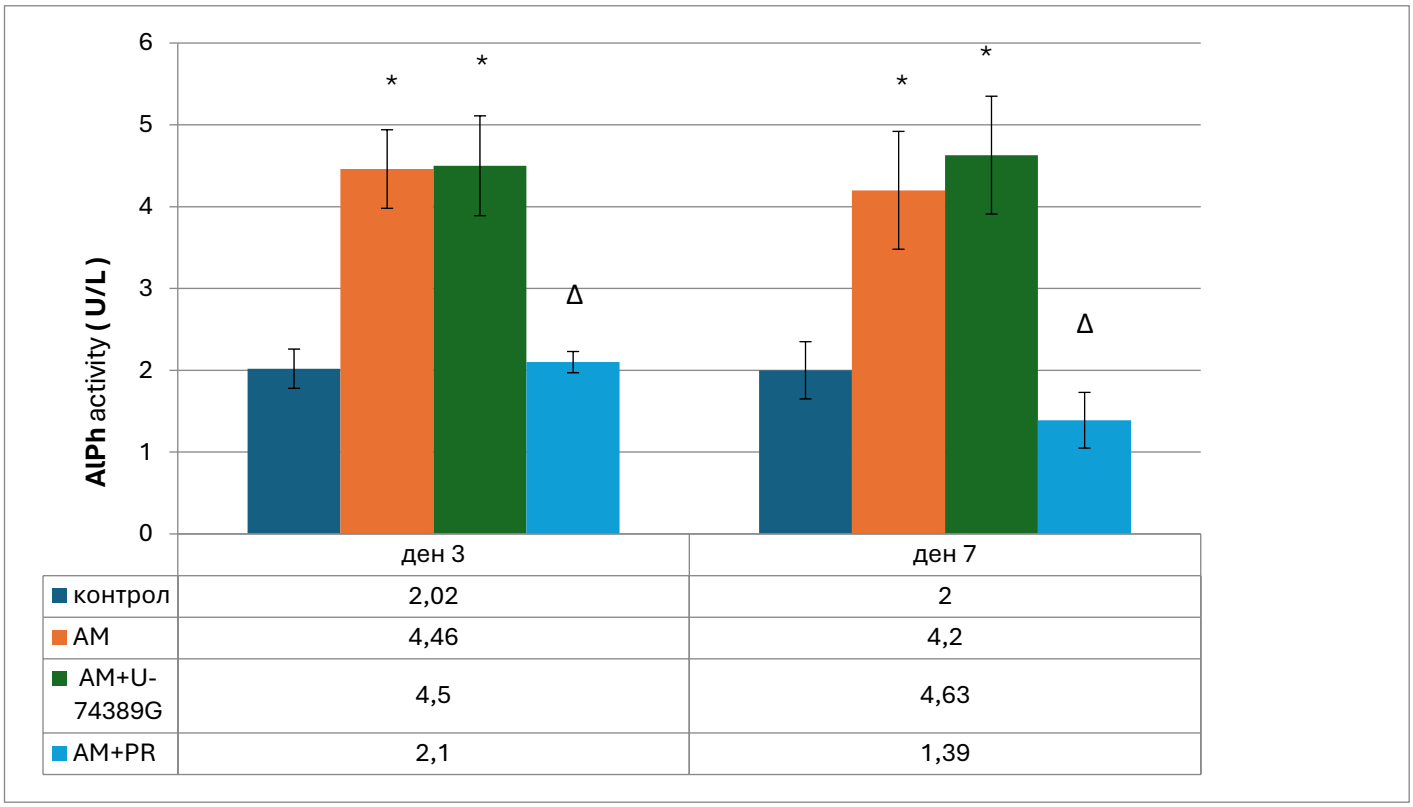
Results: Amiodarone treatment resulted in significantly increased lung weight index, protein content, total cell count, polymorphonuclear cells, alveolar macrophages , and LDH, AcPh , and AlPh activity . U-74389G and prednisolone attenuated markers of pulmonary inflammation and alveolar-capillary barrier damage compared to the amiodarone -treated group .

Conclusion: Our study showed that U-74389G reduces early amiodarone -induced lung inflammation, with a protective effect comparable to that of prednisolone .



	ден 3	ден 7
контрол	2,03	2
AM	3,74	3,18
AM+U-74389G	3,67	2,84
AM+PR	3	2,11

Fig. 21. Effect of U -74389 G and prednisolone **(** 10 mg / kg) on **acid phosphatase (AcPh)** activity in the bronchoalveolar lavage after i.t. administration of AM; * p<0.05 compared to control



	ден 3	ден 7
контрол	2,02	2
AM	4,46	4,2
AM+U-74389G	4,5	4,63
AM+PR	2,1	1,39

Fig. 22. Effect of U -74389 G and prednisolone (10 mg / kg) on **alkaline phosphatase (AlPh)** activity in bronchoalveolar lavage fluid **.** lavage after i. t. administration of AM; * p<0.05 versus control; Δ p<0.05 versus AM

Discussion. The results of our study show that U-74389G, an antioxidant with potent membrane- stabilizing effect, decreases the early AM- induced pulmonary inflammatory damage, an effect similar to that of prednisolone. Despite this, although it has been proven that as both catalytic and scavenger antioxidants decrease AM- induced pulmonary inflammation and fibrosis in animals, nor one of these molecules has not shown clear efficacy in the treatment of amiodarone - induced pulmonary toxicity (AIPT). We We hypothesized that U- 74389G might show useful activity in this model by influencing the initial inflammatory phase (Fig. 23). We chose U-74389G as a derivative of a corticosteroid core substituted with an antioxidant group. It has some studies that show that corticosteroids are effective in animal models of AIBT [Reasor MJ et al., 1996], and are recommended and most prescribed therapy in patients with AIBT [Camus P et al., 2004; Wilson BD and al., 1990].
Intratracheal administration of AM is accompanied by increased lung coefficient weight approximately 2 times compared to the control group on day 3. Histological changes show sharp perivascular and interstitial edema, as well as degenerative damage (up to necrosis) of pneumocytes type 1 and 2. The combination of AM and U-74389G reduces the relative severity of lung and cell damage.

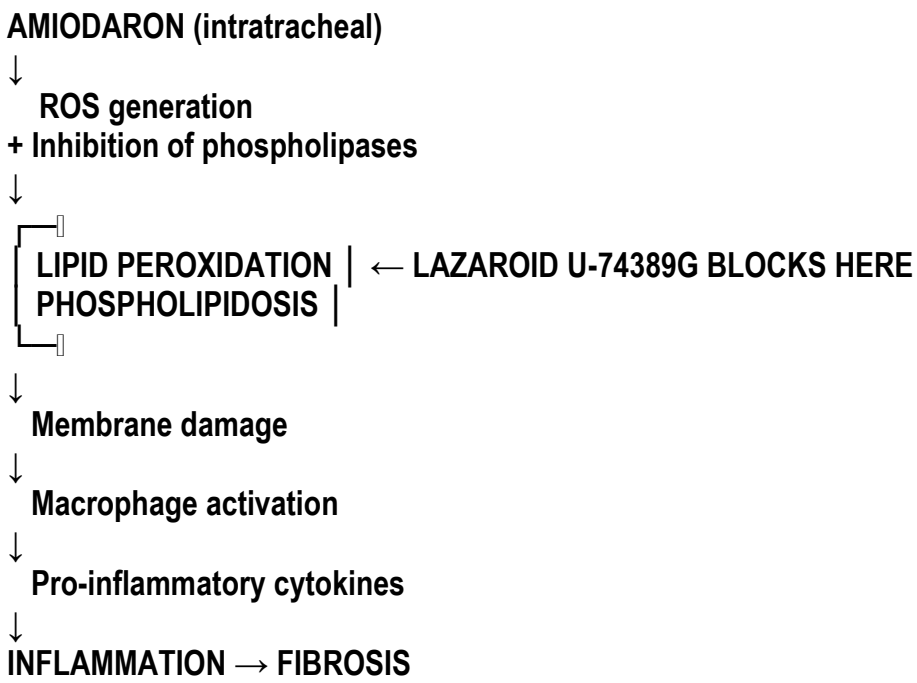


Fig. 23. Protective mechanism anti-inflammatory effect of lazaroid U-74389G in a model of amiodarone - induced pulmonary toxicity

AM- induced pulmonary disabilities cause elevated pulmonary permeability epithelium and endothelium, leading to extravasation of plasma proteins and accumulation of inflammatory cells in the alveolar space. AM causes significant increase (3.8 -fold) on day 3 in total protein content in the cell-free **bronchoalveolar lavage fluid (BALF).** U- **74389G** prevents damage to the alveolar-capillary permeability barrier, assessed by the level of protein in BALT. The total protein content in the group treated with the combination of AM plus U-74389G was comparable to the control group.
Common cells and different cellular types isolated from lavage liquid, were also tested on days 3, 7 and 28 as markers of alveolar -capillary damage barrier. The broken integrity of the air - blood barrier in the alveolar area of the lungs leads to increased number of cells in BALT. In our study The damage is most noticeable on the 3rd day and quickly resolves afterwards. U-74389G partially reduces the total cell count associated with AM administration in BALT on day 3. This aminosteroid significantly decreases PMN (polymorphonuclear leukocytes) count neutrophils - a marker of inflammation) in the lavage fluid – the percentage of PMN in the AM group was 73.0%, while in the group with combined treatment with AM and U-74389G it was 52.1%

(p=0.033). Acting on the early and transitional inflammation and damage to the alveolar-capillary barrier, U-74389G may protect the further development of pulmonary damage and fibrosis. U-74389G has a high affinity to vascular endothelium and protects against damage through its membrane stabilizing effect. The absolute The number of alveolar macrophages in the group with AM applied was slightly increased compared to control. Activated macrophages and PMN may play a role in the inflammatory response by releasing growth factors factors and cytokines, as well as the release of oxidants as part of the host defense system [Laskin DL et et al., 1996]. U-74389G did not inhibit number of alveolar macrophages after AM infusion on days 3 and 7. Mature macrophages containing two or more divided nuclei and vacuolated foamy cytoplasm, are less expressed in the group with AM and U-74389G. The cytological studies, similar to the integral characteristics, show that the aminosteroid U-74389G suppresses the initial inflammatory response, including migration and activation of inflammatory cells and the breakdown of the alveolar-capillary barrier.

The increased activity of some enzymes in BALT has long been considered a marker for toxic pulmonary lesions: **lactate dehydrogenase** (increased membrane permeability and open cell lysis), **acid phosphatase** (increased phagocytic activity or cell destruction) and **alkaline phosphatase** (toxic damage or proliferation of type 2 pneumocytes) [Dusinska M and al., 1998]. Intratracheal AM's infusion into our study increases significantly LDH activity in the wash fluid on the 3rd and 7th days and these results confirm his/her pneumotoxic effect in the early post- application stage. LDH leaks from dead or damaged cells when the integrity of the membrane is compromised. Our data are consistent with studies that demonstrate elevated enzyme levels in rats treated with amiodarone [Taylor MD et al., 2003; Wilson BD and al., 1993]. LDH activity in the AM plus U-74389G combination treatment group was elevated on days 3 and 7. The AcPh level was significantly higher compared to the control group on days 3 and 7, but U-74389G weakened this one parameter on day 7. In fact treatment with U-74389G alone results in significant increase in AcPh activity on day 3. The reason for this is unclear. A possibility is that AcPh activity as a marker for increased phagocytic activity increased, as did the number of alveolar macrophages in the U-74389G group. Alkaline phosphatase activity (AlPh) in the bronchoalveolar lavage was increased on day 3, remained higher on days 7 and 28 in both groups – with AM administration, and combined treatment with AM and U-74389G. This discovery suggests early toxic effects and proliferation of type 2 pneumocytes during the recovery period. We also observed a very slight increase in AlPh in BALT of the group of rats administered only U -74389 G on day 3 and even a drop below control levels on day 7, with a subsequent increase, but still below control on day 28 (Fig. 22). Our histological research also show well -marked proliferation of type 2 pneumocytes. U-74389G has moderate protection or does not prevent the early AM- induced increase in enzyme activities. This was surprisingly discovery. So As U-74389G has potent membrane- stabilizing and antioxidant activity, it would exhibit protective effect. Lazaroid, including U -74389G, are known to have big steady- state volume of distribution (4.9 L/ kg) and good penetration into tissue, including the lungs [Wilson JS and al., 1991]. The protocol accepted for this study, requires in advance treatment with U-74389G (2 hours before instillation AM) to ensure sufficient concentration of the aminosteroid in the lung parenchyma. One possible explanation of our the result is that U-74389G has not reached optimal pulmonary concentration to prevent early AM cytotoxic effect. According to Taylor and al. direct toxic effect of AM on epithelial cells occurs soon after it AM application – LHD activity is significantly elevated 15 minutes after AM it [Taylor MD et al., 2003]. On the other hand, it has been reported that the same dose and route of administration of the aminosteroid (before and after twice daily after the aggressive agent) limit CNS damage [Vignes JR et al., 2006] and acute necrotizing pancreatitis [Alhan E et al., 2006]. But the lungs are unique because have big epithelial a surface that is at risk of damage mediated by oxidants. [Comhair SAA et et al., 2002]. The tracheobronchial wood and the alveolar space are exposed to reactive oxidizing species and lungs need additional antioxidant resources to prevent oxidative airborne injuries noxious.

Our studies show that U-74389G has protected effect against early inflammatory response after intratracheal infusion of AM. This lazaroid inhibits AM- induced increase in inflammatory activity in the white one fraction, which may be related to its membrane- stabilizing and antioxidant properties [Hall ED et al., 1987]. It has also been shown that aminosteroids prevent the release of arachidonic acid metabolites acid [Braughler JM et al.,

1988; Richards IM and al., 1992]. Oxidative stress may be responsible for AIBT, which is maintained by increased production of superoxide anions from stimulated alveolar macrophages obtained from rats treated with AM [Vereckei A et al., 1993]. Some studies revealed that AM is metabolized to an aryl radical, which can lead to other reactive oxygen species [Taylor MD et al., 2003; Nicolescu AC and et al., 2007]. The Lazaroids neutralize the free oxygen radicals and are very effective in inhibiting iron - dependent lipid peroxidation , similar to vitamin E [Hall ED et al ., 1995; Braughler JM et al ., 1988; Clark W.M. and al ., 1995], prevent early , but not late , stages of oxygen- induced cell damage [Huang H et al ., 2001].
U-74389G has powerful stabilizing effects on cellular membranes [Jacobsen EJ et al., 1990; Clark WM et al., 1995]. Because of its lipophilicity this 21-aminosteroid has a high affinity to the lipid bilayer and is included in it, as occupies a strictly defined position and orientation [Raub TJ et al., 1993]. The interaction between piperazine derivatives nitrogen and phosphate groups of the lipid membrane increases her resistance, limits the movement of peroxyl radicals in the membrane, their interaction with fatty acids and peroxide disability [Hall ED et al., 1987].
In conclusion, the lazoroid U-74389G administered before and after AM intratracheal instillation, there is protected effect on the early one inflammatory response, assessed by cytological and biochemical markers in bronchoalveolar lavage liquid.

1.1. Effects of U- 74389G on markers of antioxidant defense system, lipid peroxidation and pulmonary fibrosis in pulmonary homogenate

Table 6 presents the antioxidant enzyme activity in lung homogenate , expressed in U/g tissue or in mcat /g, corresponding to publications in international journals. The activity of **superoxide dismutase (SOD)** compared to the control group decreased significantly on day 3 (67% compared to control) in the AM group. SOD activity did not change significantly with the combined treatment AM+U-74389G (group 3) compared to group 2 (AM group) (Fig. 24).
Catalase (CAT) activity is increasing significantly on day 3 in groups 2 and 3 compared to controls animals . CAT activity on day 3 increases less in the AM and U-74389G groups (115% vs. control) than in the AM group (123% vs. the control) (Fig. 25).
Glutathione peroxidase (GP) activity decreases significantly in both experimental groups (2 and 3) on the 3rd and 7th day compared to the control group. The reduction of the enzyme activity in group 3 (AM and U-74389G) is insignificant less than that in group 2 (AM) in the same time points. The lowest decrease in glutathione peroxidase activity in the group with isolated administration of U-74389G (Fig. 26).
Hydroperoxide concentrations (Fig. 27) are significantly increased in the plasma of rats treated with amiodarone (group 2) on day 7, when reach 146% compared to the control group ($p = 0.03$). The combination of amiodarone and U-74389G (group 3) also increases this marker, but it remains significantly lower compared to group 2 in the same time point ($p = 0.046$).
The measurement of malondialdehyde (**MDA)** content (Fig. 28) in the lung homogenate shows a similar pattern of change. The MDA content on day 7 increases more clearly only in the group treated with amiodarone (169%) compared to the control group ($p = 0.03$). The combined treatment with amiodarone and lazaroid reduce significantly this index on the same day compared to the AM group ($p = 0.037$).

Table 6. **Superoxide dismutase (SOD)** , **catalase (CAT)** , **glutathione peroxidase (GP)** activity in lung homogenate on days 3 and 7 after intratracheal administration of amiodarone (AM)

parameter / groups	**SOD**		**CAT**		**GP**	
	day 3	day 7	day 3	day 7	day 3	day 7
	U/g mean±SEM	U/g mean±SEM	mcat /g mean±SEM	mcat /g mean±SEM	U/g mean±SEM	U/g mean±SEM
Control (group 1)	325.42±54.18	325.42±54.18	9.51±0.47	9.50±0.47	60.12±5.03	62.16±7.30
AM (group 2)	217.60±51.39*	272.81±26.79	11.67±0.22*	10.96±0.46	43.21±2.78	38.84±2.03*
AM+U-74389G (group 3)	242.43±55.76*	284.57±45.68	10.89±0.47*	9.30±0.82	44.86±4.75	40.08±3.09*
U-74389G (group 4)	303.42±50.86	297.87±44.63	10.19±0.22	9.84±0.33	56.39±5.33	50.92±4.03

* P < 0.05 versus control group

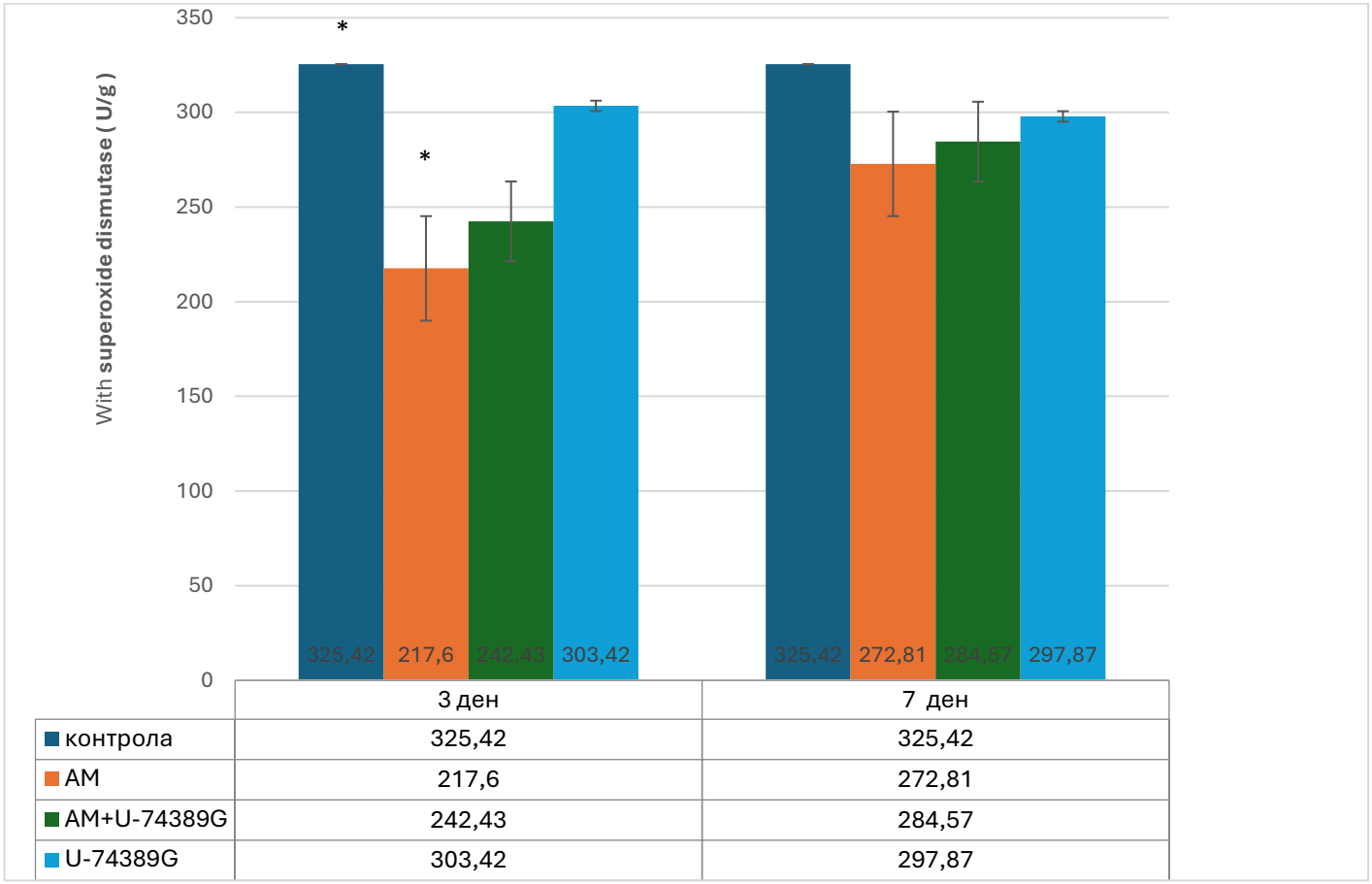
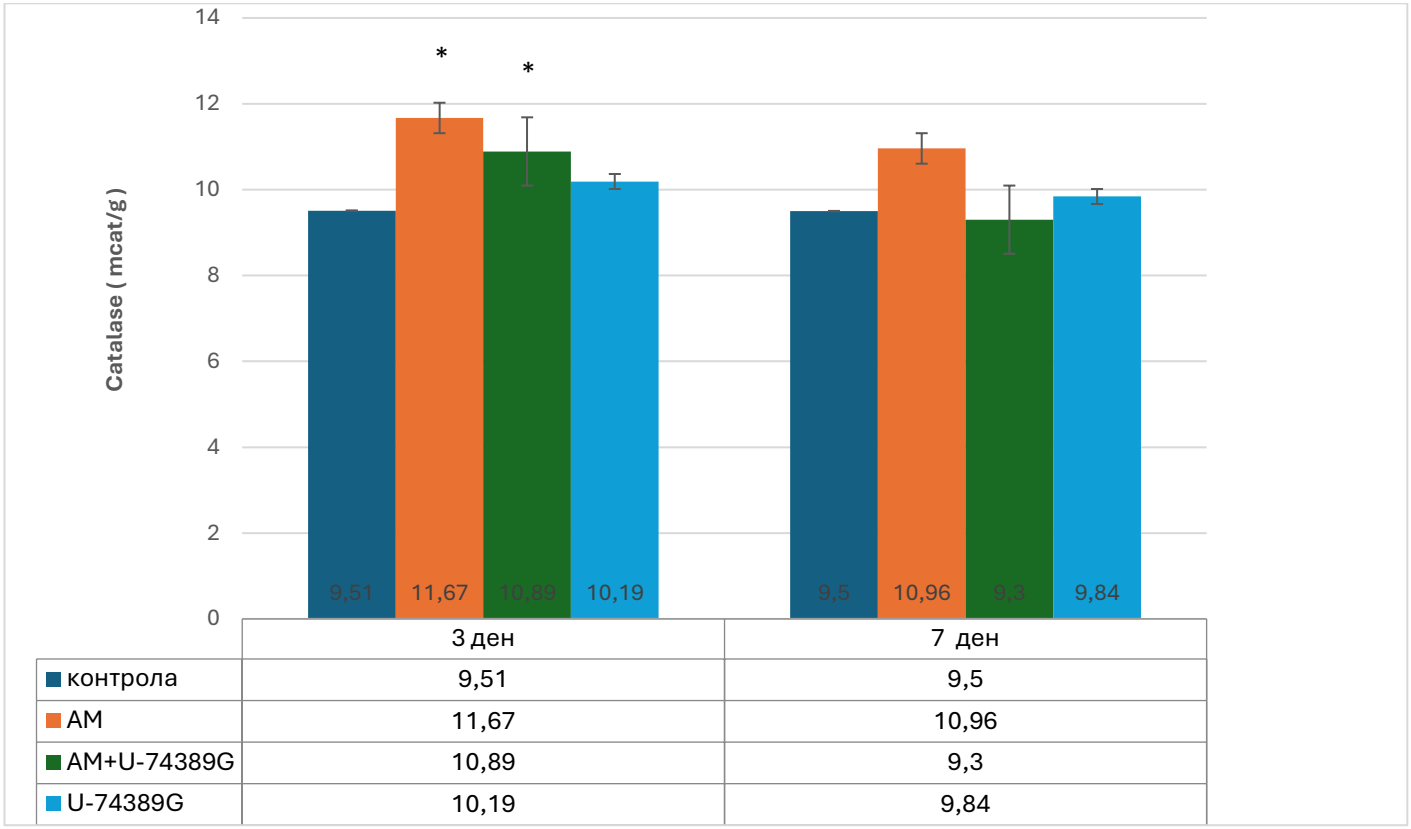
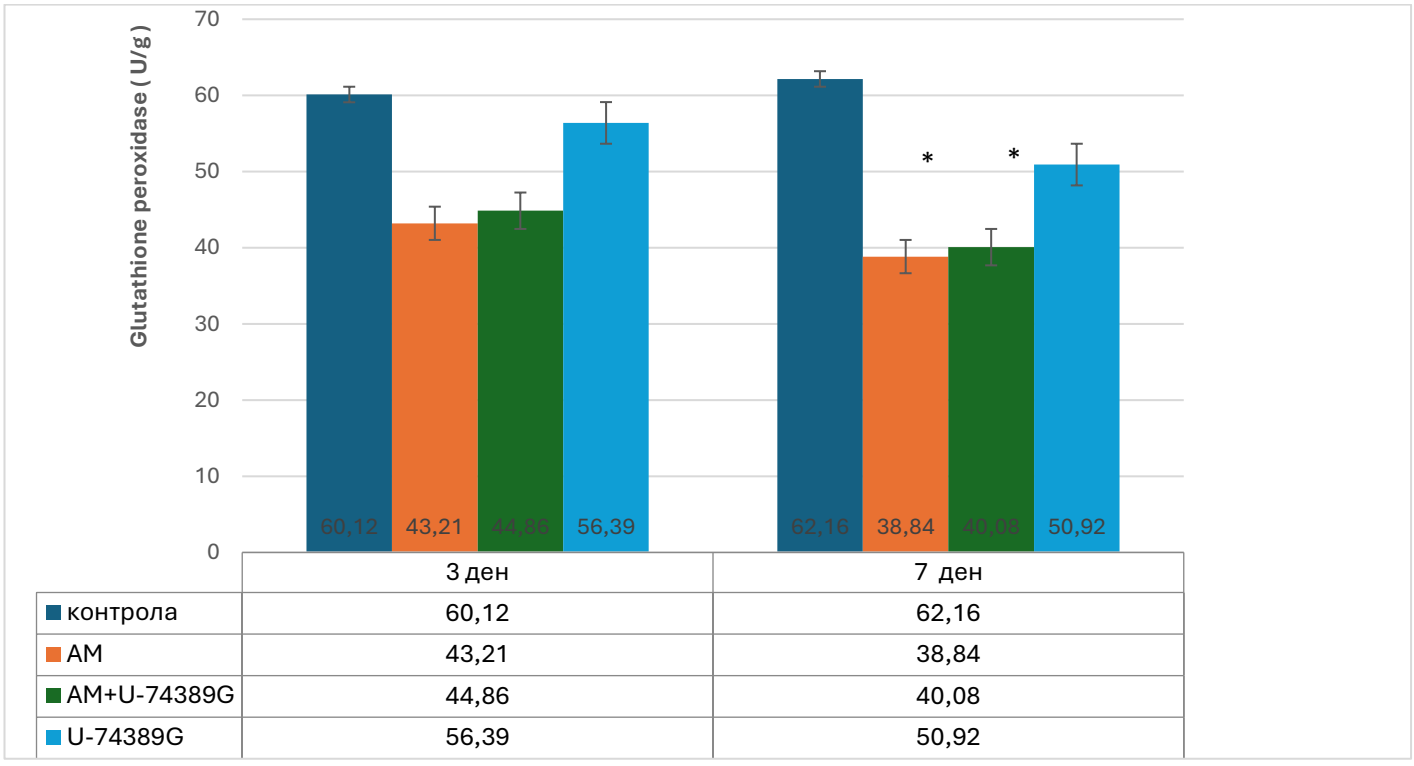


Fig. 24. Effect of U **-** 74389 G on **superoxide dismutase (SOD)** activity in the lung homogenate after i.t. administration of AM; * p<0.05 compared to control



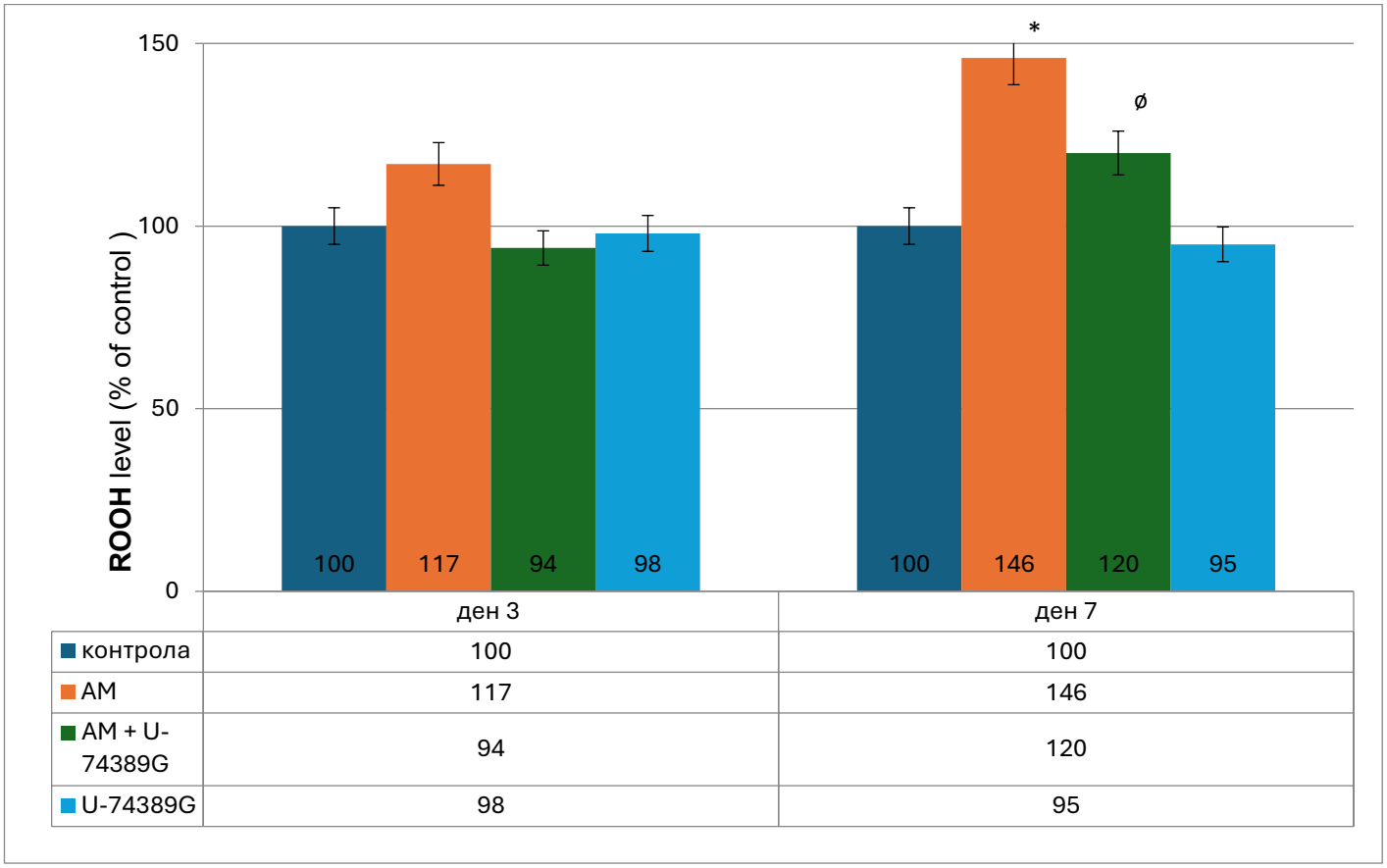
	3 ден	7 ден
контрола	9,51	9,5
AM	11,67	10,96
AM+U-74389G	10,89	9,3
U-74389G	10,19	9,84

Fig. 25. Effect of U **-** 74389 G on **catalase (CAT)** activity in the lung homogenate after i.t. administration of AM; * p<0.05 compared to control



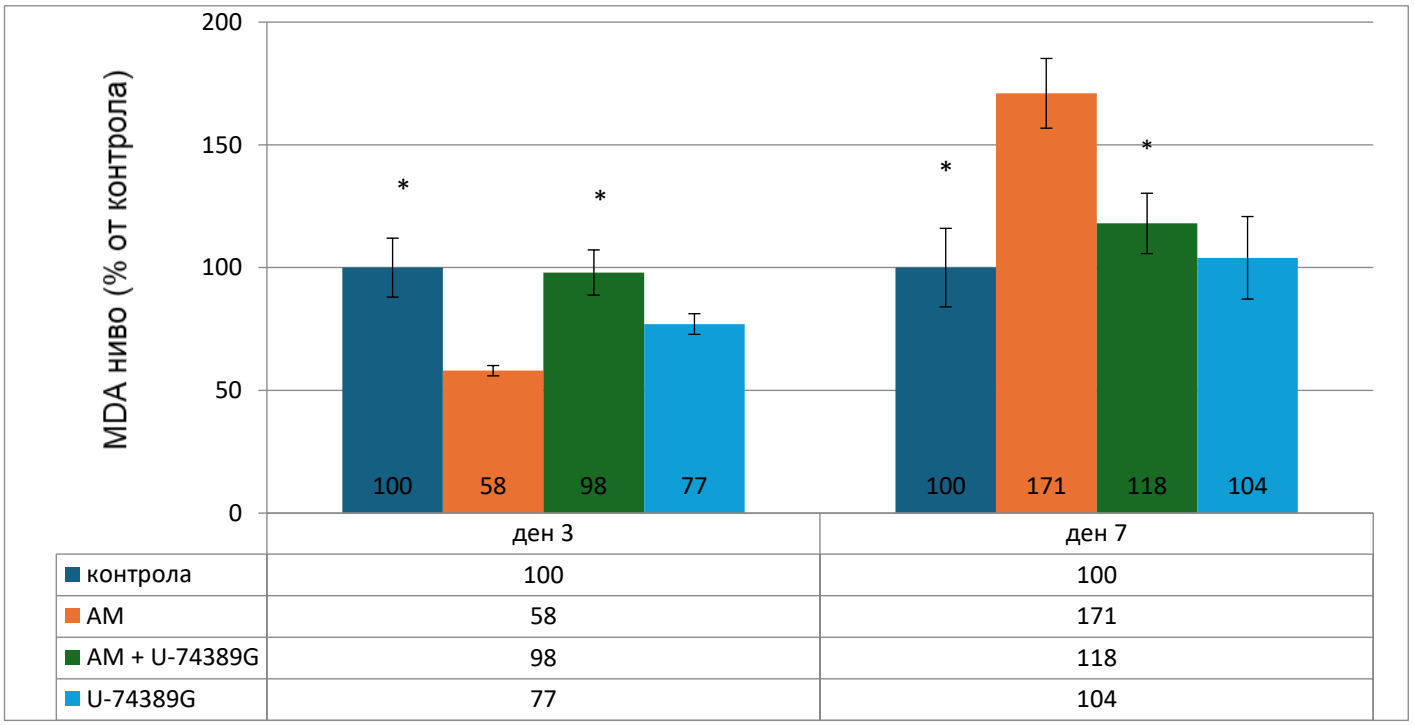
	3 ден	7 ден
контрола	60,12	62,16
AM	43,21	38,84
AM+U-74389G	44,86	40,08
U-74389G	56,39	50,92

Fig. 26. Effect of U -74389 G on **glutation peroxidase (GPX)** activity in the lung homogenate after i.t. administration of AM; * p<0.05 compared to control



	ден 3	ден 7
контрола	100	100
AM	117	146
AM + U-74389G	94	120
U-74389G	98	95

Fig. 27. Plasma **hydroperoxide** concentrations on days 3 and 7 after intratracheal administration of amiodarone. AM – amiodarone , ROOH – hydroperoxide ; * p<0.05 versus control; ø p<0.05 versus AM



	ден 3	ден 7
контрола	100	100
AM	58	171
AM + U-74389G	98	118
U-74389G	77	104

Fig. 28. **Malondialdehyde** content in lung homogenate on days 3 and 7 after intratracheal administration of amiodarone . AM – amiodarone , MDA – malondialdehyde ; * p<0.05 vs. AM

Biochemical analyses of markers for pulmonary fibrosis in lung Pulmonary fibrosis was assessed by measuring the content of hydroxyproline (HP) and collagen in lung **homogenate.** homogenate on day 14 and 28 after amiodarone administration (Figures 29, 30 and 31).
The isolated administration of amiodarone (group 2) significantly increase hydroxyproline (**HP)** content (Fig. 30) on day 14 up to 249% compared to control and on day 28 to 235%, respectively. The lazaroid U-74389G in combination with amiodarone (group 3) significantly reduce this marker (0.68±0.08 mcg / ml in lung homogenate) on day 28, compared to group 2 - amiodarone only (1.07±0.17 mcg / ml in lung homogenate). The results in group 4 (treated with U-74389G alone) were comparable to those of the control group group.
Fig. 31 shows the level of **collagen** in the lung homogenate on day 28. Collagen in the group treated with amiodarone (40.67±3.87 mcg / ml) significantly increased compared to the control (30.07±2.11 mcg / ml), while, due to the protective effect, in the group treated with AM + U-74389G, collagen even decreased moderately (26.93±2.1 mcg / ml).

Table 7. Values of hydroxyproline (on days 14 and 28) and **collagen** (on day 28) in lung **homogenate** (LH) after intratracheal administration of amiodarone .

Group	Hydroxyproline 14 day mcg / ml LH	Hydroxyproline 28 day mcg / ml LH	Collagen 28 day mcg / ml LH
Control	0.406 ± 0.03	0.456 ± 0.0 17	30.07 ± 2.11
Amiodarone	1.0 1 ± 0.0 7	1.07±0.17	40.67 ± 3.87
AM + U-74389G	0.52±0.06	0.68±0.08	26.93 ± 2.1
U-74389G	0.386±0.03	0.45±0.023	-

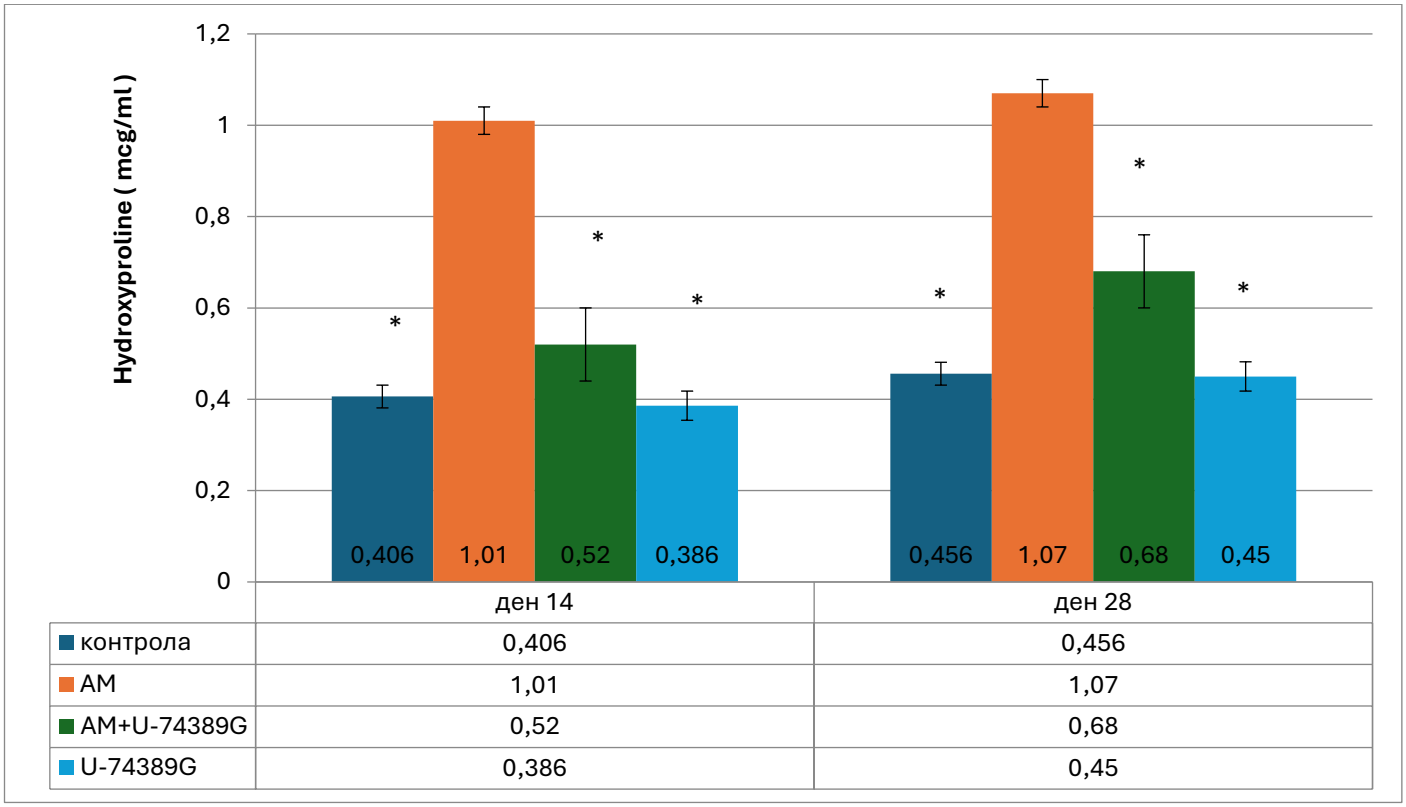
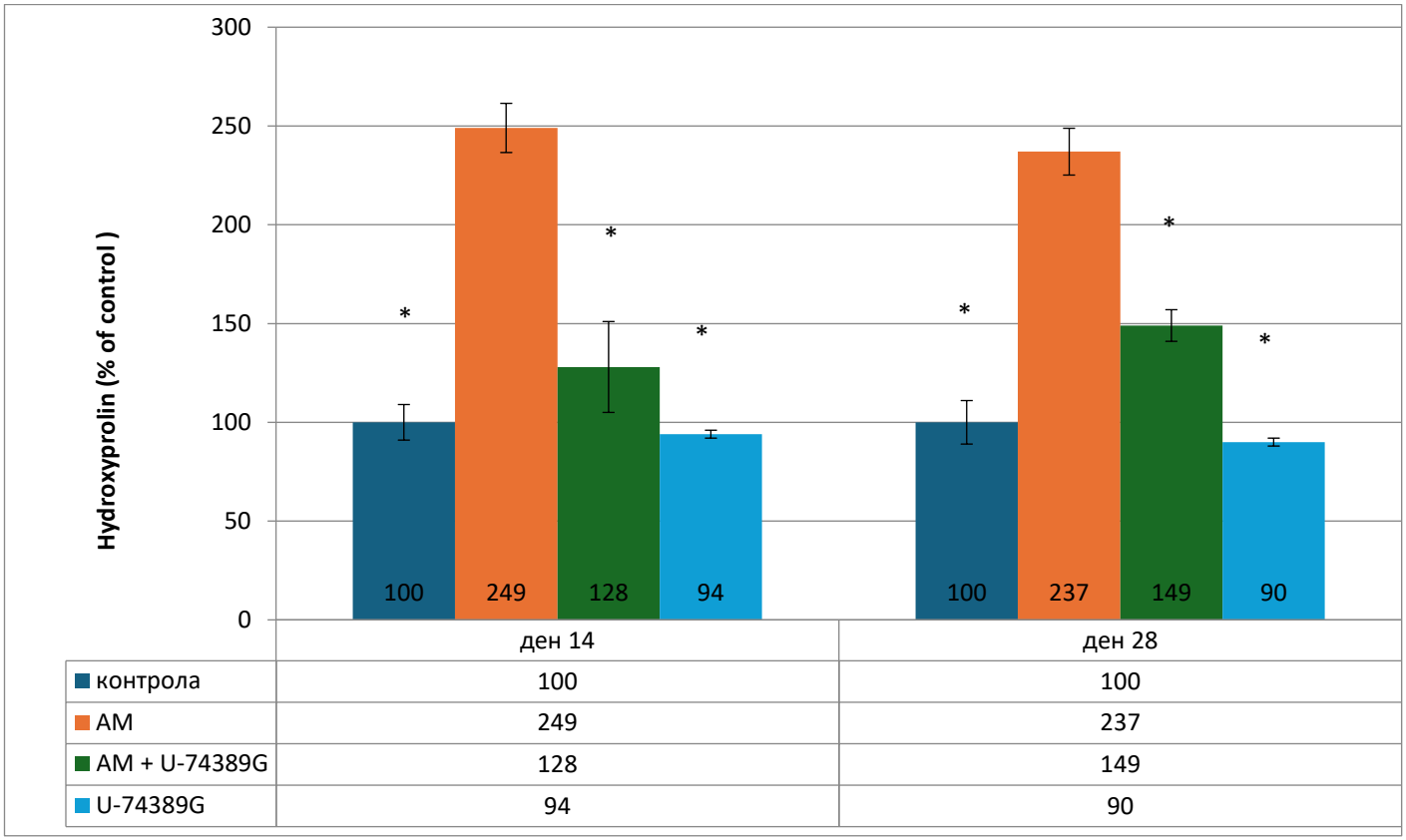
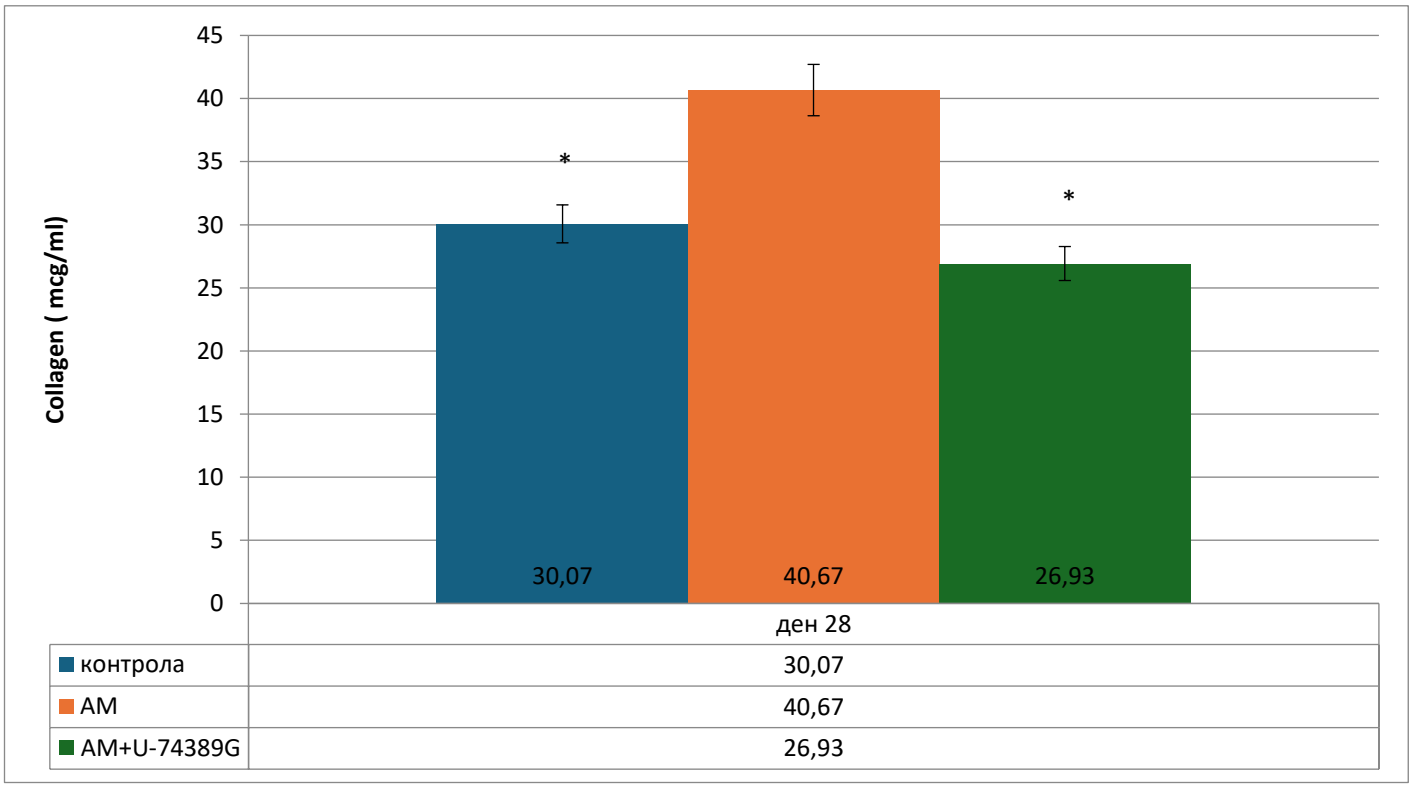


Fig. 29. Content (concentration) of **hydroxyproline (HP)** mcg / ml in lung homogenate on days 14 and 28 after intratracheal administration of amiodarone (AM); * p< 0.05 vs. AM



	ден 14	ден 28
контрола	100	100
AM	249	237
AM + U-74389G	128	149
U-74389G	94	90

Fig. 30. Percentage **hydroxyproline (HP)** content in lung homogenate on days 14 and 28 after intratracheal administration of amiodarone (AM); * p< 0.05 compared to the AM group

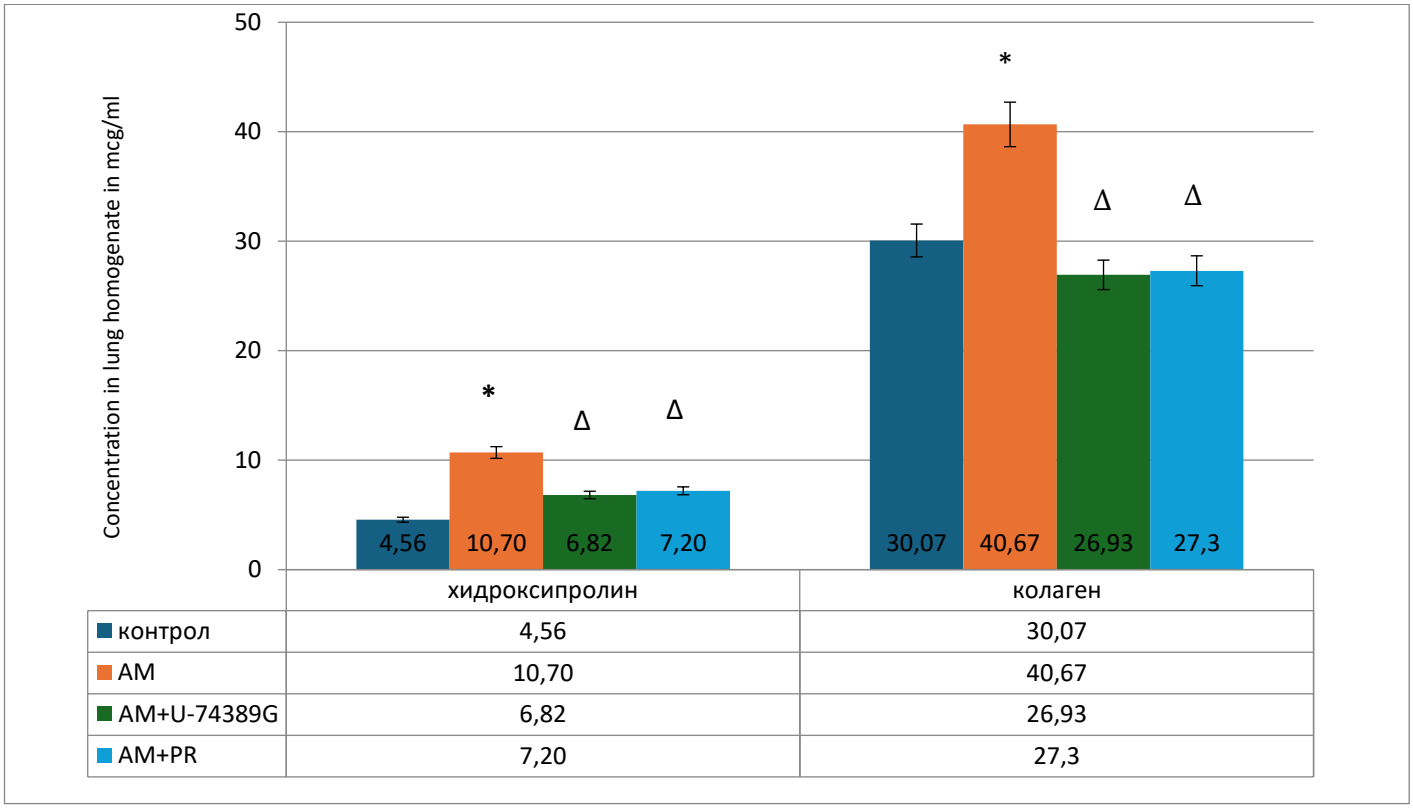


	ден 28
контрола	30,07
AM	40,67
AM+U-74389G	26,93

Fig. 31. **Collagen** content mcg / ml in lung homogenate on day 28 after intratracheal administration of amiodarone (AM); * p< 0.05 vs. AM

Comparison of the effects of U-74389G and prednisolone on markers of the antioxidant defense system and pulmonary fibrosis in lung homogenate (LH).

We were equal . the effects of U-74389G and prednisolone on markers of pulmonary fibrosis in lung homogenate . We studied 72 rats Wistar, divided into four groups: 1 – **control**; 2 – with intratracheal injection **amiodarone**; 3 – treated with **amiodarone and U -74389 G**; 4 – with administered **amiodarone and prednisolone**. Amiodarone was administered intratracheally (6.25 mg / kg ; 3.125 mg / ml) on days 0 and 2. Intraperitoneally we applied U -74389 G - 15 mg / kg / d and prednisolone - 10 mg / kg / d , three times - two hours before intratracheal application of AM, as well as on days 0 and 2 after AM. Pulmonary fibrosis was assessed by the content of hydroxyproline and collagen in lung tissue. homogenate on day 28 after amiodarone administration (Fig. 32) **.** Hydroxyproline content in lung homogenate on day 28 after intratracheal administration of AM was as follows: control group – 4.56 mcg / ml, AM group – 10.7 mcg / ml, AM + U-74389G – 6.82 mcg / ml, AM + PR – 7.2 mcg / ml (fig. 32).
Intratracheal AM application increased significantly collagen content **in** the lung homogenate on day 28 (40.67 mcg / ml), while combined administration of AM with U-74389G and AM with prednisolone even reduced the amount of collagen (AM + U-74389G – 26.93 mcg / ml and AM + PR – 27.3 mcg / ml , respectively). In the control group collagen content in the lung homogenate was 30.07 mcg / ml (Fig. 32). The results show that amiodarone leads to moderate interstitial and perivascular fibrosis, thickening of the interstitial spaces, and cellular infiltration, especially expressed on day 28. The isolated Intratracheal administration of amiodarone is also associated with significantly increase hydroxyproline and collagen content in the lung homogenate on day 28, expression of pulmonary fibrosis. U -74389 G and prednisolone significantly and comparable decrease the values of these markers, proving their inhibitory effect on development of pulmonary fibrosis after intratracheal administration of amiodarone. It is also evident that the protective effect of U-74389G on pulmonary fibrosis at day 28 slightly surpasses that of prednisolone.



	хидроксипролин	колаген
контрол	4,56	30,07
AM	10,70	40,67
AM+U-74389G	6,82	26,93
AM+PR	7,20	27,3

Fig. 32. Effect of U -74389 G and prednisolone on the amount of **hydroxyproline** and **collagen** in lung tissue homogenate on day 28 after i. t. administration of AM
* p< 0.05 versus control; Δ p< 0.05 vs. AM

Histology. Histological image of amiodarone- induced pulmonary fibrosis on day 28 after amiodarone administration and the recovery group changes treated with U -74389 G (Fig. 33-36).

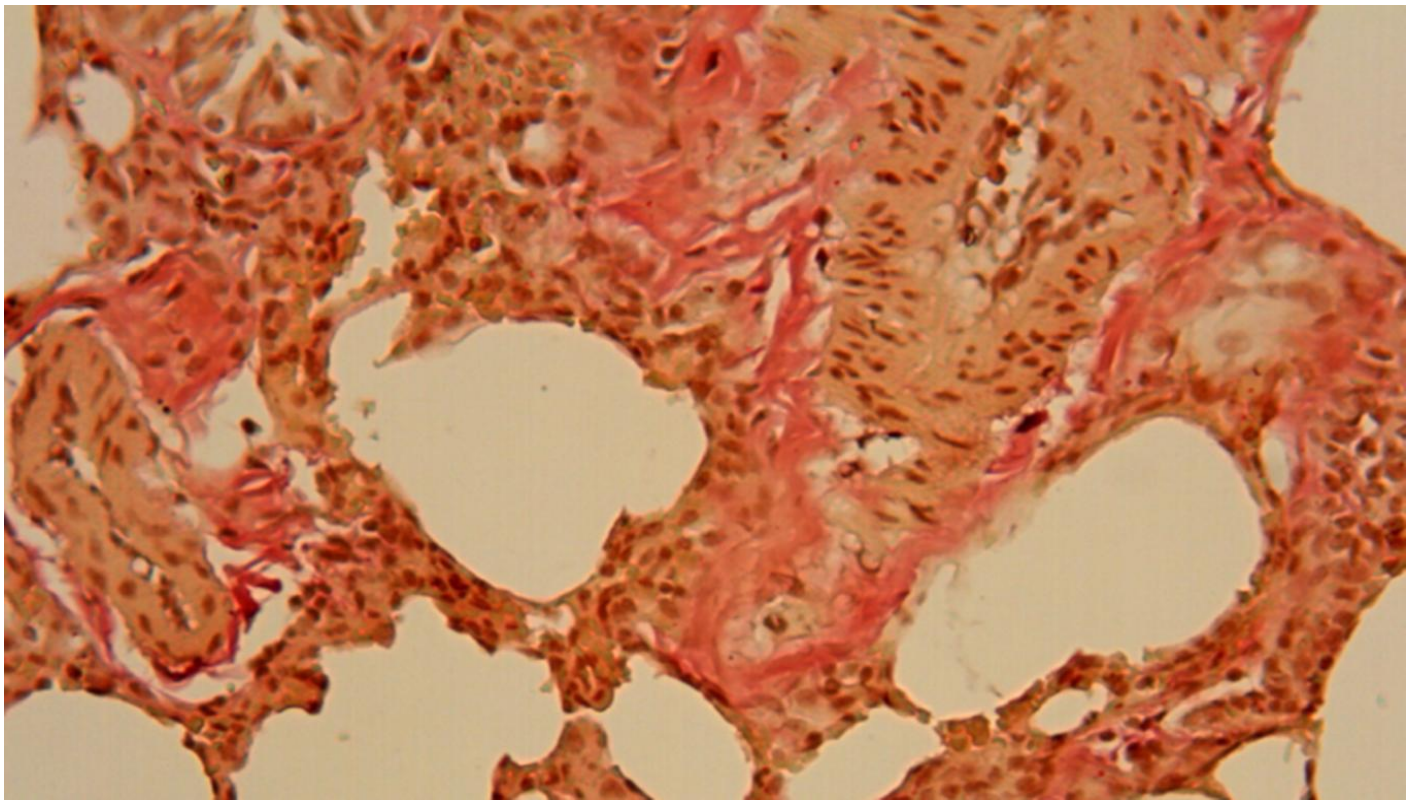


Fig. 33. Histological changes on day 28 after intratracheal Amiodarone application: moderate interstitial and perivascular fibrosis, thickening of the interstitial spaces (staining Van Gieson, x100)

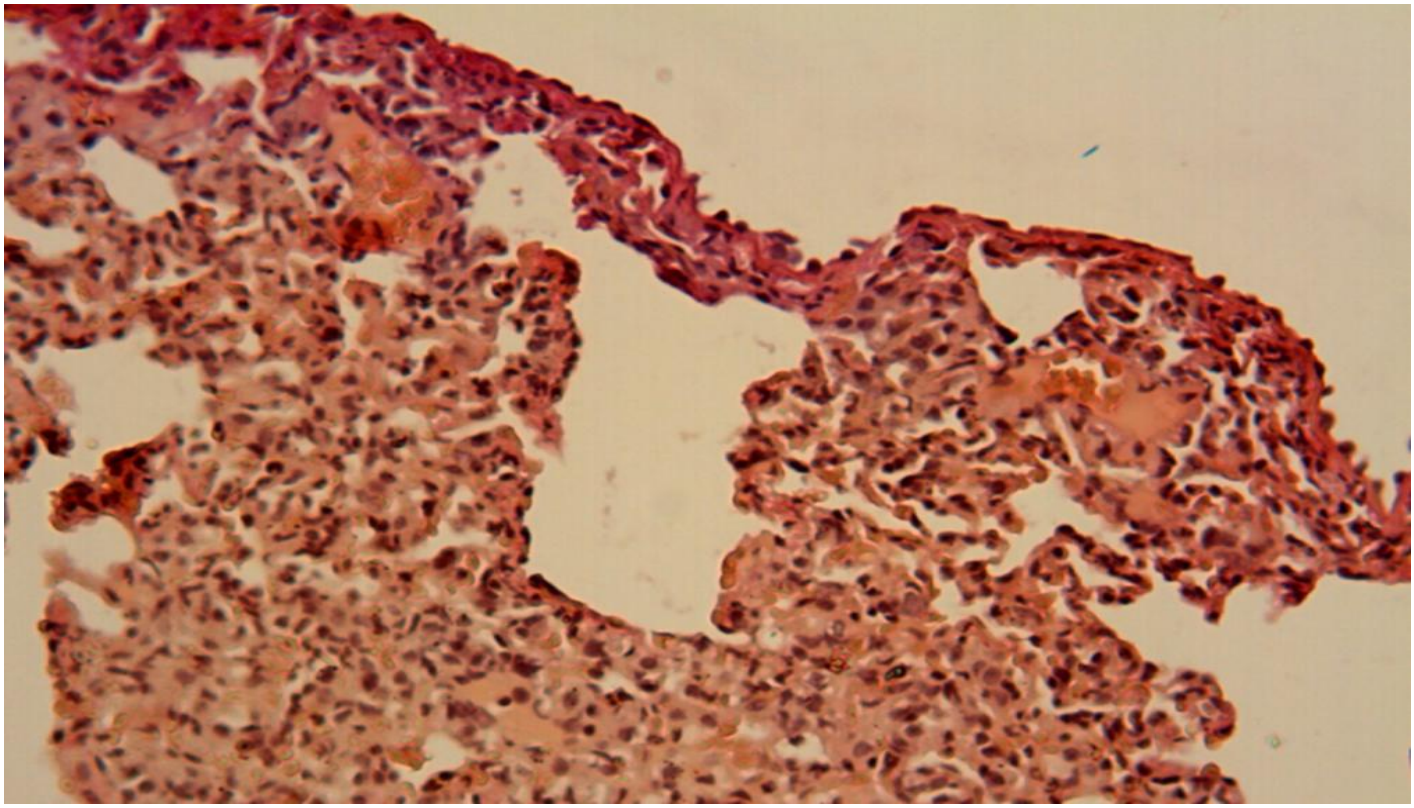


Fig. 34. Histological changes on day 28 after intratracheal amiodarone application: involved subpleural fibrosis, cellular infiltration (staining) Van Gieson, x100)

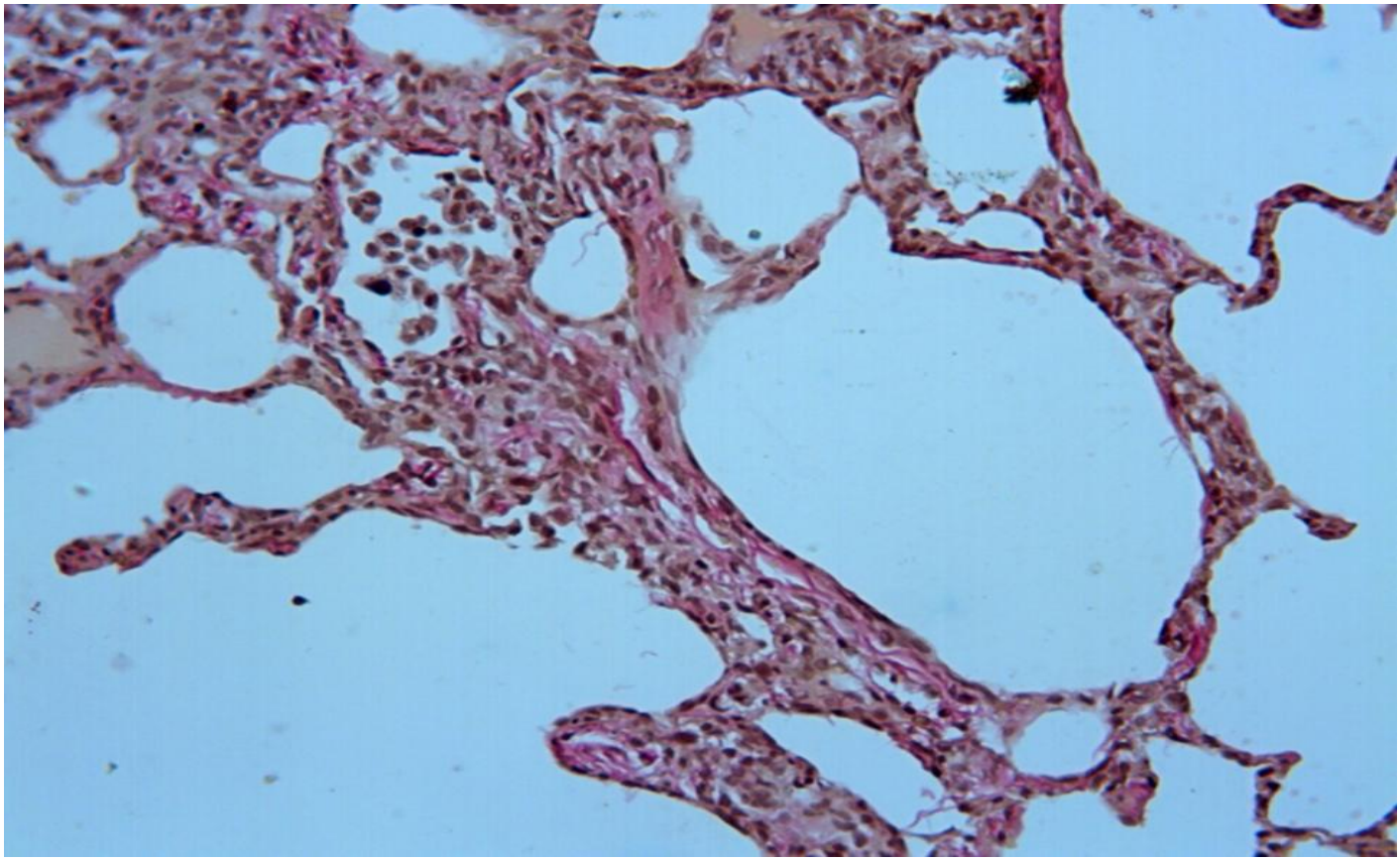


Fig. 35. Histological changes on day 28 after intratracheal Amiodarone application: moderate interstitial and perivascular fibrosis, thickening of the interstitial spaces (coloring) Van Gieson, x 100)

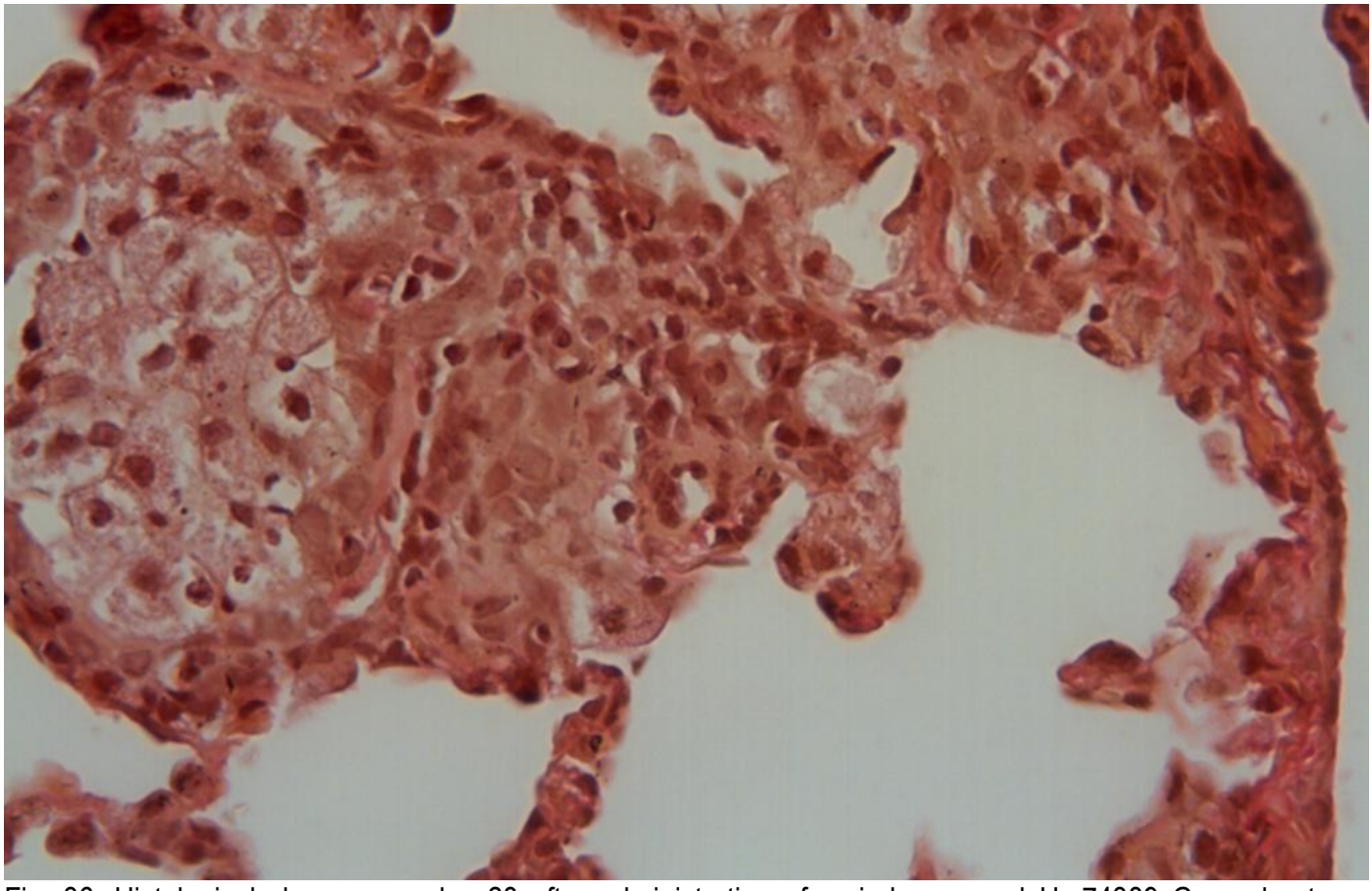


Fig. 36. Histological changes on day 28 after administration of amiodarone and U -74389 G: moderate interstitial fibrosis, focal profiled alveolar macrophages (staining Van Gieson, x 400).

Discussion: Pulmonary fibrosis is a chronic progressive condition with a serious prognosis. Its treatment is symptomatic and poorly effective [Crouch E , 1990; 1995; Freis KM and al ., 1994] . Pulmonary fibrosis is a potential unwanted drug reaction in more than 25 drugs – cytostatics, antiepileptics, antifungals, antibacterials, etc. Amiodarone is a benzofuran derivative and is one of the most widely used antiarrhythmic drugs for the treatment of supraventricular and ventricular arrhythmias [Connolly, SJ 1999]. Although the therapeutic his effectiveness is undeniable, the application is limited due to safety profile – toxic effects on the lungs, thyroid gland, eye, liver, pancreas [Connolly, SJ 1999]. About 6-8% of patients treated with AD develop severe lung damage , with mortality in 5-10% of those affected . sick [Pitcher WD and al, 1992]. The changes in amiodarone- induced pulmonary toxicity (AIPT) are well described, while the pathogenetic mechanisms are incompletely understood [Wilson BD and al ., 1990; Reasor MJ and al., 1996], despite the multitude in live and in in vitro experiments. AIBT is expressed in : direct damaging effect [Wilson BD and al ., 1990], induction of apoptosis or necrosis of alveolar epithelial cells type II [Bargout R and al ., 2000], endothelial cells [Futamura Y , 1996; Kachel DL and al ., 1990] and alveolar macrophages [Reinhart and al ., 1996], which generate products as free radicals and fibronectin [Vereckei and et al ., 1993]; pulmonary phospholipidosis [Reasor MJ and al ., 1996]; influencing the properties of cellular membranes and increase in intracellular free calcium [Powis , G. Et al ., 1990]; mitochondrial dysfunction [Card JW and al ., 2003]; damage to the epithelium and basal cells membranes , followed by migration of inflammatory cells such as macrophages, lymphocytes and neutrophils into the lung parenchyma, release of cytokines (TGF-ß1, TNF-α, Il-1, Il-6), activation of fibroblasts , modeling of the extracellular matrix, including collagen deposition [Card JW and al ., 2003; 1995; Reinhart PG and al ., 1997] .

Numerous models of AIBT have been developed in hamsters [Cantor and al ., 1984; Daniels and al ., 1989; Wang and al ., 1992; Blake et al.,1995] and rats [Reinhart et al ., 1997] in order to clarify the pathophysiological mechanisms of its development. Oral administration of AM in animals leads to pulmonary phospholipidosis without inducing a pronounced inflammatory and fibrotic response [Heath and al ., 1985; Riva and al ., 1987]. When very high doses of AM were administered for a long time (13 months), inflammatory changes and pulmonary fibrosis were described [Vereckei, 1993; Carvalho and al ., 1996]. Intratracheal administration of AM leads to the development of an inflammatory reaction [Cantor and al ., 1984; Blake and Reasor , 1995] and subsequent fibrosis in hamsters and rats [Reinhart et al ., 1997; Taylor et al. al ., 2000; 2003]. According to Taylor , a single intratracheal administration of AM , at a dose of 6.25 mg / kg as aquatic A solution with a concentration of 3.125 mg / ml in rats did not increase the level of hydroxyproline , used as a marker of pulmonary fibrosis. Its introduction on days 0 and 2 led to more pronounced fibrosis, maximally expressed on day 28 [Taylor et al.,2002].

The experimental data show that intratracheal infusion of 6.25 mg / kg amiodarone on days 0 and 2 causes pulmonary toxicity The results of the study give us reason to assume that amiodarone may affect antioxidant defense system in the lungs. The research on **pulmonary homogenate** show that the activity of the key antioxidant enzyme **superoxide dismutase (SOD)**, decreases significantly on the 3rd day after treatment with amiodarone (group 2). At the same time, SOD activity after combined treatment with amiodarone and U-74369G (group 3) decreased less than in the AM group.

Changes in catalase **(CAT)** activity show different kinetics. CAT levels in groups 2 and 3 increase significantly on day 3 compared to controls. This index in group 3 remained below the enzymatic activity measured in the amiodarone group but not established significant difference between the two treatment groups (2 and 3).

The activity of another key antioxidant enzyme, **glutathione peroxidase (GPX)**, decreases significantly in both groups 2 and 3, on the 3rd and 7th day, compared to the control group. The dynamics of enzyme activities prove that U-74389G mitigates toxic effects of amiodarone on the activity of key antioxidant enzymes.

On day 7, in the amiodarone-treated group, it was found that increase in plasma **hydroperoxide** concentrations and malondialdehyde **(MDA)** content, which shows possible increase in lipid levels peroxidation and oxidant - induced damage in the lungs at this time. The literature shows contradictory data on the effect of amiodarone on lipid peroxidation. Some authors assume that the oxidative stress plays key role in AM- induced pneumotoxicity [Jessurum GA and al., 1997; Leeder RG and a., 1994] and that it causes

membrane destabilization Leeder RG and al ., 1994]. Investigating the protective effect of L-carnitine in amiodarone- induced pulmonary changes, Gado and Aldamash found that AM increases glutathione reductase levels and decreases levels of SOD, catalase and MDA content in the lung homogenate [Gado AM and al., 2013]. Amiodarone increases the enzyme activity of SOD, catalase, as well as lipid peroxidation in others organs of rats (live, kidneys, testes) after oral administration [Chakraborty A and al., 2014]. Intratracheal amiodarone infusion in hamsters induces increase in lipid peroxidation in the bronchoalveolar lavage fluid and lung homogenate Blake TL and al., 1995]. Ribeiro et al. demonstrated that amiodarone does not affect antioxidant enzymatic activity and inhibits about 70% of the lipid peroxidation [Ribeiro SM and al., 1997]. Conversely, other researchers discover production of free radicals in AM solutions and formation of aryl radicals in photolysis of water solutions of amiodarone or N- desethylamiodarone [Nicolescu AC and al., 2007]. It is also suggested that the generation of hydroxyl radicals in concentrated aquatic solution of AM is related to the presence of a C- centered radical in the amiodarone molecule. Nicolescu and al. suggest that mitochondrial dysfunction in peripheral pulmonary epithelial cells in the early phase is a more significant cause of AM toxicity than overproduction of ROS [Nicolescu AC and al., 2007].

It has been shown that alveolar macrophages are the first cell type affected in the white blood cell liver, leading to a " foamy" appearance, a process associated with the initiation of phospholipidosis [Reasor MJ and al., 1996; Bargout R and al., 2000]. It was found that **"foamy" alveolar macrophages** are prone to change pulmonary recovery and/or progression of fibrosis after AM administration. In our a study found There was a significant increase in the number of macrophages in the amiodarone-treated group. The cytoplasm in these macrophages was foamy and vacuolated (Figures 8 and 9. Results consistent with Nacca and al. [Nacca N and al., 2012], which mentioned that the classic finding is lipid - laden macrophages in the alveolar spaces, known like "sparkling" cells, in pulmonary amiodarone- induced toxicity. This drug also so encourages the accumulation of phospholipids in tissues and has a strong inhibitory effect on lysosomal phospholipase, leading to accumulation of phospholipids in membrane -rich structures [Jessurum GA and al., 1997; Bargout R and a., 2000]. Amiodarone affects production of toxic oxygen radicals and can induce the accumulation of phospholipids in tissues with direct cytotoxic effect on the alveolar-capillary membrane in the white liver.

Although the inflammation is not noticeable process, the inflammatory response is an important feature of pulmonary amiodarone - induced toxicity. Inflammation includes the recruitment of specific cells, including polymorphonuclear neutrophils, lymphocytes, plasma cells and eosinophils [Halliwell WHY, 1997]. Inflammatory cells can induce tissue damage through the release of toxic substances such as oxygen free radicals, proteases and inflammatory mediators as cytokines and chemokines. An early inflammatory response has been demonstrated after amiodarone administration to the lungs with a fourfold increase in total cell count and protein content in BALT on day 3 after AM treatment, as well as a significant increase in neutrophil count, which remains elevated to 28th day [Stavreva GT, Shopova VL, Dancheva VY, 2008].

Amiodarone- induced pulmonary fibrosis is a serious complication, but the mechanism her remains no completely clarified. Through electron - microscopic and histological studies, Mahdi demonstrated expressed pulmonary fibrosis after chronic oral treatment with AM in rats [ahdy AA and al., 2014]. Recurrent changes associated with inflammatory process, are considered catalysts of the progression of the fibrotic response. The increase in hydroxyproline content on days 14 and 28 after amiodarone treatment supports the claim that AM can lead to pulmonary fibrosis.

The 21-aminosteroid U-74389G loses weight the effects of amiodarone on lipid peroxidation. Lazaroids inhibit membrane lipid peroxidation by trapping peroxyl radicals, a mechanism similar to that of vitamin E. They potentiate antioxidant efficacy of vitamin E by slowing down the oxidation of vitamin E during linoleic peroxidation acid [Braughler JM and et al., 1987]. The Lazaroids inhibit oxidant- mediated damage in isolated rat lungs after ischemia / reperfusion [Haynes J and al., 1970]. They also so have high affinity to membrane lipids and by inclusion in the lipid bilayer because of their lipophilicity, the lazaroids produce stabilizing cell membrane effects. The protonated piperazine nitrogen interacts negatively with charged phosphate-containing main groups of the membrane lipid This action limits movement of lipids peroxyl radicals in the membrane, thus reducing their interaction with fatty acids acids and inhibits lipid peroxidation. Saija 's

research and al. prove the strong antioxidant activity of U-74389G in a membrane system [Saija A and al., 2001]. They suggested that U-74389G may act as chain catcher spreading lipids peroxyl radicals in membranes. The Lazaroid protects not only cellular membranes from peroxidative stress, but also intracellular components.

The results obtained from our study, show that the development of pulmonary fibrosis is significantly inhibited by U-74389G on days 14 and 28 after amiodarone instillation, based on the reduced hydroxyproline content and the finding in the morphological research.

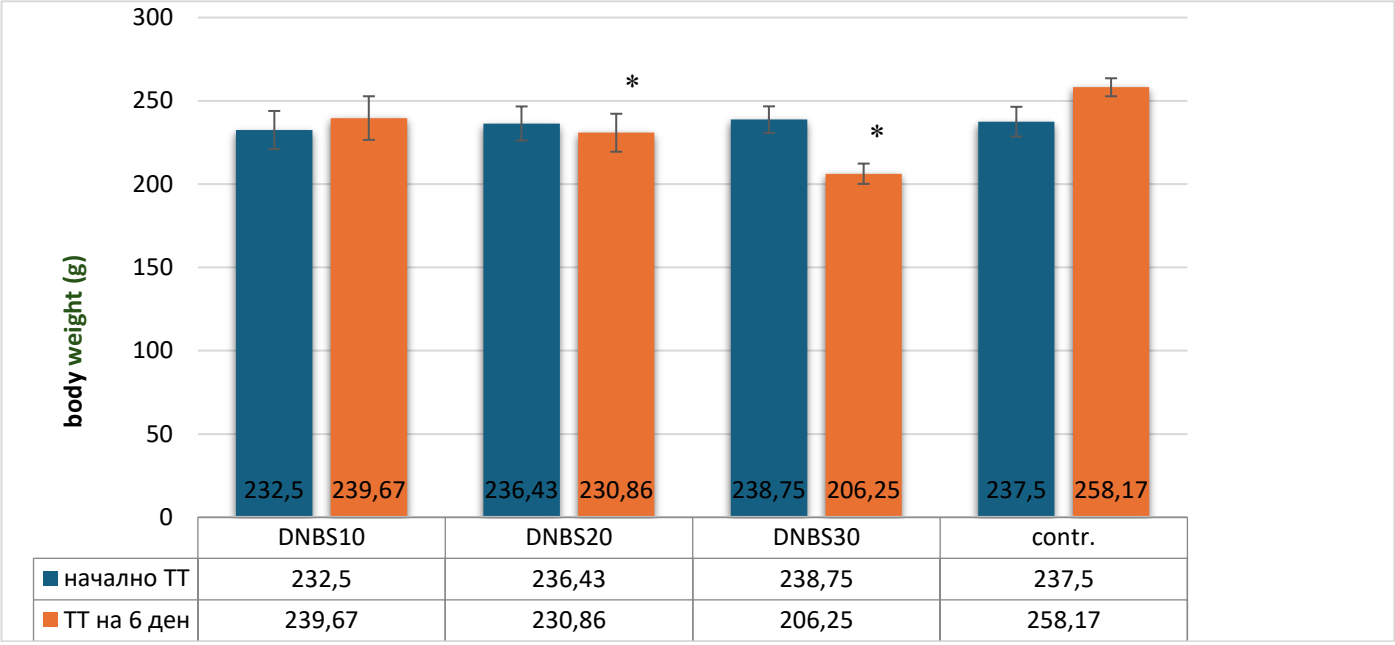
In conclusion, intratracheal administration of amiodarone results in severe toxic damage to the lung parenchyma in rats, assessed by biochemical markers in the lung homogenate and plasma. Amiodarone affects antioxidant defense system in the lungs, promotes lipid peroxidation and causes interstitial and perivascular pulmonary fibrosis, also associated with significantly increase hydroxyproline and collagen content in the lung homogenate on day 28. The 21-aminosteroid U-74389G significantly inhibits lipid peroxidation and softens fibrous changes in the lungs of rats provoked by amiodarone

2. Effect of U-74389G in an experimental model of colitis

2.1. Selection of an appropriate experimental model of IBD (inflammatory bowel disease).

The aim of the study was to establish a reproducible and suitable experimental model of chemical colitis for studying the effect of medicinal substances. The experiments were conducted on 32 male white Wistar rats , divided into 4 groups: control, treated with 0.25 ml of 50% alcohol, rectally, introduced with a probe 8 cm into the colon and three experimental groups, treated with the ulcerogenic agent dinitrobenzenesulfonic acid (2,4-Dinitrobenzenesulfonic acid hydrate , DNBS): DNBS 10 – with experimental colitis induced by 10 mg DNBS dissolved in 0.25 ml of 50% alcohol was administered rectally, with a probe 8 cm into the colon; DNBS 20 and DNBS 30, with a DNBS dose of 20 and 30 mg , respectively . Body weight, food intake and fecal consistency were monitored as clinical markers of induced colitis. On day 6, the colon was removed and the severity of intestinal inflammation was assessed macroscopically and histologically, according to the criteria reported by Antonioli and colleagues [Antonioli and al ., 2007]. The criteria for macroscopic scoring of colon damage are as follows: presence of adhesions between the colon and other organs (0 – none, 1 – small and 2 – large adhesions); presence of ulceration (0 – none, 1 – hyperemia , 2 – ulceration without inflammatory reaction, 3 – wounding with inflammation in 1 area, 4 – 2 areas with ulcers and inflammation, 5 – large lesions with an area < 2 cm , 6 – large lesions with an area > 2 cm); the result increased a in a by 1 unit for each millimeter of column wall thickness. Microscopic examination was performed by light microscopy on sections stained with hematoxylin and eosin and Van Gieson , taken from an area of inflamed distal colon ; and immunological studies on the level of IL -1, IL -6, IL -10 and TNF - alpha in the serum of the experimental animals by ELISA.

Results and discussion. Rats treated with DNBS at doses of 20 and 30 mg , dissolved in 0.25 ml of 50% alcohol, administered rectally, with a probe 8 cm into the colon, significantly reduced **their body weight** and food intake compared to control animals (Fig. 37). The most significant decrease in weight compared to the beginning of the experiment and the control was recorded in the DNBS 30 group from 238.75±7.9 g at the beginning of the experiment to 206.25±6.09 g on the 6th day (p=0.04).



	DNBS10	DNBS20	DNBS30	contr.
начално ТТ	232,5	236,43	238,75	237,5
ТТ на 6 ден	239,67	230,86	206,25	258,17

Fig. 37. **Body weight (BW)** of the animals at the beginning of the experiment and on the 6th day after intrarectal administration of DNBS at a dose of 10, 20 and 30 mg . * p<0.05 compared to the control group .

The index reflecting macroscopic changes upon administration of the ulcerogenic agent DNBS at a dose of 10 mg was 1.33±0.08 (37.5% of the animals were free of ulcer defects); at a dose of 20 mg – 2.83±0.48 (also 3 out of 8 rats – 37.5% were free of ulcer defects); at a dose of 30 mg – 3.87±0.6 (Fig. 38). Histological studies showed thickened with the tone of the colon intestine with multiples mosaic scattered areas of fibrinoid necrosis (ulcers) in the mucosa reaching the lamina own . Next door mucous membrane had a changed architectonics – deformed crypts, reduction of goblet cells and single crypt abscesses; and infected inflammatory infiltrate in lamina own, m. mucosae; and thickening of the lamina propria. Immunological studies showed a statistically significantly higher level of proinflammatory cytokines of **IL -1** (Fig . 39), **IL -6** (Fig. 40) and **TNF- alpha** (Fig. 41) in the serum of rats treated with DNBS doses of 20 and 30 mg, while the level of anti-inflammatory **IL -10** was significantly lower in all groups with induced colitis compared to those in the controls animals (Fig. 42).

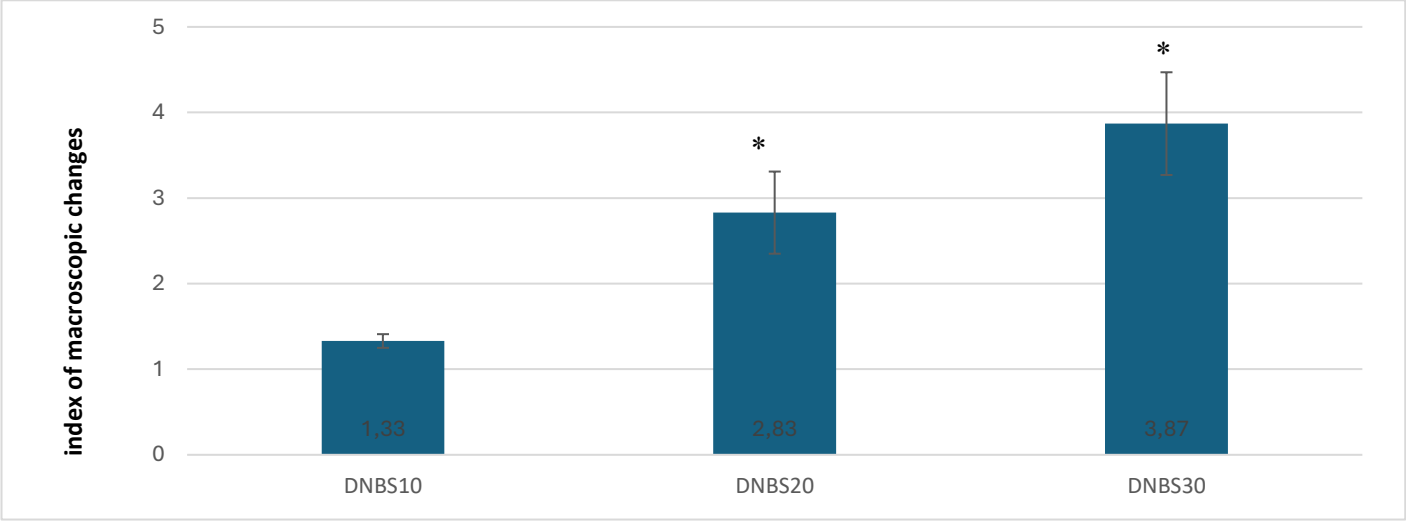


Fig. 38. **Index reflecting macroscopic changes** in distal colon on the 6th day after intrarectal administration of DNBS at a dose of 10, 20 and 30 mg . * p<0.05 compared to the control group.

The studied experimental pattern leads to inflammation of the gastrointestinal mucosa, ulceration, edema, bleeding, weight loss, characteristic of inflammatory bowel disease changes in serum cytokines and clinical manifestations [Cohen and al. 2004; Thoreson Culle et al., 2007; Wells Blennerhassett et al., 2004]. Hapten-induced 2,4- dinitrobenzenesulfonic acid hydrate (DNBS) colitis is a simple and effective model for acute and chronic inflammation and/or ulceration of the colon. The ulcerogenic agent DNBS , administered intrarectally at doses of 10 and 20 mg, led to ulcerative changes in 62.5% of the animals, therefore the experimental colitis induced by these doses of the aggressive substance was deemed unsuitable for studying the effect of substances with an expected protective effect.

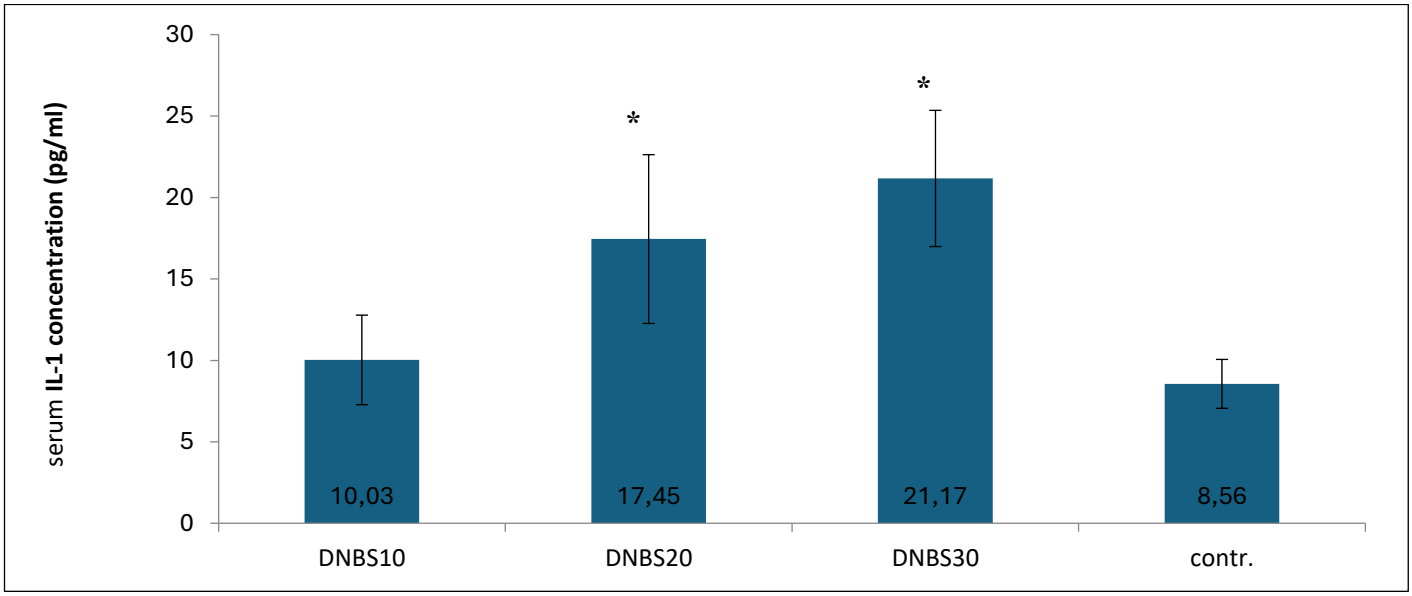


Fig. 39. Serum concentrations of **IL -1** on the 6th day after intrarectal administration of DNBS at a dose of 10, 20 and 30 mg . * p<0.05 compared to the control group.

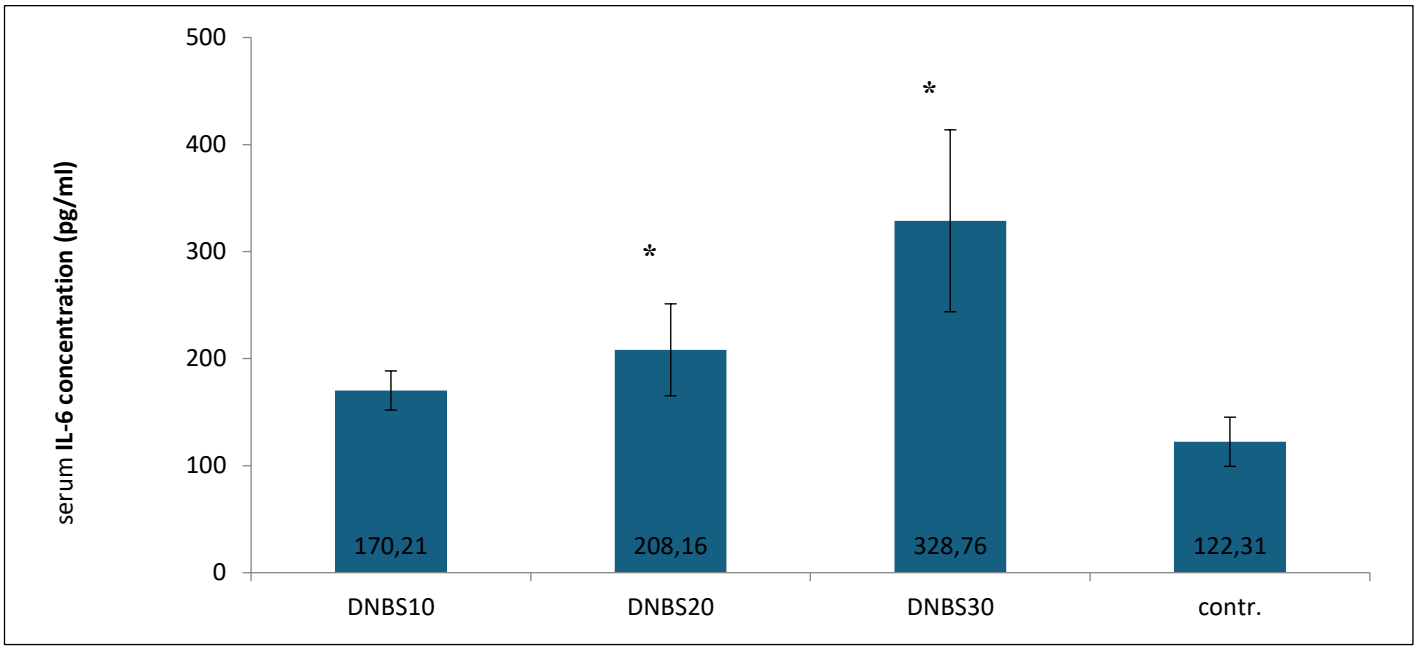


Fig. 40. Serum concentrations of **IL -6** on the 6th day after intrarectal administration of DNBS at a dose of 10, 20 and 30 mg . * p<0.05 compared to the control group.

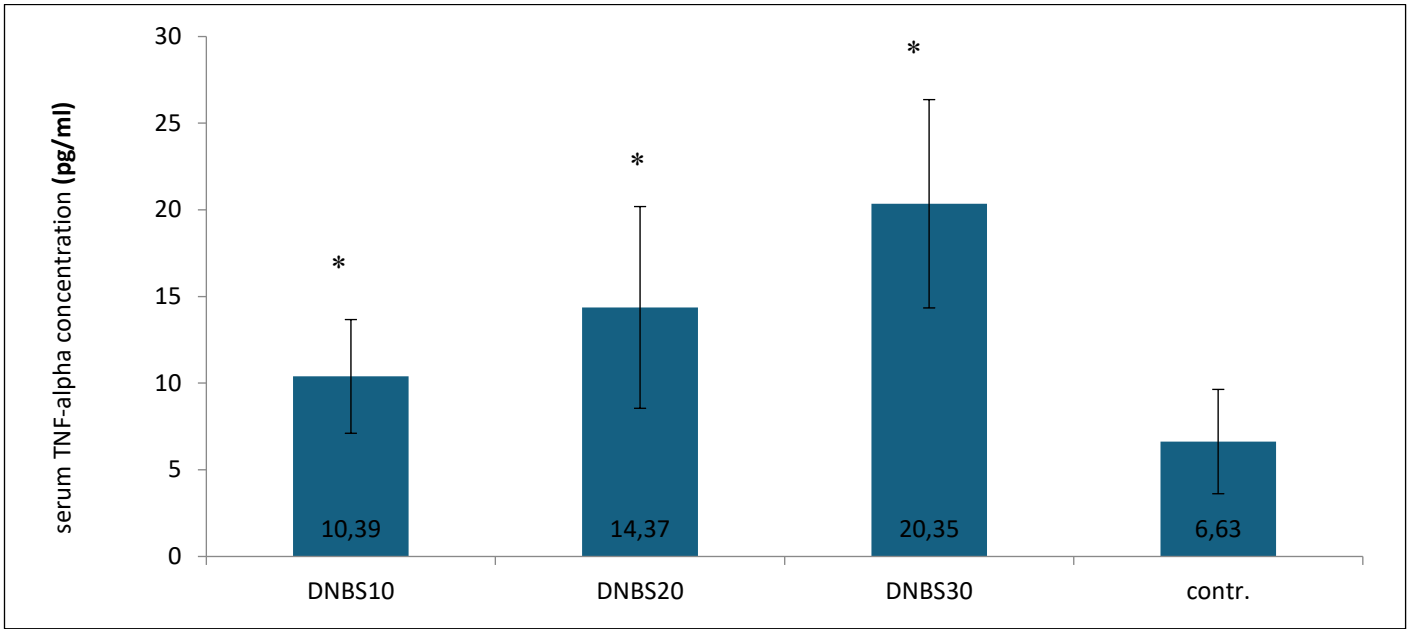


Fig. 41. Serum concentrations of **TNF - alpha** on the 6th day after intrarectal administration of DNBS at a dose of 10, 20 and 30 mg . * p<0.05 compared to the control group.

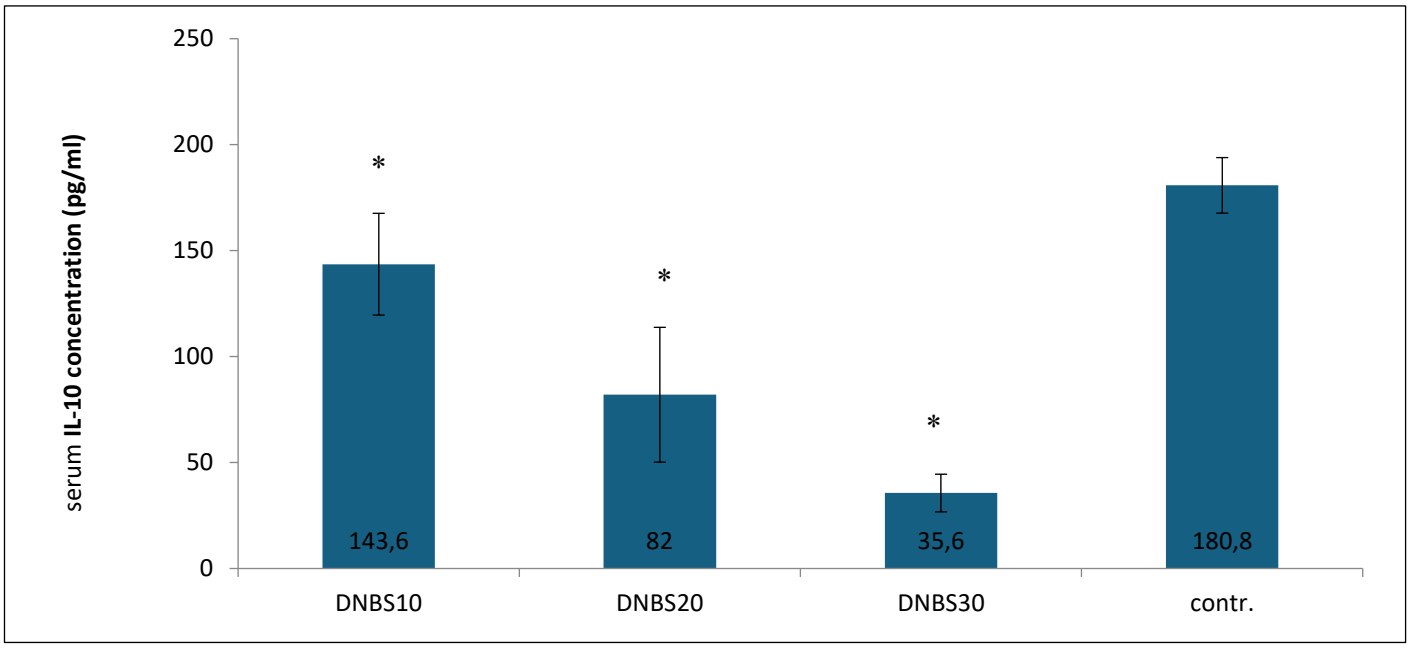


Fig. 42. Serum concentrations of **IL-10** on the 6th day after intrarectal administration of DNBS at a dose of 10, 20 and 30 mg . * p<0.05 compared to the control group.

DNBS administered at a dose of 30 mg intrarectally leads to the development of experimental colitis of varying severity in all tested animals and this dose was considered appropriate in an experiment to prove the effect of substances with anti-inflammatory and/or protective effects.

DNBS dose of 20 mg was considered appropriate for inducing inflammatory changes in experiments involving the study of motor activity, because in histological studies, ulcerative changes did not penetrate to the muscular layer of the colon wall.

Conclusion. The results of the conducted experiments show that DNBS at a dose of 30 mg induces reproducible colitis in all experimental animals and is a suitable model for studying the effect of potential drugs, such as U-74389G.

1.3. Study of the effect of U-74389G on an experimental model of inflammatory bowel disease

The experienced animals are divided into 4 groups of 8 animals randomly: 1. **Control room group** - rats are treated intrarectally with saline solution. 2. **DNBS group** - the dose of 30 mg DNBS, dissolved in 0.25 ml 50% ethanol, is inserted into the colon intestine once with a rubber catheter. 3. **U-74389G group** – the lazaroid is injected intraperitoneally at a dose of 5 mg / kg for 6 days, starting 1 day before causing colitis. 4. **Sulfasalazine (SS) group** – rats with experimental colitis were treated with Sulfasalazine, orally, at a dose of 300 mg / kg, once daily for 6 days, starting 1 day before Induction of colitis. On day 6, animals were sacrificed and colon segment samples were obtained.

Results. Changes in body weight (Fig. 43), food intake (Fig. 44) and diarrhea status were taken as indicators of disease activity. During the 5 days after colitis induction, DNBS-treated rats showed a significant decrease in body weight, most pronounced on day 3 ($P<0.05$). Food consumption was significantly lower than that of control rats, as well as in the U-74389G and ulfasalazine -treated groups. during the first 3 days after DNBS placement (Table 8).

Table 8. Changes in body weight and food intake

groups	Body weight (g)			Food intake (g/100 g body weight)		
	day 1	day 3	day 6	day 1	day 3	day 5
Control group	235.63±5.93	249.37±3.59	255.00±4.23	5.02±0.09	5.12±0.07	5.27±0.08
DNBS group	237.00±2.52	207.80±4.75*	212.50±6.25*	5.05±0.08	2.15±0.17*	3.82±0.19*
U-74389G group	243.37±4.62	221.75±3.65*ø	237.37±5.25*ø	5.1±0.09	4.46± 0.04ø	4.54± 0.22*ø
Sulfasalazine group	233.13±4.90	209.13±3.97*	221.37±2.42*	5.04±0.07	4.19±0.07*ø	4.26±0.12*

Each result is given as the mean of eight rats ± SEM; (*) indicates a significant difference compared to control animals; (ø) indicates a significant difference compared to DNBS -treated animals.

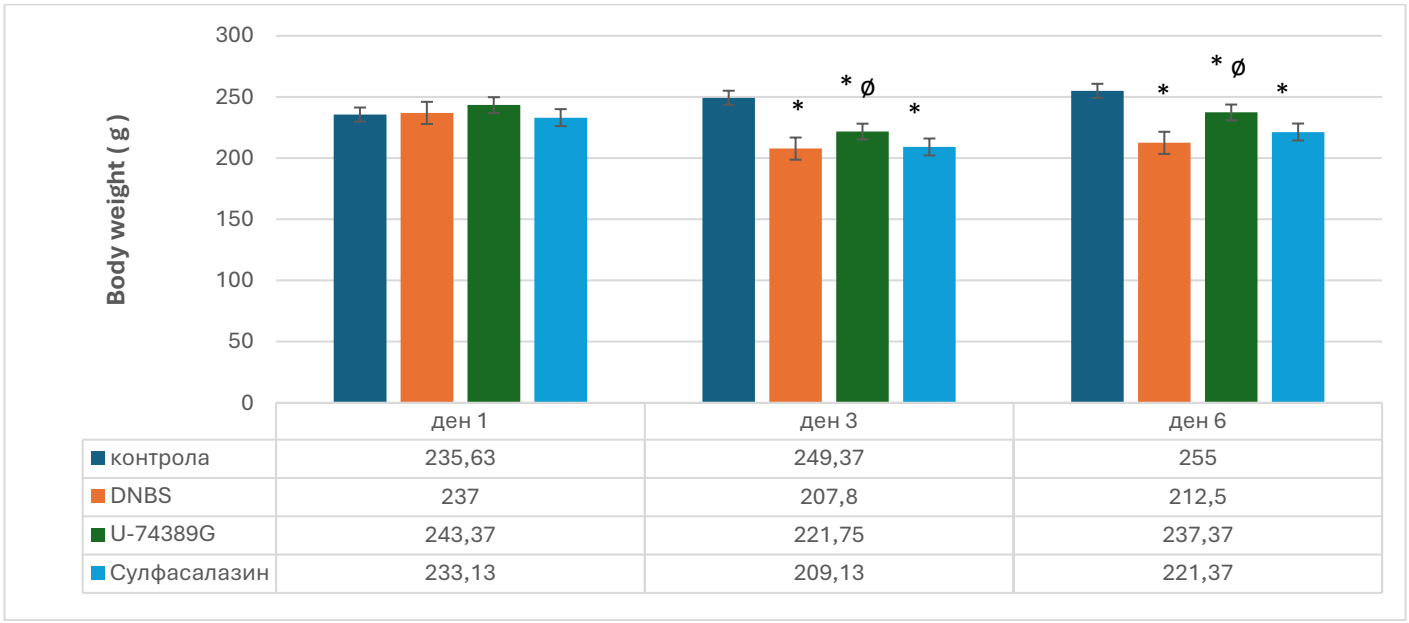
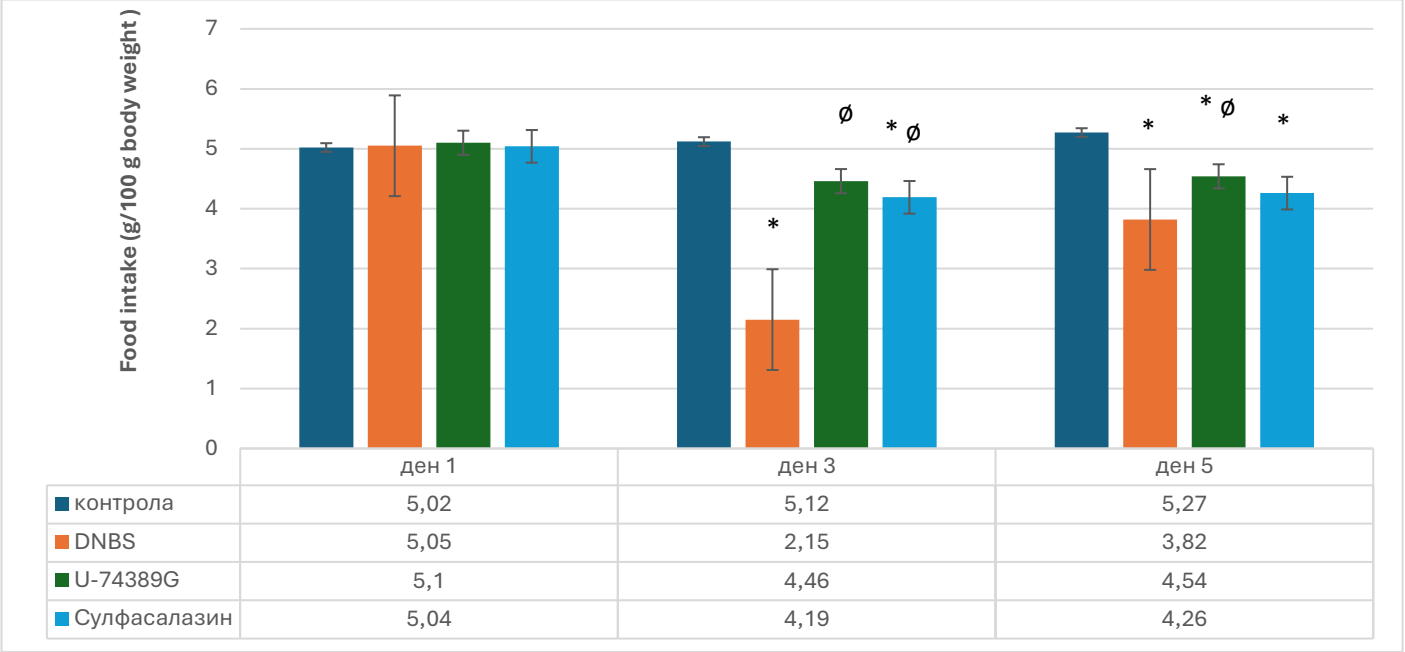


Fig. 43. Change in **body weight** . Each result is given as the mean of eight rats ± SEM . (*) indicates a significant difference compared to control animals; (ø) indicates a significant difference compared to DNBS -treated animals.



	ден 1	ден 3	ден 5
контрола	5,02	5,12	5,27
DNBS	5,05	2,15	3,82
U-74389G	5,1	4,46	4,54
Сулфасалазин	5,04	4,19	4,26

Fig. 44. Change in **food intake** . Each result is given as the mean of eight rats ± SEM . (*) indicates a significant difference compared to control animals; (ø) indicates a significant difference compared to DNBS -treated animals.

The diarrheal status was assessed based on the consistency of the feces and the presence of blood in them (Fig. 45). Changes in the diarrhea index were observed in all animals with induced colitis. The highest **diarrheal index (diarrheal index)** with a value of 3.1 was assessed on day 3 in the DNBS group. No significant differences in diarrhea status and food intake were observed on day 6 between the control group and the colitis rats treated with U-74389G at a dose of 5 mg / kg .

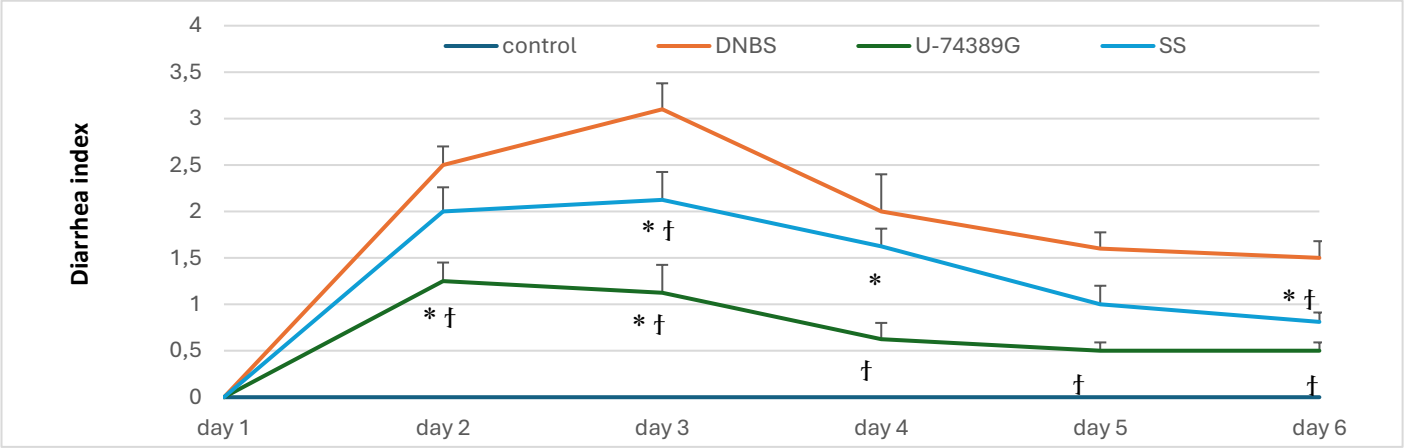


Fig. 45. Daily diarrhea status for 6 days after colitis induction. Each result is given as the mean of eight rats ± SEM. (*) indicates a significant difference compared to control animals; (†) indicates a significant difference compared to DNBS -treated animals.

Macroscopic examination. DNBS treatment induced severe inflammation and ulceration in rats with colitis (Fig. 46 and Fig. 47), expressed by **ulceration index (index)** 3.87±0.61 and **macroscopic assessment of colon damage (macroscopic scoring of colon damage)** - 5.7±0.74 (presence of adhesions, ulcers and thickening of the colon wall). Macroscopic evaluation of tissues obtained from animals treated with U-74389G revealed a significant beneficial effect (P<0.05) on the symptoms of IBD (ulceration index 1.25±0.25 and macroscopic assessment of colon damage 1.625±0.41). The effect of sulfasalazine on the studied markers was less pronounced compared to the effect of U-74389G, as can be seen in Figures 46 and 47.

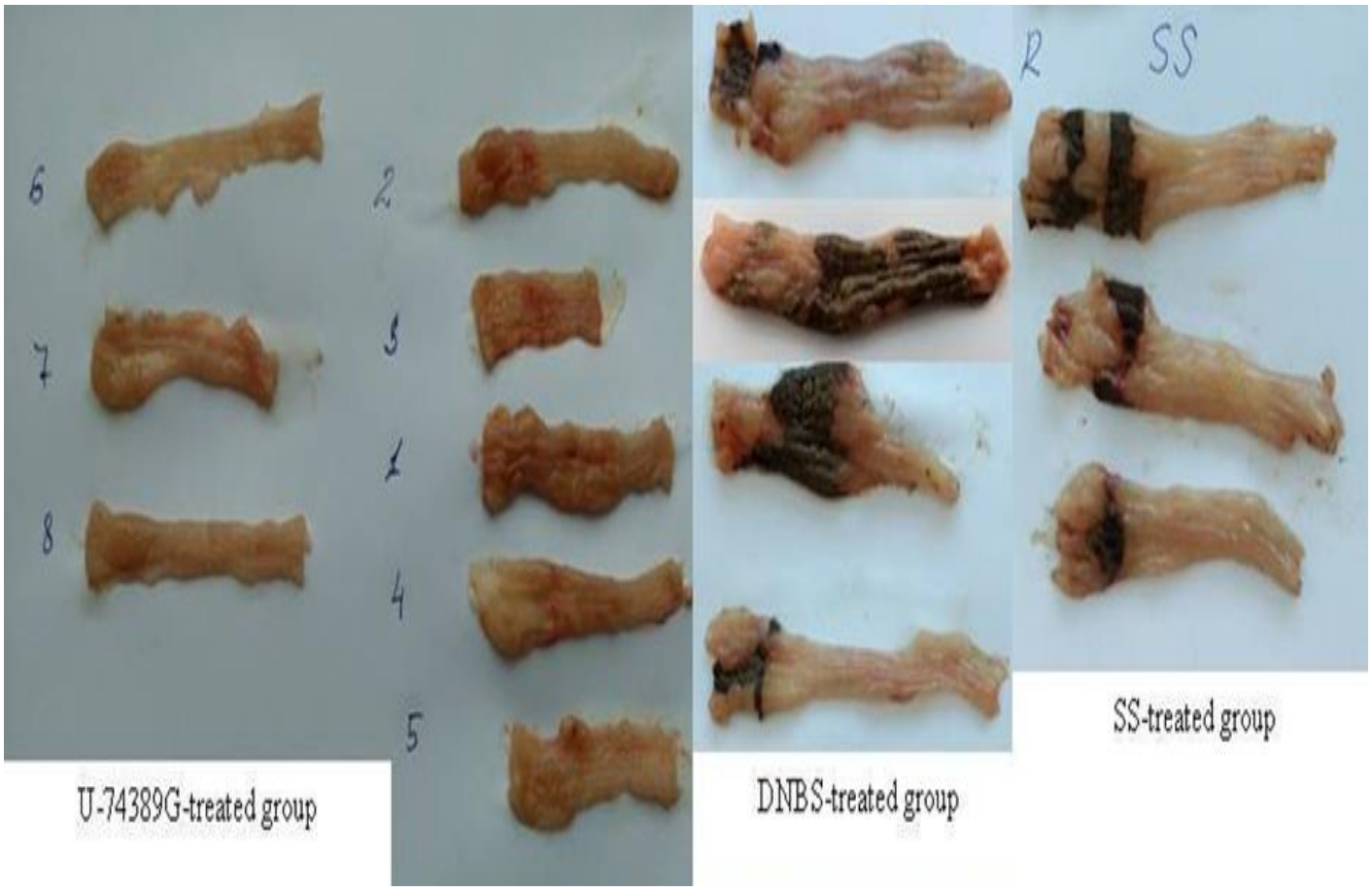


Fig. 46. Macroscopic changes in the colon on day 6 after induction of colitis in animals treated with U-74389G, DNBS and SS.

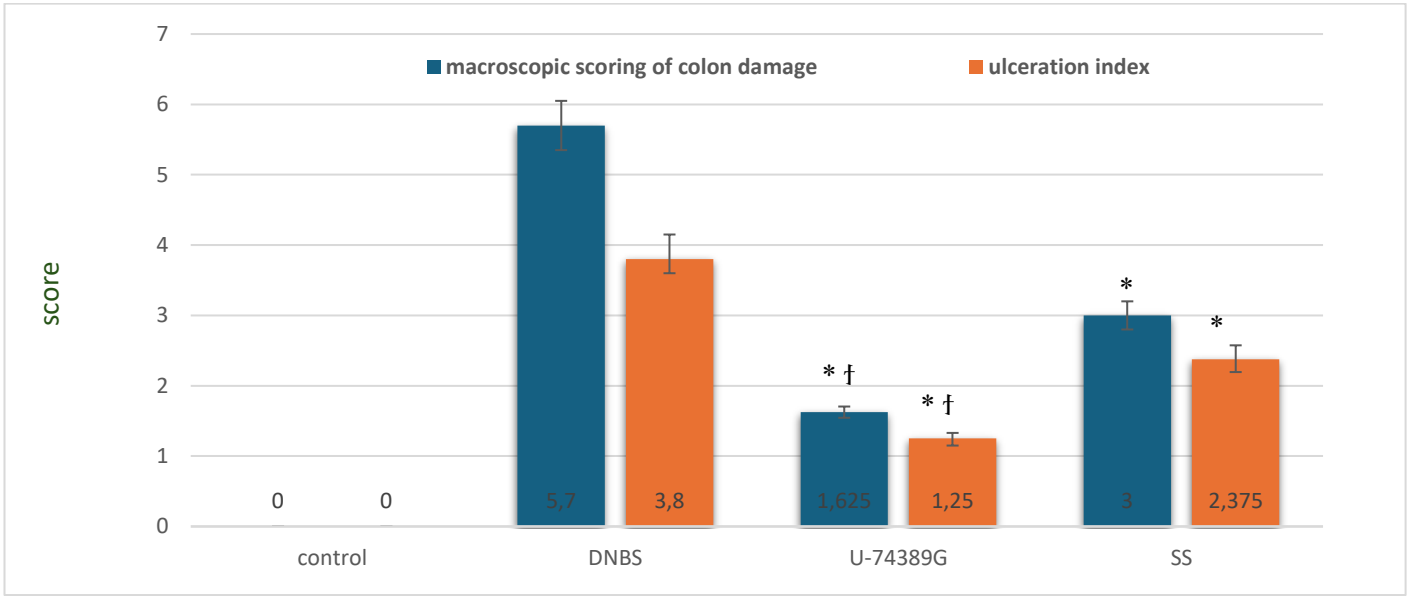
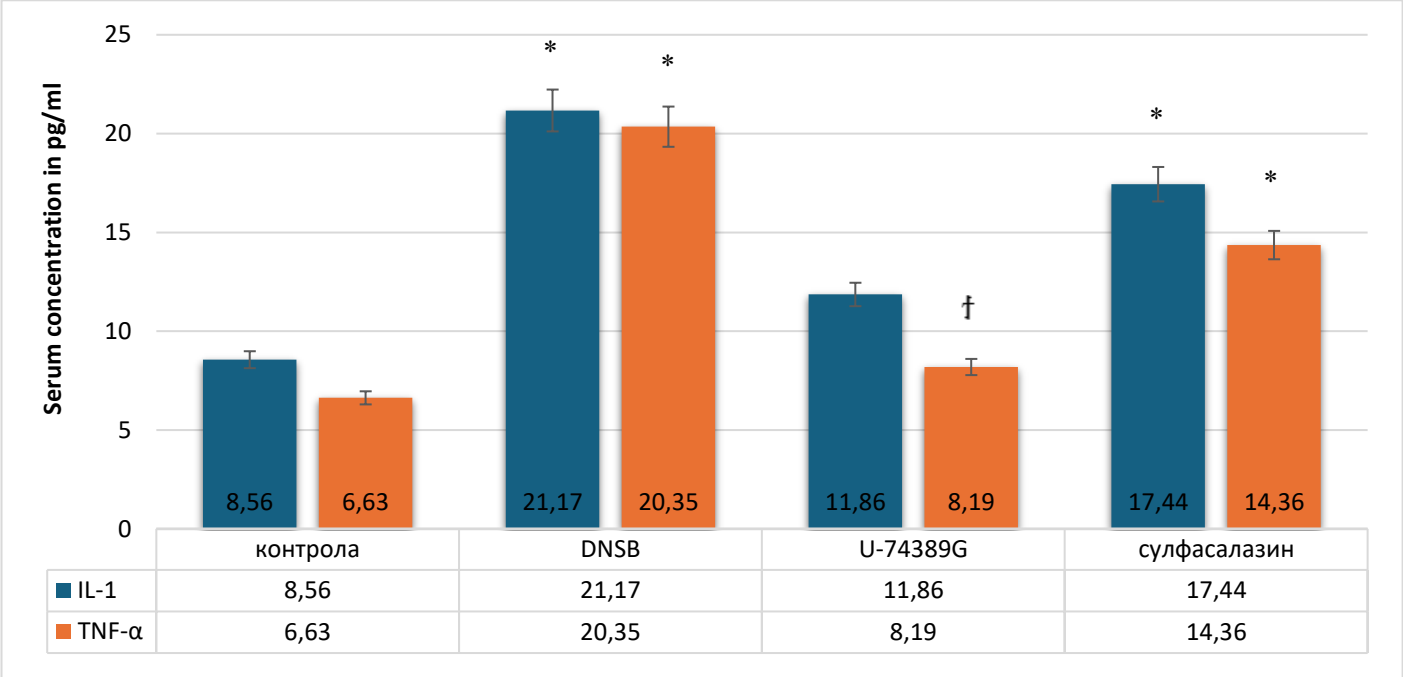


Fig. 47. Macroscopic lesion score assessed on day 6 after DNBS colitis induction. Each column represents mean ± SEM (n=). Control groups show a zero score as no disease developed. (*) indicates a significant difference compared to DNBS -treated animals ; (†) indicates a significant difference compared to ulfasalazine (SS) -treated animals.

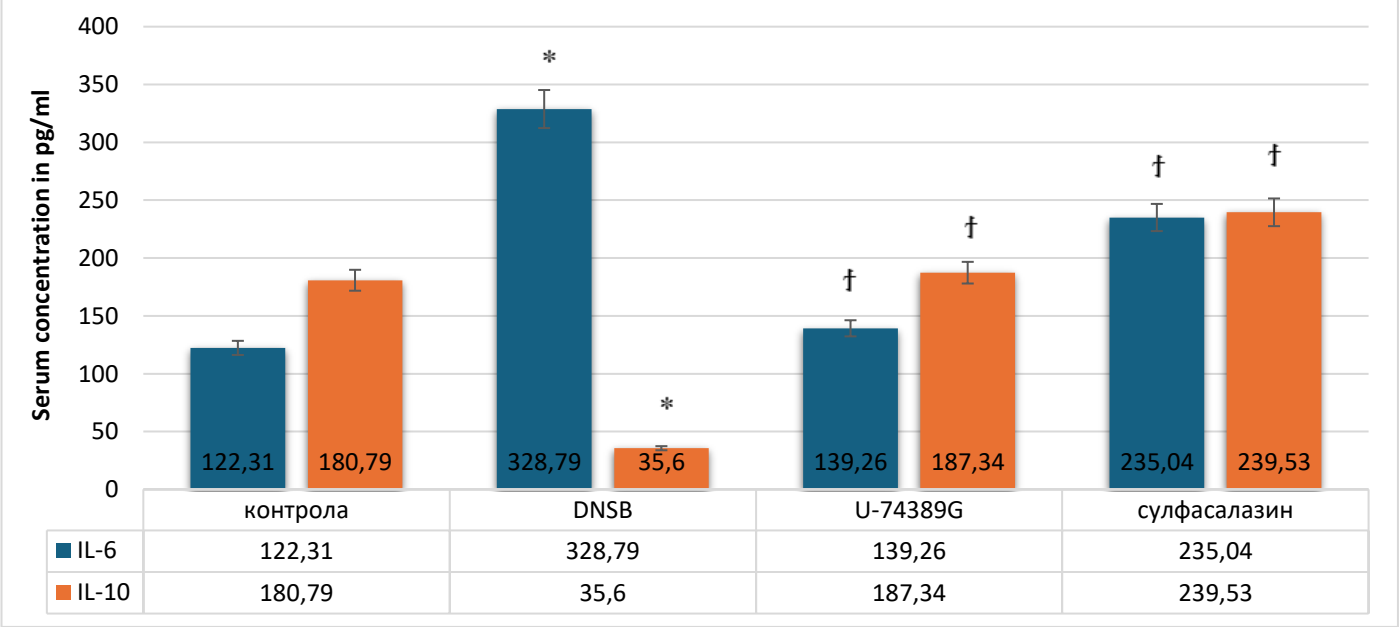
Immunological studies. Immunological assessment of cytokine levels .

Serum levels of inflammatory cytokines **IL-1** , **IL-6** and tumor necrosis TNF **-α** levels in the U-74389G-treated group were significantly lower than those in the DNBS group, while the level of anti-inflammatory **IL-10** was significantly increased by U-74389G (Fig. 48 and Fig. 49).



	контрола	DNSB	U-74389G	сулфасалазин
IL-1	8,56	21,17	11,86	17,44
TNF-α	6,63	20,35	8,19	14,36

Fig. 48. Serum concentrations of inflammatory cytokines **IL-1** and **TNF-α** on day 6 after induction of colitis with DNBS. Each result is given as the mean of eight rats ± SEM. (*) indicates a significant difference compared to control animals; (†) indicates a significant difference compared to DNBS-treated animals.



	контрола	DNSB	U-74389G	сулфасалазин
IL-6	122,31	328,79	139,26	235,04
IL-10	180,79	35,6	187,34	239,53

Fig. 49. Serum inflammatory cytokine concentrations **IL -6** and anti-inflammatory cytokine **IL -10** on day 6 after induction of colitis with DNBS. Each result is given as the mean of eight rats ± SEM. (*) indicates a significant difference compared to control animals; (†) indicates a significant difference compared to DNBS - treated animals.

Determination of malonate Dialdehyde (MDA in serum of rats with DNBS- induced colitis as a marker of increased oxidative stress in colitis

MDA mean, standard deviation, median and range for each group. Note: The standard deviation for the DNBS group was significantly higher than the other groups, reflecting variability in the severity of induced colitis.

Group	Average (ng/ml)	SD	SE	Median	Min - Max	n
DNBS (colitis)	390.75	212.99	86.95	380.81	105.85 - 685.39	6
Control	152.26	42.31	17.27	137.36	115.04 - 218.68	6
U-74389G (treatment)	143.96	65.16	26.60	123.14	94.99 - 273.17	6

After treatment with DNBS, which induces acute colitis in rats, malonate levels were measured on day 6. dialdehyde (MDA) in serum, as a marker of increased oxidative stress in colitis. The DNBS -induced colitis group showed a significantly higher mean MDA value (390.75 ± 213.0 ng / ml) compared to the control group (152.26 ± 42.3 ng / ml). This difference was statistically significant ($p < 0.05$), confirming that DNBS -induced colitis leads to pronounced lipid peroxidation and oxidative damage. The U-74389G-treated **group showed a mean** MDA value (143.96±65.2 ng / ml) — statistically significantly lower than the DNBS group ($p < 0.05$) and indistinguishable from the control ($p = 1.000$, ns). This means that treatment with U-74389G **normalizes malonate** levels **dialdehyde** to baseline values (Fig. 50).

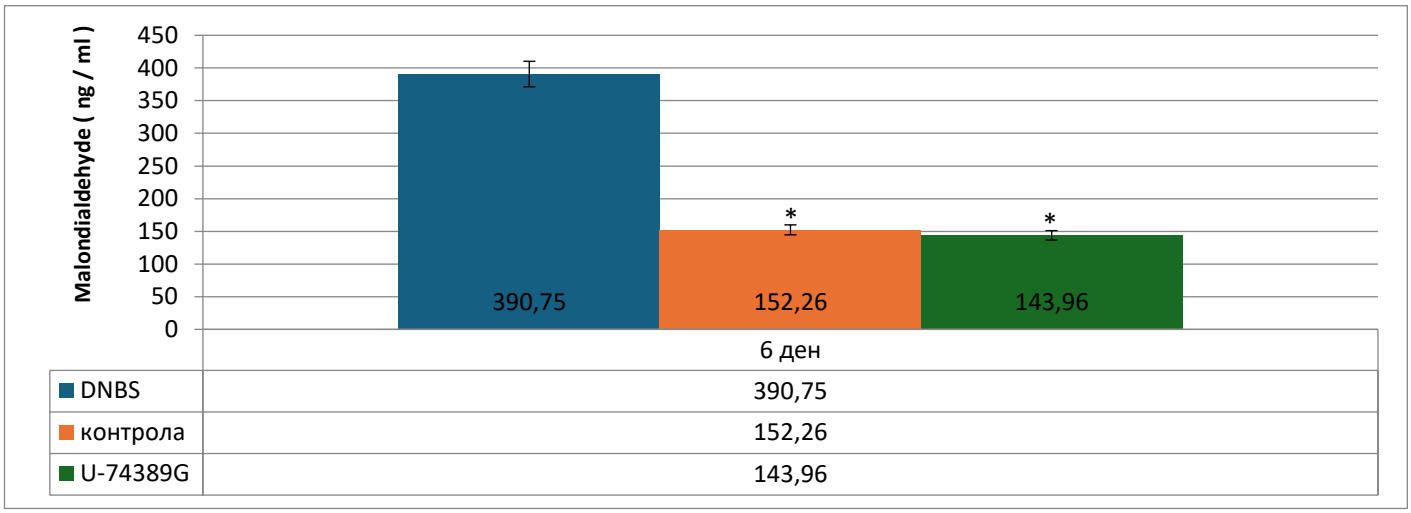


Fig. 50. Concentrations of **malon dialdehyde (MDA)** on day 6 in serum of rats with DNBS -induced colitis; * $p < 0.05$ versus DNBS group

The present data indicate that substance U-74389G exerts significant antioxidant activity. protective effect in DNBS -induced colitis, reducing lipid peroxidation to levels indistinguishable from healthy controls. The results support its potential as an anti-inflammatory and antioxidant agent in intestinal inflammation.

Histology. Histological evaluation of the colon from the DNBS group showed extensive damage, with some areas of ulceration, transmural infiltration with neutrophils and lymphocytes, mucus depletion, hemorrhage, and edema. The DNBS group had a greater degree of ulceration, followed by the SS group , while U -74389 G showed significantly reduced hemorrhage, inflammatory cell infiltration, and tissue necrosis (Fig. 51, Fig. 52, and Fig. 53).

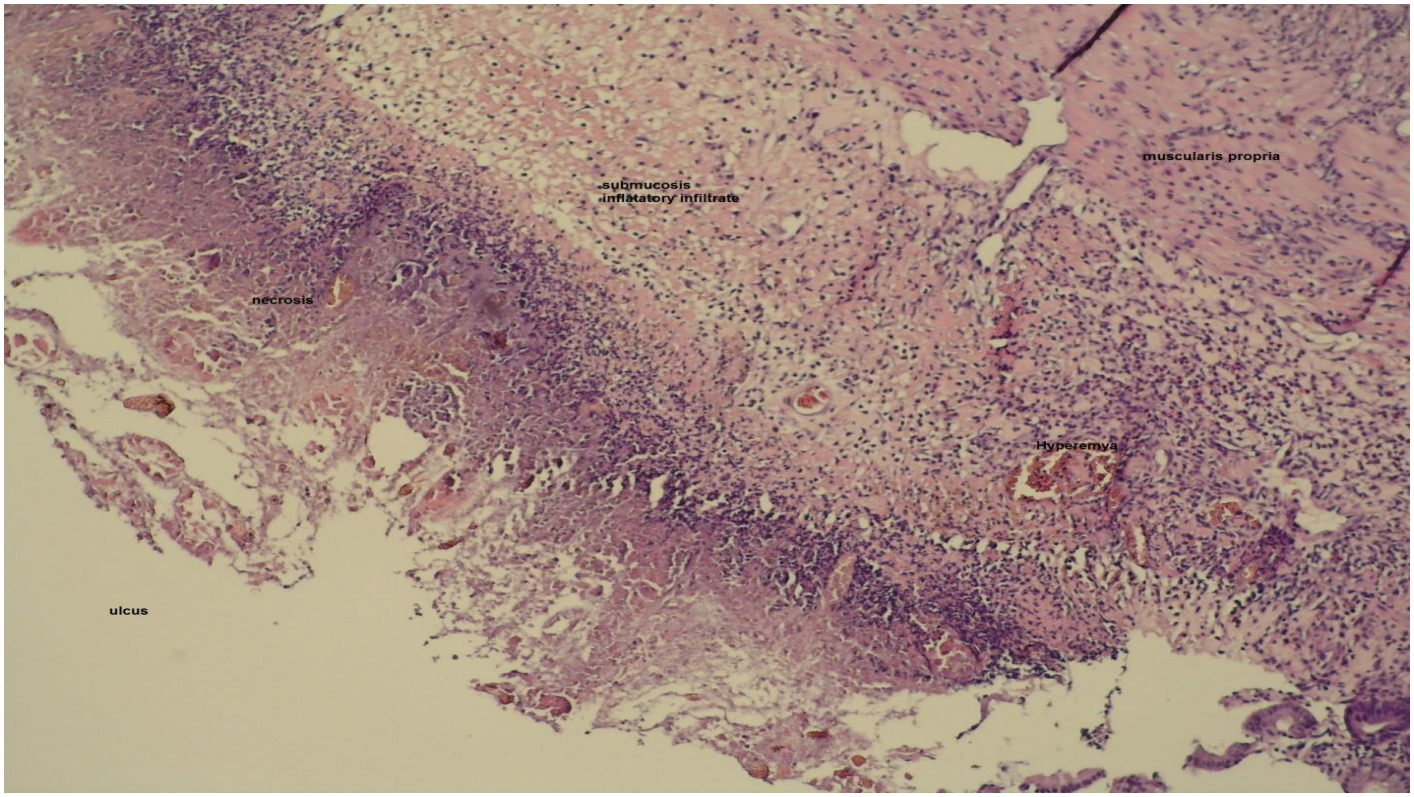


Fig. 51. Histological examination of DNBS-treated rats shows large areas of mucosal necrosis where the glandular architecture is completely destroyed; the submucosa is thickened with edema and marked infiltration with inflammatory cells associated with vasodilation; changes are also observed in the muscularis layer, which also appears thickened (hematoxylin-eosin stain, magnification x 100).

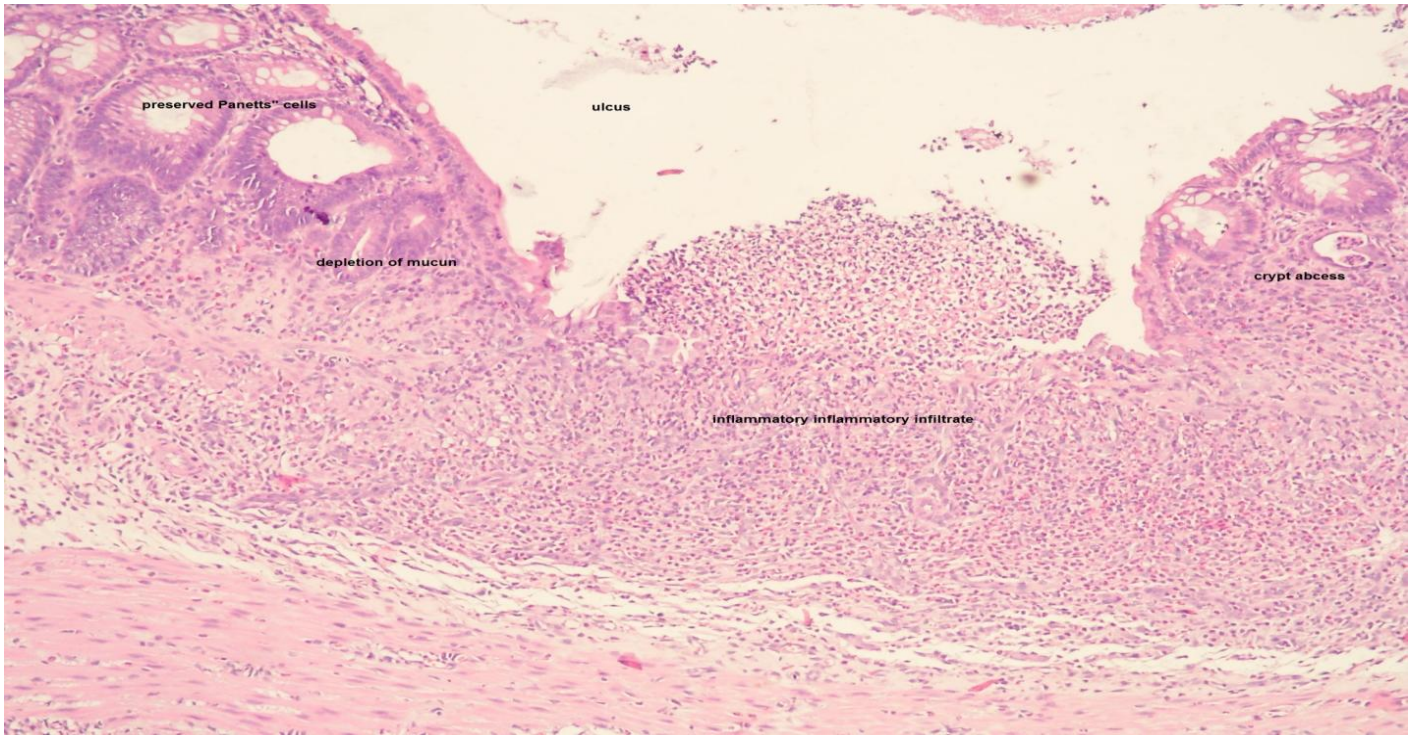


Fig. 52. Histological examination of rats treated with DNBS (hematoxylin-eosin staining, magnification x 100). Colon wall with multiple mosaic scattered areas of fibrinoid necrosis - ulcers in the mucosa reaching the muscularis propria. The adjacent mucosa has altered architectonics - deformed crypts, reduction of goblet cells and single crypt abscesses. Pronounced inflammatory infiltrate in the lamina propria , muscularis mucosae and submucosa and thickening of the m. propria. The inflammatory infiltrate is chronically exacerbated with a predominance of Eo leukocytes.

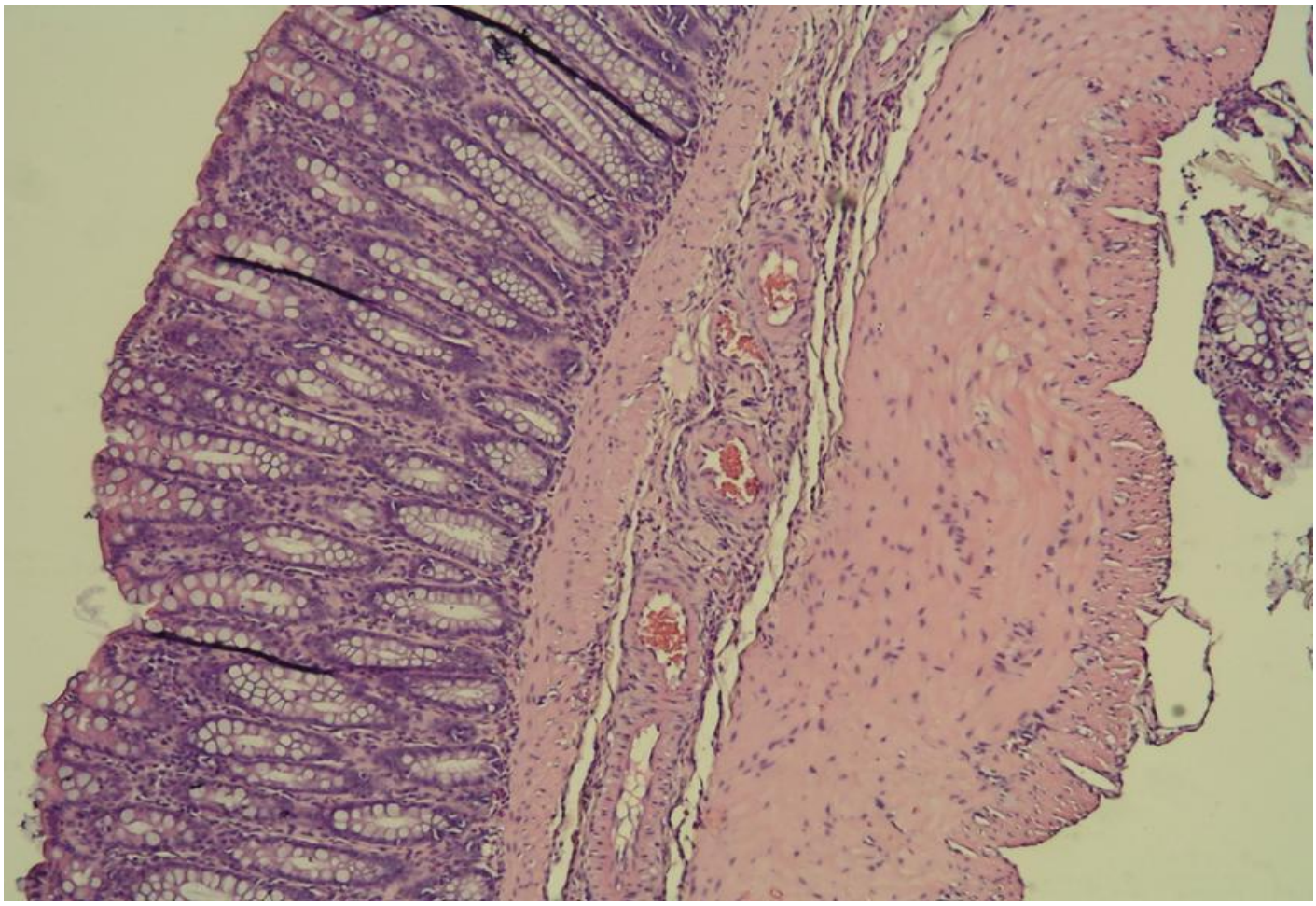


Fig. 53. Histological examination of rats treated with DNBS followed by U-74389G administration showed restoration of mucosal architecture and reduction of cellular infiltrate, crypt abscesses, and goblet cell depletion (hematoxylin-eosin stain, magnification x 100).

Discussion. In the present study, we evaluated the protective effect of the 21-aminosteroid **U -74389 G on** DNBS -induced colitis in rats. Our data show that U -74389 G ameliorates clinical symptoms, macroscopic and histological lesions of the colon.

IBD is considered a multifactorial disease due to the interaction between the immune system, genetic predisposition, environmental responses, and microbial factors [Farzaei MH and al ., 2016]. There is evidence in the literature supporting the significant role of free radicals, both in the development of IBD and in pharmacological targets to limit their effect in the development of anti-IBD drugs [Piechota - Polanczyk A and al ., 2014]. Oxidative imbalance induces and exacerbates disease in two ways, including oxidative damage to intestinal mucosal cells and increased production of inflammatory cytokines . A hapten- induced experimental DNBS colitis model was used . The aggressive agent was dissolved in alcohol, which enhanced the penetration of DNBS into the lamina own [Kim JJ and al ., 2012]. Then DNBS haptenizes local microbial proteins, they become immunogenic and provoke host immune responses, infiltration of neutrophils , macrophages and T-lymphocytes.

DNBS - hapten- induced inflammation involves specific cells, including polymorphonuclear neutrophils, lymphocytes, plasma cells and eosinophils [Sanchez - Muñoz F and al., 2008]. Inflammatory cells can cause tissue damage by releasing oxygen free radicals, proteases , and inflammatory mediators such as cytokines and chemokines .

The results of our study show that intrarectal administration of DNBS at a dose of 30 mg , dissolved in 0.25 ml of 50% ethanol, induces inflammation resembling IBD. The clinical course, immunology and histopathology of the changes gave us reason to assume that this model is suitable for studying the effect of potential therapeutic agents.

We observed that DNBS caused significant weight loss, reduced food intake, and severe changes in stool consistency, including the presence of blood, in all colitis rats, with no mortality among the animals. During the 5 days after colitis induction, DNBS -treated rats showed a significant decrease in body weight and food consumption, most pronounced on day 3 ($P < 0.05$). At the same time, these parameters responded favorably to U -74389 G and their values were significantly increased compared to the DNBS group . On the 3rd day after the introduction of the aggressive agent, loose stools were observed in 33%, diarrhea in 67% of the animals, and the presence of blood in the stool in 37.5% of the colitis rats. Only one rat in the U -74389 G - treated group had diarrhea and was positive for blood in the stool on day 3.

Macroscopic evaluation of tissues obtained from animals treated with U -74389 G showed that 50% (n =4) of the animals had hyperemia and 3 rats had ulcers without inflammatory reaction. The changes were significantly more severe in the animals with colitis, with 7 of 8 animals having ulcerative changes with inflammation and large lesions.

Cytokines are important in the pathogenesis of IBD and regulation of their synthesis successfully reduces disease severity and maintains remission. Activated immune cells secrete several cytokines that actively regulate the inflammatory response in induced colitis. Once secreted by antigen presenting cells, these cytokines prime and differentiate many T cells, activating the adaptive immune response. TNF - α expression in macrophages has been detected in colonic tissues and serum TNF - α levels correlate with clinical and laboratory indicators of intestinal disease activity [Sanchez - Muñoz F and al., 2008]. In addition to TNF - α , IL -1 and IL -6 have pronounced proinflammatory activity. They are produced by various cell types through the initiation of cyclooxygenase type 2, phospholipase A and inducible nitric oxide synthase and are involved in immunological reactions in the development of IBD. IL -10 has anti-inflammatory and immunosuppressive properties. It reduces both the presentation of antigens and the subsequent release of proinflammatory cytokines. cytokines , thereby reducing mucosal inflammation. Increased serum concentrations of inflammatory cytokines TNF - α , IL -1 and IL -6 and decreased anti-inflammatory cytokine levels IL -10 on day 6 after colitis induction was detected in DNBS -treated rats . U -74389 G significantly affected the concentration of cytokines supporting the claim that this aminosteroid produces anti-inflammatory effects.

In our study, we compared the effects of U -74389 G with those of **sulfasalazine (SS)** on markers of disease activity, macroscopic assessment of colonic damage, immunological parameters, and histological changes. Sulfasalazine has been studied in IBD and has demonstrated efficacy in inducing remission in mild to moderate disease, as well as in maintaining remission [Das KM and al ., 2000]. SS has anti-inflammatory, immunosuppressive and antibacterial effects, which are mainly due to its breakdown products sulfapyridine and 5-aminosalicylic acid. The effects of SS on the studied markers were less pronounced compared to those of U -74389 G . These experimental findings can be explained by the fact that it may take weeks for the effects of SS to occur .

The goal of IBD therapy is to modulate the inflammatory response. Despite the use of various therapeutic groups, including biologicals, not all patients receive a satisfactory result from therapy [Tanida Set al., 2015]. Glucocorticosteroids (GCS) are effective in the treatment of IBD to control disease exacerbations and induce remission. GCS, interacting with their receptors, modulate the immune response. They inhibit the expression of adhesion molecules and the movement of inflammatory cells to target tissues, including the intestine [Hayashi R and al., 2004; Braughler JM and al., 1988]. GCS cannot be recommended for the prevention of disease recurrence due to their safety profile and uncertain efficacy data [Marehbian J and al., 2009].

The 21-aminosteroids, analogs of methylprednisolone , have been developed as compounds with free radical scavenging and membrane stabilizing activities and lack of glucocorticoid activity due to the substitution of the 11- b - hydroxy group. 21-aminosteroids include tirilazad , U -74389 G (16-desmethyl-tirilazad), etc. They have been tested for their ability to inhibit lipid peroxidation in tissues after various types of injury [Jacobsen

EJ and al., 1990; Hall ED and al., 1994; Muller TB and al., 1995]. U -74389 G has been shown to have neuroprotective properties in a stroke model [Fabian RH and al., 1998] and brain damage [Shi F and al., 1995]. It also inhibits lipid peroxidation in cultured endothelial cells [Antonini JM and al., 1995], and has protective effects in other models of multiple organ injury, such as in the lungs, heart, and skeletal muscle reperfusion injury [Remmers D and al ., 1996]. Lazaroids have a high affinity for membrane lipids and by incorporating into the lipid bilayer due to their lipophilicity , they exert cell membrane stabilizing effects [Kavanagh RJ and al., 2000]. The protonated The piperazine nitrogen interacts with negatively charged phosphate-containing head groups of the membrane lipid bilayer. This action limits the movement of lipid peroxyl radicals into the membrane, thereby reducing their interaction with fatty acids and inhibiting lipid peroxidation .
U -74389 inhibits initial inflammation, including the migration and activation of inflammatory cells [Huang H and al., 2001]. New experimental results show that lazaroids are potent, time-dependent inhibitors of caspase-1, which convert inactive IL -1 β into an active cytokine [Kawarski M and al., 2015].

In conclusion, intrarectal administration of DNBS induced severe colonic damage in rats as assessed by disease activity markers, macroscopic assessment of colonic damage, immunological parameters and histological changes. Our study showed that the beneficial effects of the 21-aminosteroid U -74389 G may be due to the membrane-stabilizing activity, anti-inflammatory and antioxidant properties of lazaroid .

2. CONCLUSIONS

I. Model on amiodarone-induced pulmonary toxicity

1. Double intratracheal administration of amiodarone (6.25 mg / kg , days 0 and 2) induces a reproducible pattern of pulmonary toxicity with lower lethality compared to a single administration (M1) and is a recommended model for further studies.
2. The Lazaroid U -74389 G dose 5 mg / kg , administered before and after intratracheal Instillation of amiodarone significantly reduces the early pulmonary inflammatory response – it reduces the protein content and the number of polymorphonuclear leukocytes in BALT on the 3rd and 7th days, an effect comparable to that of prednisolone .
3. U -74389 G did not prevent the early increase in LDH and AlPh activity in BALT, which is probably due to the direct cytotoxic effect of amiodarone, occurring before optimal pulmonary concentration of the aminosteroid is achieved.
4. Intratracheal administration of amiodarone suppresses the activity of superoxide dismutase and glutathione peroxidase , as U -74389 G mitigated these changes, indicating moderate antioxidant protection of the enzymatic defense system in the lung parenchyma.
5. U -74389 G significantly inhibited amiodarone -induced lipid peroxidation – reducing MDA levels in lung homogenate and hydroperoxide in plasma on day 7.
6. On days 14 and 28, U -74389 G significantly reduced the hydroxyproline and collagen content in the lung homogenate compared with the amiodarone alone group , showing a significant inhibitory effect on the development of pulmonary fibrosis, slightly superior to the effect of prednisolone .
7. Histopathological examination confirmed the biochemical data – in the AM + U -74389 G group there was significantly less pronounced interstitial and perivascular fibrosis on day 28 compared to the amiodarone alone group .

II. Model of DNBS -induced colitis

1. DNBS at a dose of 30 mg, dissolved in 0.25 ml of 50% ethanol and administered intrarectally, induces reproducible colitis in all experimental animals and is a suitable model for studying the effect of potential drugs. The doses of 10 mg and 20 mg DNBS did not induce colitis in 37.5% of animals and is unsuitable for this purpose.
2. U -74389 G at a dose of 5 mg / kg, administered intraperitoneally for 6 days, significantly improved clinical indicators of disease activity – body weight, food intake and diarrhea index in rats with DNBS -induced colitis.
3. U -74389 G significantly reduced macroscopic signs of colonic damage (ulcer index and total macroscopic score), with an effect superior to that of sulfasalazine under the conditions of the 6-day protocol.
4. U -74389 G suppresses serum levels of proinflammatory cytokines TNF - α, IL - 1β and IL -6, and significantly increased the levels of the anti-inflammatory IL -10 compared to the DNBS group, demonstrating a pronounced immunomodulatory effect.
5. U -74389 G normalizes malonate levels dialdehyde (MDA) in serum to values indistinguishable from those in the control group, demonstrating a potent antioxidant effect in DNBS -induced colitis.
6. Histological examination revealed significantly less pronounced mucosal necrosis, cellular infiltration and edema in animals treated with U -74389 G compared to the DNBS group, with evidence of partial restoration of mucosal architecture.

4. THE CONCLUSION

The present study is the first to systematically investigate the effects of lazaroid U -74389 G in two different experimental models – amiodarone -induced pulmonary toxicity and DNBS -induced colitis in rats.

The results show that U -74389 G exerts significant anti-inflammatory, antioxidant and antifibrotic effects in both models. In pulmonary toxicity, the aminosteroid effectively limits the early inflammatory response and reliably inhibits the development of pulmonary fibrosis – an effect comparable to the standard corticosteroid treatment with prednisolone , without the adverse effects inherent to glucocorticosteroids . In colitis, U -74389 G demonstrates a complex protective effect – clinical, macroscopic, immunological, biochemical and histological – superior to the effect of sulfasalazine within the applied protocol.

The mechanisms underlying these effects include: inhibition of lipid peroxidation by scavenging peroxyl radicals, stabilization of cell membranes through interaction with the phospholipid bilayer, bilayer , suppression of pro-inflammatory mediators and immunomodulatory effects. These properties position U -74389 G as a promising molecule for further investigation in chronic oxidative- mediated and fibro-inflammatory diseases.

SCIENTIFIC CONTRIBUTIONS OF THE DISSERTATION

Contributions of an original nature

1. lazaroid has been systematically compared U -74389 G and prednisolone in amiodarone -induced pulmonary toxicity in rats – it was found that the protective effect of U -74389 G with respect to markers of pulmonary fibrosis (hydroxyproline , collagen) was comparable, and in some indicators slightly superior to the effect of prednisolone .
2. U -74389 G was studied in a DNBS -induced colitis model in rats, demonstrating a complex protective effect - clinical, macroscopic, immunological (cytokines), antioxidant (MDA) and histological.
3. U -74389 G was found to normalize serum malonate levels. dialdehyde in DNBS- induced colitis to values indistinguishable from the control, demonstrating complete antioxidant protection against DNBS -induced oxidative stress.
4. It has been documented that U -74389 G significantly suppresses pro-inflammatory cytokines (TNF - α , IL - 1β , IL -6) and increased IL -10 in DNBS -induced colitis, with an effect superior to sulfasalazine in the 6-day protocol used.

Contributions of a confirmatory and scientifically applied nature

5. It has been confirmed that a double intratracheal administration of amiodarone (days 0 and 2, 6.25 mg / kg) represents a safer and more appropriate model of AIBT compared to a single administration at a higher concentration.
6. It has been shown that DNBS at a dose of 30 mg , intrarectally , is the optimal dose for inducing reproducible and severe colitis in Wistar rats , suitable for pharmacological studies, while doses of 10 and 20 mg provide incomplete penetrance .
7. the antifibrotic potential of lazaroids in lung injury have been expanded , with U -74389 G being found to significantly inhibit collagen deposition and hydroxyproline elevation after intratracheal administration of amiodarone .

LIST OF SCIENTIFIC PUBLICATIONS

1. Plamen K. Krastev, Alexander B. Blazhev, Galya Ts. Stavreva. EFFECT OF AMINOSTEROID U-74389G IN A MODEL OF INFLAMMATORY BOWEL DISEASE IN RATS. Journal of Biomedical and Clinical Research (ISSN 1313-6917), Vol.14, Number 2, 2021 (eISSN 1313-9053), pp. 131-139
2. Кръстев Пламен, Виолета Данчева, Галя Ставрева. Сравнително изследване на ефекта на U-74389G и преднизолон при индуцирана от амиодарон белодробна токсичност. VIII национален конгрес по фармакология, клинична фармакология и терапия с международно участие, 15-17 ноември 2024, МУ-Плевен, Сборник доклади, стр.194-204
3. Galya Ts. Stavreva, Violeta Y. Dancheva, Lyudmil G. Terziev, Plamen K. Krastev. Effects of 21-aminosteroid U-74389g on amiodarone-induced pulmonary toxicity in rats. Archives of the Balkan Medical Union vol. 54, no. 3, pp. 404-410 September 2019