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Effects of Inhalation Therapy on Apoptosis-Related Proteins in Experimental Acute Lung Injury in Rats

Denis S. Ziablitsev

denis898@gmail.com

Bogomolets National Medical University

Vitalii O. Kostenko

Poltava State Medical University

Victoria V. Mykhaylovska

Bogomolets National Medical University

Mykola S. Babenko

Bogomolets National Medical University

Andrii I. Kurchenko

Bogomolets National Medical University

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Abstract

Introduction:

Acute lung injury (ALI) remains a critical condition associated with high mortality despite advances in intensive care. Its pathogenesis involves diffuse bronchoalveolar damage that frequently progresses to fibrotic remodeling of lung tissue. Dysregulated apoptosis of pulmonary parenchymal cells represents a key mechanism linking inflammation to fibrosis and is largely controlled by the balance between proapoptotic and antiapoptotic Bcl-2 family proteins, including Bax and Bcl-xL, as well as activation of caspase-3. Modulation of these pathways represents a promising therapeutic strategy.

Aims

To evaluate the effects of inhalation therapy with camostat mesylate, methylprednisolone, and enoxaparin sodium on the tissue content of apoptosis-related proteins (caspase-3, Bax, and Bcl-xL) and lung morphology in a rat model of experimental acute lung injury.

Material and methods

Acute lung injury was induced in male Wistar rats (n = 45) by a combination of aspiration bronchopneumonia and systemic/intratracheal lipopolysaccharide administration. From day 5 to day 21, animals received inhalation therapy with the studied agents or saline (placebo control). On day 21, lung tissue was examined using histological and immunohistochemical analyses to assess caspase-3–positive cells. Tissue levels of caspase-3, Bax (monomer and dimer), and Bcl-xL were quantified by immunoblotting.

Results

Placebo-treated rats demonstrated pronounced fibrotic remodeling (carnification) accompanied by a high proportion of caspase-3–positive macrophages and fibroblasts (35–75%). This was associated with a marked increase in active caspase-3 (9.3-fold) and Bax monomer (2.8-fold), along with a significant decrease in Bcl-xL (3.2-fold) compared with intact controls ($p < 0.05$). All inhalation therapies significantly attenuated fibrotic changes, reduced the number of caspase-3–positive cells, and decreased tissue levels of active caspase-3 (1.3–2.1-fold), Bax monomer (1.3–1.9-fold), and Bax dimer (1.3–2.4-fold), while restoring Bcl-xL expression ($p < 0.05$). Methylprednisolone exerted the strongest effect on Bax dimer reduction, camostat mesylate most effectively increased Bcl-xL levels, and enoxaparin sodium showed the greatest suppression of active caspase-3. Immunohistochemically, methylprednisolone predominantly reduced apoptosis of macrophages and type II alveolocytes, enoxaparin targeted fibroblasts, whereas camostat demonstrated a more uniform antiapoptotic effect.

Conclusions

Experimental acute lung injury is characterized by excessive apoptosis driven by Bax /Bcl-xL imbalance and caspase-3 activation. Inhalation therapy with camostat mesylate, methylprednisolone, and enoxaparin sodium effectively corrected these alterations through complementary mechanisms, attenuating fibrotic remodeling. These findings support the potential clinical relevance of combined or targeted antiapoptotic strategies in acute lung injury.

INTRODUCTION

Acute lung injury (ALI), particularly acute respiratory distress syndrome, remains a leading cause of mortality in intensive care with fatality rates of 35–46% despite modern protocols [1]. This pathology gained exceptional significance during the COVID-19 pandemic, when diffuse alveolar damage frequently progressed to pulmonary fibrosis [1]. Identifying therapeutic approaches to interrupt the "acute inflammation–cell death–fibrogenesis" pathway is therefore of high clinical relevance.

Dysregulated apoptosis plays a central role in lung injury pathogenesis. Excessive apoptosis of alveolar epithelium and endothelium disrupts barrier function and activates fibroblasts, while apoptosis resistance in fibroblasts and myofibroblasts sustains fibrosis progression [1, 2]. Thus, apoptosis represents not merely a consequence but a key driver of lung tissue remodeling.

At the molecular level, apoptosis regulation involves the Bcl-2 protein family. Shift toward pro-apoptotic Bax causes mitochondrial membrane permeabilization and caspase cascade activation, culminating in effector caspase-3 activation [3]. Conversely, reduced anti-apoptotic protein expression (Bcl-2, Bcl-xL) correlates with lung parenchymal damage progression and fibrotic changes [3, 4]. Experimental models demonstrate that Bax/ Bcl-xL-2 ratio alterations directly correlate with inflammation severity and alveolar damage extent [3].

Given the multifactorial pathogenesis, promising strategies employ multi-targeted approaches: glucocorticoids suppress inflammation and stabilize the epithelial barrier [4], anticoagulants address microthrombosis and ischemia, while modulation of the local renin-angiotensin system (RAS) may inhibit pro-apoptotic and pro-fibrotic pathways [1]. However, comparative efficacy of these approaches in correcting apoptotic protein content remains incompletely characterized.

The aim was to investigate the effects of inhaled camostat mesylate (serine protease inhibitor), methylprednisolone (glucocorticoid), and enoxaparin sodium (low-molecular-weight heparin) on tissue content of key apoptotic proteins (caspase-3, Bax, Bcl-xL) and lung morphology in rats with experimental ALI.

MATERIALS AND METHODS

The study was conducted in accordance with EU Directive 2010/63 on the protection of animals used for scientific purposes and current Ukrainian legislation. Animals were housed under standard vivarium conditions at O.O. Bogomolets National Medical University. The study was approved by the Ethics and Academic Integrity Committee of O.O. Bogomolets National Medical University.

Acute lung inflammation was modeled using a previously described method [4, 5] by intratracheal insertion of a capron thread (aspiration bronchopneumonia) combined with systemic inflammatory response induction. Lipopolysaccharide (LPS; Sigma-Aldrich, USA) was administered intraperitoneally at 2.5 mcg/kg on days 1 and 2 before surgery, followed by endotracheal injection of 0.125 mcg/kg in 50 µL saline immediately after thread insertion.

Forty-five male Wistar rats (180–190 g) were obtained from the institutional animal facility (vivarium) of Bogomolets National Medical University (Kyiv, Ukraine). No privately owned animals were used. The animals were randomly assigned to groups: sham-operated (all surgical procedures except thread and LPS administration; n = 5) and experimental ALI (n = 40). By day 5, eleven animals (27.5%) had died. Surviving animals (n = 29) were randomly divided into four subgroups receiving daily inhalations from day 5 onward: subgroup 1 – saline (placebo control; n = 8); subgroup 2 – camostat mesilate, a serine protease inhibitor affecting ACE2-associated TMPRSS2 pathway (ACE2 Inhibitor MLN-4760; EMD Millipore, USA; n = 7); subgroup 3 – methylprednisolone (Solu-Medrol; Pfizer, USA; n = 7); subgroup 4 – enoxaparin sodium (Clexane; Sanofi, France; n = 7).

Inhalations were performed using an original apparatus delivering humidified air through heated saline containing the respective drug via a mask secured to the animal's head. Under conditions of 750–755 mmHg atmospheric pressure, 55–60% humidity, and 37°C solution temperature, 0.04 g solution was delivered as aerosol over 15 minutes, corresponding to 4 mcg camostat mesilate, 5 mcg methylprednisolone, and 4 mcg enoxaparin sodium per inhalation. Body weight-adjusted doses were 20 mcg/kg for camostat and enoxaparin, and 25 mcg/kg for methylprednisolone, selected based on respective pharmaceutical guidelines. Inhalations were administered daily from day 5 post-surgery.

Animals were monitored for general condition, rectal temperature, cyanosis, dyspnea, respiratory rate, and auscultatory findings throughout the 21-day observation period. On day 21, animals were deeply anesthetized with sodium thiopental (50 mg/kg, i.p.) until the loss of corneal and pedal reflexes was confirmed. Following the induction of deep anesthesia, euthanasia was performed by decapitation to facilitate rapid tissue collection. Lungs were excised and fixed in 10% neutral buffered formalin (pH 7.4) for 24–36 hours, then paraffin-embedded. Serial 2–3 µm sections were prepared using a rotary microtome HM 325 (Thermo Shandon, UK).

Immunohistochemistry was performed using caspase-3 monoclonal antibodies (clone 74T2; ThermoFisher Scientific, USA) on Super Frost Plus adhesive slides (Menzel, Germany). Antigen retrieval employed citrate buffer (pH 6.0) or EDTA buffer (pH 8.0). Detection utilized Master Polymer Plus Detection system (peroxidase, DAB chromogen; Master Diagnostica, Spain). Microscopy was performed using ZEISS microscopes (Axio Imager A2 with ERc 5s and Axiocam 105 cameras; Carl Zeiss Primo

Star) and Olympus BX40 with C3030-ADU camera and DP-Soft software, at 50×–400× magnification. Positively stained cells were counted per 100 cells (macrophages, fibroblasts, type II alveolar cells) and expressed as percentage.

For immunoblotting, tissue samples were pulverized in liquid nitrogen, homogenized in 50 mM Tris-HCl buffer (pH 7.4) with phosphatase and protease inhibitors (ThermoScientific, USA), and subjected to electrophoresis in 8% SDS-polyacrylamide gel (BioRad, USA). Proteins were transferred to nitrocellulose membranes by electroblotting and incubated with primary antibodies: anti-caspase-3 (ABM1C12, Abcam, USA, ab208161, mouse, 1:5000), anti-Bax (Sigma-Aldrich, USA, B3428, rabbit, 1:2000), anti-Bcl-xL (Sigma-Aldrich, USA, SAB4502623, rabbit, 1:1000), and anti-β-actin (Invitrogen, USA, MA5-15739, 1:5000) as loading control. Following primary incubation, membranes were washed and treated with species-specific horseradish peroxidase-conjugated secondary antibodies (Invitrogen, USA). Semi-quantitative analysis was performed densitometrically using TotalLab software (TL120, Nonlinear Inc, USA). Results were expressed in arbitrary units normalized to β-actin content.

Statistical analysis was performed using Statistica 10 (StatSoft Inc., USA). Data are presented as means ± standard errors. Group comparisons employed ANOVA, with $p < 0.05$ considered statistically significant.

RESULTS

From the first hours after modeling acute lung injury, animals developed pronounced dyspnea with involvement of accessory respiratory muscles and diaphragm, accompanied by moist rales on auscultation. From days 2–3, breathing became shallow, labored, and tachypneic (up to 102–150/min), with distant wheezes, crepitation, cyanosis, lethargy, reduced motor activity, and hyperthermia (37.9–38.9°C).

In treated animals, the postoperative period was characterized by milder clinical manifestations compared to the placebo group. Although hyperthermia, cyanosis, dyspnea, and moist rales were still observed, crepitation was absent. The least pronounced symptoms were noted in animals receiving methylprednisolone and enoxaparin sodium.

Overall mortality reached 27.5% (11 animals) within the first five postoperative days. After initiation of inhalation therapy, no deaths were recorded in any treatment subgroup.

The dynamics of morphological changes during acute lung inflammation have been described previously [5]. Endotracheal lipopolysaccharide administration rapidly induced acute exudative–alterative inflammation in the lungs. Against the background of a systemic inflammatory response triggered by prior intraperitoneal LPS injection, local histiovascular reactions developed, characterized by increased vascular permeability and interstitial and intra-alveolar edema. The pathological process progressed toward acute exudative–hemorrhagic inflammation with features of hyperimmune vascular injury. By days 14–21, productive inflammation predominated, with diffuse parenchymal fibrosis.

In our earlier studies using this experimental model, a progressive increase in lung tissue levels of Bax (monomeric and dimeric forms) and caspase-3 (pro-caspase-3 and active caspase-3) was demonstrated, with peaks at days 5–7 and day 21 [6]. These changes reflected activation of apoptosis during key trigger phases of lung inflammation, corresponding to the development of exudative–hemorrhagic pneumonia and subsequent fibrotic remodeling.

In the present study, lung morphology in the placebo-control group on day 21 was consistent with previously reported findings [6]. Extensive areas of dense fibrosis (carnification) were observed (Fig. 1A), along with fibrotic changes in medium- and small-caliber vessel walls, including focal luminal obliteration. Diffuse fields of chronic productive inflammation with fibrosis contained numerous immunopositive macrophages and fibroblasts, as well as positive staining of type II alveolar cells and, in some areas, vascular endothelium.

Inhalation of the studied agents resulted in less severe pathological changes (Fig. 1B–D). Camostat mesylate was associated with reduced fibrosis; alveoli were free of debris, and only a small number of caspase-positive macrophages and fibroblasts were present (Fig. 1B). Methylprednisolone attenuated lung injury, with increased aeration, limited consolidation, and reduced fibrosis; caspase-positive cells were fewer and predominantly fibroblasts (Fig. 1C). Following enoxaparin sodium, vascular changes persisted, but caspase-positive cells were low, mainly isolated macrophages and type II alveolocytes (Fig. 1D).

Quantitative assessment confirmed a significant reduction in caspase-positive cells in all treated groups (Fig. 2). Camostat mesylate reduced total positive cells, methylprednisolone decreased positive macrophages and type II alveolocytes, whereas enoxaparin sodium mainly reduced positive fibroblasts.

Reduced staining intensity in treated groups compared with placebo is illustrated in Fig. 3

In the placebo group, bronchial walls were markedly thickened due to fibrosis, with dystrophic–atrophic changes of ciliated epithelium and irregular goblet cell distribution, indicating impaired bronchial drainage (Fig. 3A). In contrast, methylprednisolone preserved lung architecture and promoted hyperplasia of ciliated epithelium and goblet cells, with a markedly lower proportion of caspase-positive cells (Fig. 3B).

Overall, inhalation therapy substantially improved lung morphology, reducing fibrosis and enhancing regenerative processes. These changes were accompanied by a significantly lower proportion of caspase-positive cells, suggesting decreased apoptotic activity.

To elucidate apoptotic mechanisms, lung tissue levels of caspase-3, Bax, and Bcl-xL were assessed by immunoblotting (Fig. 4). Procaspase-3 (35 kDa) was detected only in control samples (Fig. 4A). Active caspase-3 (17 kDa) was markedly increased in ALI, peaking in the placebo group (9.3-fold vs control; $p < 0.05$), whereas all treatments significantly reduced its level (1.3–2.1-fold vs placebo; $p < 0.05$), with the lowest values observed after enoxaparin sodium.

Therefore, three main trends in caspase-3 content changes in lung tissue during acute pulmonary inflammation could be identified: disappearance of pro-caspase-3, significant accumulation of active caspase-3, and reduction of its content under the influence of inhalation treatment. These shifts in caspase-3 content corresponded to the described features of cellular distribution of its tissue expression. Bcl-2 family proteins, including the major proapoptotic protein Bax and antiapoptotic Bcl-xL, are key players in the mitochondrial apoptosis pathway [7]. Initially, Bax monomer, which has constitutive expression and localizes in the cytoplasm, oligomerizes in the mitochondrial membrane under proapoptotic stimuli, forming pores through which cytochrome C is released into the cell cytoplasm [8]. In our studies, the content of monomeric Bax protein (22 kDa) under inflammation conditions with saline inhalations was higher than in controls (2.8-fold; $p < 0.05$), indicating its significant accumulation in cells on day 21 of pulmonary inflammation. In groups receiving inhalation treatment, Bax monomer content compared to placebo-control was lower (1.3-1.9-fold; $p < 0.05$), with minimal values observed with camostat mesilate. The content of Bax dimer (45 kDa), its main oligomeric form in the mitochondrial membrane [8], remained nearly unchanged with saline inhalations compared to controls. With inhalation treatment, its content was lower than placebo-control by 1.3-2.4-fold ($p < 0.05$), with the lowest values observed with methylprednisolone. Accordingly, the treatment reduced the content of the main proapoptotic protein—Bax dimer—with the greatest effect for methylprednisolone. The content of antiapoptotic Bcl-xL protein, which can disrupt activated Bax oligomers in the membrane [8], expectedly decreased during pulmonary inflammation (3.2-fold vs. control; $p < 0.05$), but showed clear recovery tendency with inhalation treatment. The smallest shift compared to placebo-control was observed with enoxaparin sodium (1.4-fold; $p > 0.05$), and the largest with camostat mesilate (8.3-fold; $p < 0.05$). Methylprednisolone also significantly increased Bcl-xL protein content (5.0-fold; $p < 0.05$). Corresponding to Bax protein changes, its interaction with Bax oligomers occurred—the 55 kDa fraction on the blot represented the Bcl-xL/Bax protein complex. During acute inflammation, its content decreased to minimum levels. Under inhalation treatment, complex content recovered in groups receiving camostat mesilate and methylprednisolone (exceeding placebo-control by 17.0 and 15.5-fold; $p < 0.05$), corresponding to Bcl-xL tissue accumulation dynamics. Unexpectedly, Bcl-xL/Bax complex content increased significantly with enoxaparin sodium (34.6-fold vs. placebo-control; $p < 0.001$). This maximal accumulation in lung tissue corresponded to the lowest Bcl-xL protein content among study groups.

In summary, pulmonary inflammation on day 21 was characterized by dense fibrosis and high proapoptotic protein activity with suppressed antiapoptotic proteins. Inhalation administration of camostat mesilate, methylprednisolone, and enoxaparin sodium prevented lung injury and fibrosis development, and prevented overexpression of active caspase-3 forms and Bax protein while restoring antiapoptotic Bcl-xL protein activity.

DISCUSSION

Molecular profile of apoptotic dysregulation and its correction by inhalation therapy

Our results confirm that dysregulated apoptosis is a key driver of late fibrotic remodeling following acute lung injury (ALI). By day 21, animals in the placebo group demonstrated a pronounced pro-apoptotic shift, characterized by accumulation of Bax monomer and active caspase-3 together with a marked reduction in the anti-apoptotic protein Bcl-xL. Morphologically, this imbalance was reflected by numerous caspase-3-positive cells within fibrotic regions, indicating sustained activation of mitochondrial apoptosis pathways. These findings are consistent with contemporary concepts of ALI pathogenesis, where excessive activation of caspase-dependent apoptosis contributes to persistent inflammation, epithelial loss, and subsequent fibrotic remodeling of lung tissue [9].

The implemented inhalation therapy effectively corrected this apoptotic imbalance. Camostat mesylate, methylprednisolone, and enoxaparin sodium significantly reduced levels of active caspase-3 and Bax while restoring Bcl-xL expression ($p < 0.05$). These molecular changes closely parallel experimental evidence demonstrating that therapeutic modulation of the Bax/Bcl-2 family and downstream caspase activation attenuates lung inflammation and apoptosis, thereby limiting structural lung damage [10]. Importantly, the observed anti-apoptotic effects were directly associated with morphological improvement, including reduced fibrosis, preservation of alveolar architecture, and a lower proportion of apoptotic cells.

As a result, suppression of excessive caspase-regulated apoptosis appears to represent a common final mechanism underlying the anti-fibrotic protection provided by these pharmacologically distinct agents, despite their different primary targets.

Morphological correlates and cellular specificity of therapeutic effects

The immunohistochemical findings bridge the molecular data with structural outcomes. The dramatic reduction in caspase-3-positive cells across all treatment groups corresponded with improved tissue architecture, including less fibrosis and signs of regeneration (e.g., epithelial hyperplasia with methylprednisolone).

Quantitative analysis revealed a cell-type-specific therapeutic influence. Methylprednisolone most effectively suppressed apoptosis in macrophages and type II alveolar cells, aligning with its primary anti-inflammatory mechanism. Enoxaparin sodium showed the strongest effect on fibroblasts, consistent with its role in improving microcirculation and reducing ischemic injury. Camostat mesylate exerted a more uniform anti-apoptotic effect across all cell populations, likely reflecting its systemic modulation of protease activity and the local renin-angiotensin system.

The protection of type II alveolar cells is of particular importance, as their role in surfactant production and epithelial regeneration makes them critical for preventing aberrant repair and fibrosis [11, 12]. Concurrently, the reduction in fibroblast apoptosis, especially with enoxaparin, may indicate stabilization of the interstitial environment, preventing pathological remodeling.

Comparative analysis of molecular mechanisms of drug action on the apoptotic cascade

Comparative immunoblot analysis revealed a clear mechanistic specificity of each drug acting on distinct components of the apoptotic cascade. Methylprednisolone exerted the most pronounced suppression of the pro-apoptotic protein Bax, producing the greatest reduction in its active dimeric form. This aligns with its core anti-inflammatory mechanism via glucocorticoid receptors and suppression of NF-κB, thereby removing upstream inflammatory triggers of Bax-mediated apoptosis [13, 14].

Camostat mesylate demonstrated the strongest stimulation of anti-apoptotic defense, markedly increasing Bcl-xL levels and restoring the Bcl-xL/Bax complex. This effect may be explained by its anti-protease activity [15]. Camostat-mediated modulation of the local renin–angiotensin system likely involves a shift toward the protective ACE2/angiotensin-(1–7)/Mas receptor axis, promoting expression of anti-apoptotic Bcl-2 family proteins [16, 17]. The favorable effects observed in our study may be attributable to inhalation delivery, enabling direct local action, in contrast to mixed outcomes of systemic administration in clinical trials [18].

Enoxaparin sodium proved the most effective inhibitor of the execution phase, reducing active caspase-3 levels to a minimum. This likely results from its pleiotropic properties: inhibition of neutrophil elastase and HMGB1, attenuation of thrombin-mediated apoptotic signaling, and improved microcirculation reducing ischemic stress [19, 20].

Collectively, the three drugs act on complementary levels of the apoptotic cascade: methylprednisolone targets inflammatory inducers, camostat promotes an anti-apoptotic microenvironment, and enoxaparin acts from inflammation suppression to direct caspase inhibition. This multilevel intervention effectively limits excessive apoptosis and fibrotic remodeling, supporting further evaluation of their potential rational combination.

Integration of evidence levels and pathophysiological model

The synthesis of our results forms a coherent multilevel model where molecular shifts directly translate into structural tissue changes. The correlative analysis confirms dysregulated apoptosis as a critical link between acute inflammation and chronic fibrosis [1].

The key finding is the specificity of drug mechanisms, positioning them for targeted intervention at different stages of the "inflammation–apoptosis–fibrosis" cascade. Methylprednisolone acts proximally by suppressing the inflammatory driver [4]. Camostat mesylate attenuates proteolytic and RAS-mediated signals, shifting the balance toward anti-apoptotic defense [1, 21]. Enoxaparin sodium exerts a complex effect combining improved microcirculation with direct suppression of the execution phase [20].

This differential action provides a rationale for developing combined therapeutic strategies in acute lung injury. Simultaneous intervention at multiple levels could yield synergistic effects. Our data also highlight

the potential of the inhalation route to achieve high local bioavailability. Future studies should define optimal combinations, dosing regimens, and therapeutic windows for clinical translation.

Clinical relevance, limitations, and future perspectives

The translational significance of these findings lies in substantiating a novel therapeutic strategy for preventing pulmonary fibrotic remodeling through selective modulation of apoptosis in distinct lung cell populations. The demonstrated ability of drugs with different primary targets—anti-inflammatory, anticoagulant, and local renin–angiotensin system modulation—to suppress excessive apoptosis by acting on specific links of the pathological cascade provides a theoretical framework for the development of rational combination therapies. Such strategies may yield synergistic benefits in acute respiratory distress syndrome and other conditions with a high risk of progression to fibrosis [21]. The inhalation route is of particular translational importance, as it enables high local drug concentrations while minimizing systemic adverse effects [20].

Several limitations of the current study should be acknowledged, as they define clear directions for future research. First, the experimental model combining aspiration bronchopneumonia with systemic lipopolysaccharide exposure reproduces key features of severe sterile inflammation but does not fully recapitulate all etiological variants, such as viral lung injury. Second, the analysis at a single late time point (day 21) allowed for the assessment of fibrotic outcomes but not the dynamic progression of early apoptotic activation and its resolution. Third, the relatively small sample size per treatment group limits the statistical power to detect more subtle, yet potentially important, differential effects. Therefore, the comparative efficacy data should be regarded as preliminary.

Future research should be directed towards experimental testing of combined inhalation regimens, studying early apoptotic markers to define optimal therapeutic windows, expanding the panel of target cell types, and undertaking the necessary translational steps of pharmacokinetic and pilot clinical studies to advance this multilevel therapeutic model.

CONCLUSIONS

1. On day 21 of experimental acute lung injury, rats receiving placebo (saline inhalation) exhibited extensive dense fibrotic zones (carnification) containing a large proportion (35–75%) of caspase-3-positive macrophages and fibroblasts.

2. Inhalation therapy administered from day 5 to 21 prevented the development of inflammatory damage and fibrotic remodeling of the lungs. Camostat mesylate reduced the overall number of caspase-3-positive cells, methylprednisolone predominantly decreased the proportion of caspase-3-positive macrophages and type II alveolar cells, while enoxaparin sodium had the most pronounced effect on fibroblasts.

3. The placebo group demonstrated a significant increase in the content of active caspase-3 and Bax protein, concomitant with a decrease in Bcl-xL and the Bcl-xL/Bax complex. Inhalation therapy prevented the overexpression of pro-apoptotic proteins and restored anti-apoptotic defenses. Specifically, enoxaparin sodium was most effective in reducing active caspase-3, methylprednisolone in reducing the Bax dimer, and camostat mesylate in decreasing the Bax monomer while increasing Bcl-xL and the Bcl-xL/Bax complex.

Declarations

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding: This research received no external funding.

Ethics approval and Consent to Participate: The animal study protocol was approved by the Ethics and Academic Integrity Committee of O.O. Bogomolets National Medical University (Kyiv, Ukraine) and was conducted in accordance with Directive 2010/63/EU and relevant Ukrainian legislation. Consent to participate is not applicable as the study did not involve human participants.

Consent to Publish: Not applicable.

Competing Interests: The authors declare that they have no competing interests.

References

1. Wang J, Li K, De H, Li X, Zhu Y, Yu H, Chen H. Pulmonary fibrosis: pathogenesis and therapeutic strategies. MedComm (2020). 2024;5(10):e744. doi: 10.1002/mco2.744.
2. Gu W, Zeng Q, Wang X, Jasem H, Ma L. Acute lung injury and the NLRP3 inflammasome. J Inflamm Res. 2024;17:3801-3813. doi: 10.2147/JIR.S464838.
3. Chen S, Bai Y, Xia J, Zhang Y, Zhan Q. Rutin alleviates ventilator-induced lung injury by inhibiting NLRP3 inflammasome activation. iScience. 2023;26(10):107866. doi: 10.1016/j.isci.2023.107866.
4. Kutsuzawa N, Ito Y, Kagawa S, Kohno C, Takiguchi H, Asano K. Dexamethasone restores TNFα-induced epithelial barrier dysfunction in primary rat alveolar epithelial cells. PLoS One. 2023;18(12):e0295684. doi: 10.1371/journal.pone.0295684.
5. Ziablitsev DS, Dyadik OO, Tikhomirov AO, Tsvetkova MM, Ziablitsev SV. Changes in the content and features of localization of angiotensin-converting enzyme-2 (ACE2) in acute experimental bronchopneumonia. Fiziol Zh. 2022;68(3):24-34. Ukrainian.
6. Ziablitsev DS, Tykhomyrov AO, Dyadik OO, Kolesnikova SV, Ziablytsev SV. Localization and level of proapoptotic protein regulators in rat lung tissue during the development of acute experimental

bronchopneumonia. Ukr Biochem J. 2022;94(4):36-46. doi: 10.15407/ubj94.04.036.

7. Chopra M, Reuben JS, Sharma AC. Acute lung injury: apoptosis and signaling mechanisms. Exp Biol Med (Maywood). 2009;234(4):361-371. doi: 10.3181/0811-MR-318.
8. Subburaj Y, Cosentino K, Axmann M, Pedrueza-Villalmanzo E, Hermann E, Bleicken S, Spatz J, García-Sáez AJ. Bax monomers form dimer units in the membrane that further self-assemble into multiple oligomeric species. Nat Commun. 2015;6:8042. doi: 10.1038/ncomms9042.
9. Xiao J, Wang L, Zhang B, Hou A. Cell death in acute lung injury: caspase-regulated apoptosis, pyroptosis, necroptosis, and PANoptosis. Front Pharmacol. 2025;16:1559659. doi: 10.3389/fphar.2025.1559659.
10. Emre AS, Mehtap S, Cem D, İlter İ, Melih A, Özlem Ö, Serdar S, Ekrem ÇH, Rasih Y. Cannabidiol protects lung against inflammation and apoptosis in a rat model of blunt chest trauma via Bax/Bcl-2/Cas-9 signaling pathway. Eur J Trauma Emerg Surg. 2025;51(1):95. doi: 10.1007/s00068-025-02767-0.
11. Sun W, Zhao B, He Z, Chang L, Song W, Chen Y. PLAC8 attenuates pulmonary fibrosis and inhibits apoptosis of alveolar epithelial cells via facilitating autophagy. Commun Biol. 2025;8(1):48. doi: 10.1038/s42003-024-07334-8.
12. Sun Z, He W, Meng H, Ji Z, Qu J, Yu G. Lactate activates ER stress to promote alveolar epithelial cells apoptosis in pulmonary fibrosis. Respir Res. 2024;25(1):401. doi: 10.1186/s12931-024-03016-5.
13. Meduri GU, Confalonieri M, Chaudhuri D, Rochwerg B, Meibohm B. Prolonged glucocorticoid treatment in ARDS: pathobiological rationale and pharmacological principles. In: Fink G, editor. Stress: Immunology and Inflammation. Amsterdam: Academic Press; 2024. p. 289-324.
14. Patel BV, Wilson MR, O'Dea KP, Takata M. TNF-induced death signaling triggers alveolar epithelial dysfunction in acute lung injury. J Immunol. 2013;190(8):4274-4282. doi: 10.4049/jimmunol.1202437.
15. Kawase M, Shirato K, van der Hoek L, Taguchi F, Matsuyama S. Simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors prevents severe acute respiratory syndrome coronavirus entry. J Virol. 2012;86(12):6537-6545. doi: 10.1128/JVI.00094-12.
16. Santos RAS, Sampaio WO, Alzamora AC, Motta-Santos D, Alenina N, Bader M, Campagnole-Santos MJ. The ACE2/Angiotensin-(1-7)/MAS Axis of the Renin-Angiotensin System: Focus on Angiotensin-(1-7). Physiol Rev. 2018;98(1):505-553. doi: 10.1152/physrev.00023.2016.
17. Ambrocio-Ortiz E, Pérez-Rubio G, Del Ángel-Pablo AD, Buendía-Roldán I, Chávez-Galán L, Hernández-Zenteno RJ, Ramírez-Venegas A, Rojas-Serrano J, Mejía M, Pérez-Padilla R, Guadarrama-Pérez C, Falfán-Valencia R. Angiotensin-Converting Enzyme 2 (ACE2) in the Context of Respiratory Diseases and Its Importance in Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infection. Pharmaceuticals (Basel). 2021;14(8):805. doi: 10.3390/ph14080805.
18. Khan U, Mubariz M, Khlidj Y, Nasir MM, Ramadan S, Saeed F, Muhammad A, Abuelazm M. Safety and Efficacy of Camostat Mesylate for Covid-19: a systematic review and Meta-analysis of Randomized controlled trials. BMC Infect Dis. 2024;24(1):709. doi: 10.1186/s12879-024-09468-w.

19. Shute J, Puxeddu E, Calzetta L. Heparin, low molecular weight heparin, and non-anticoagulant derivatives for the treatment of inflammatory lung disease. Pharmaceuticals (Basel). 2023;16(4):584. doi: 10.3390/ph16040584.
20. Mauri V, Frantzeskaki F, Karvouniaris M, Tsaganos T, Raftogiannis M, Poulakou G, Giamarellos-Bourboulis EJ, Spyridopoulos I. Low Molecular Weight Heparin inhibits thrombin-mediated endothelial inflammation and apoptosis in sepsis. Thromb Haemost. 2022;122(11):1947-1959. doi: 10.1055/a-1893-1864.
21. Zhang H, Li Y, Wang C, Zhang Y, Wei S, Li L, Zhang L, Yang J, Wang Y, Li X, Zhang W. The ACE2/Ang-(1-7)/Mas axis attenuates LPS-induced alveolar epithelial cell apoptosis by inhibiting the JNK pathway. Eur J Pharmacol. 2023;950:175742. doi: 10.1016/j.ejphar.2023.175742.

Figures

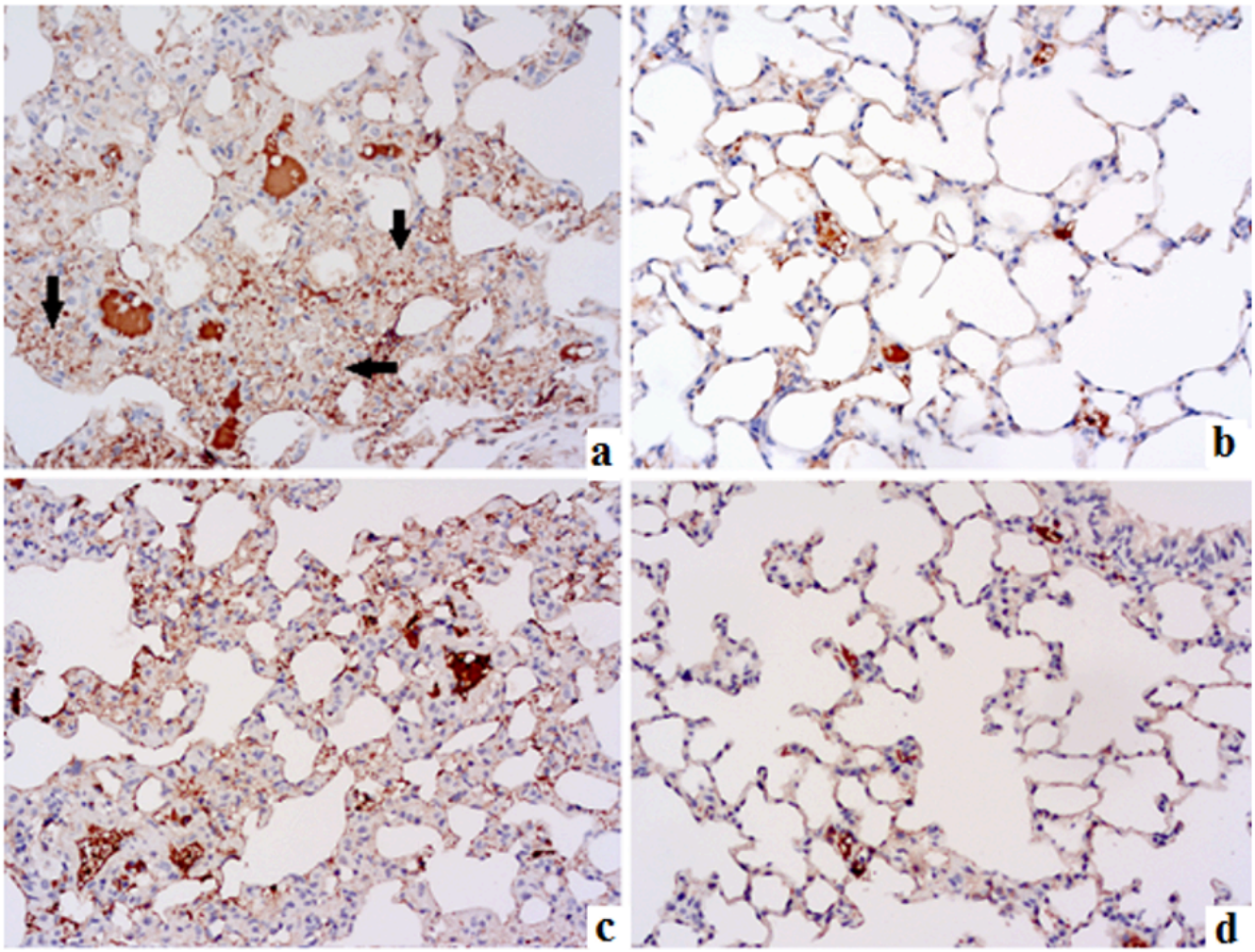


Figure 1

Lung tissue from rats with acute lung injury. Representative IHC staining for caspase-3, counterstained with hematoxylin; ×200;

a – Inhalation of saline (placebo control). Arrows indicate fields of chronic inflammation with fibrosis;

b – Inhalation of camostat mesylate;

c – Inhalation of methylprednisolone;

d – Inhalation of enoxaparin sodium.

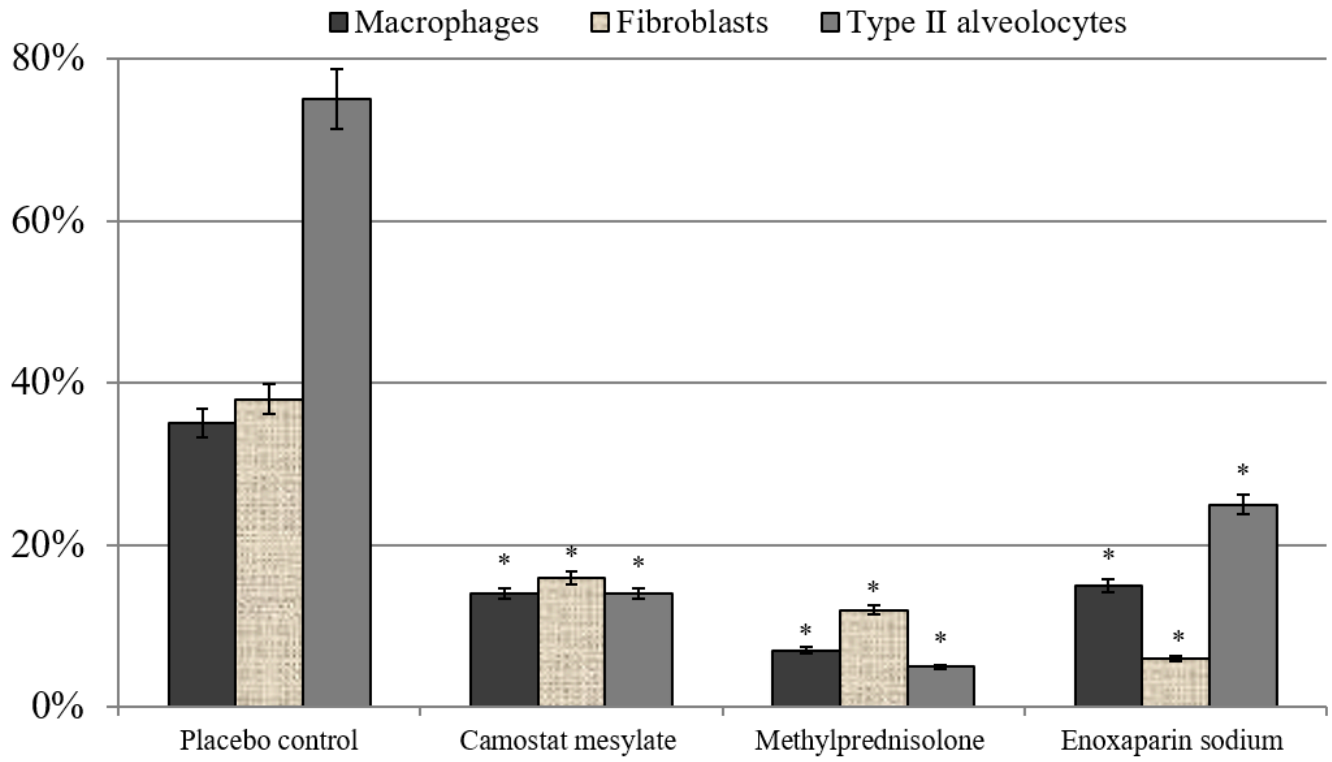


Figure 2

Proportion of caspase-3-immunopositive cells in lung tissue by experimental group. * – statistically significant difference from the placebo-control group ($p<0.05$).

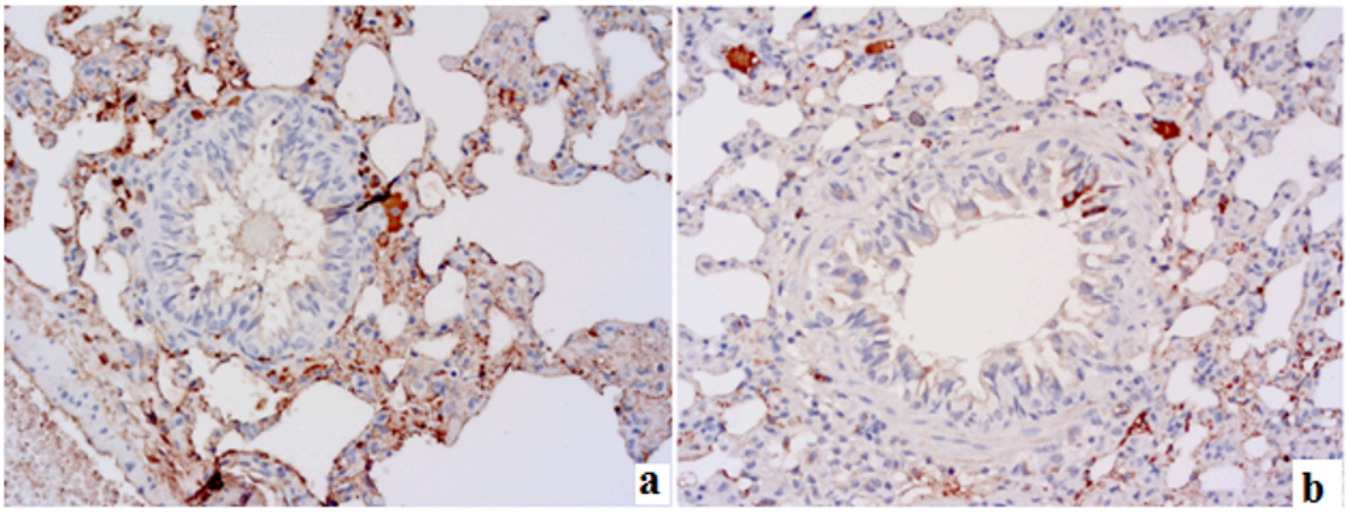
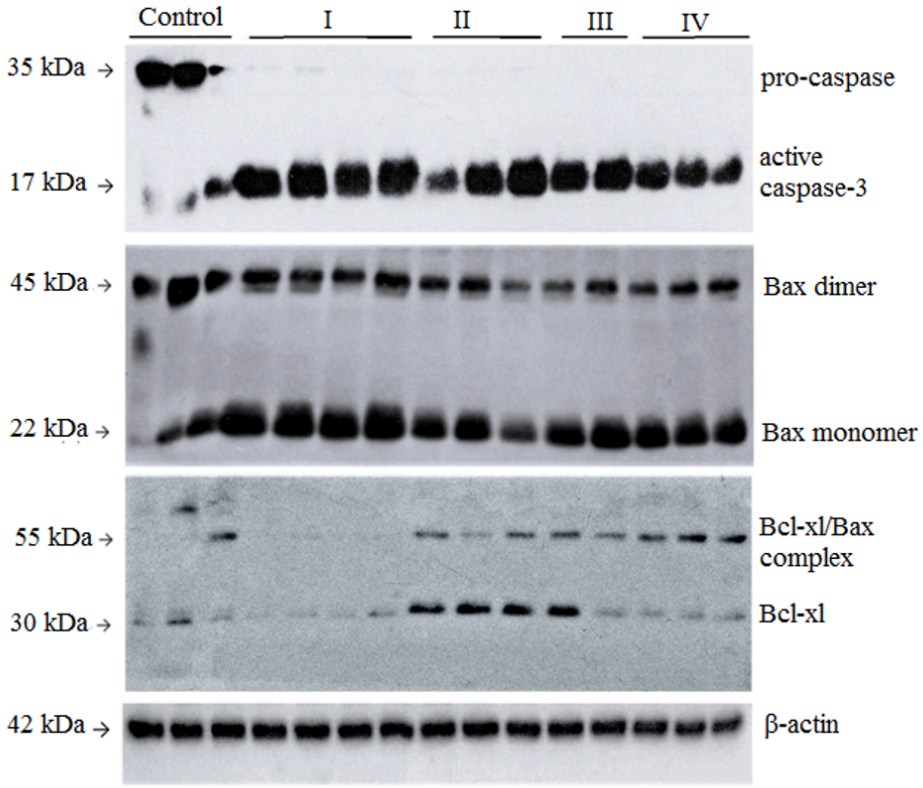


Figure 3

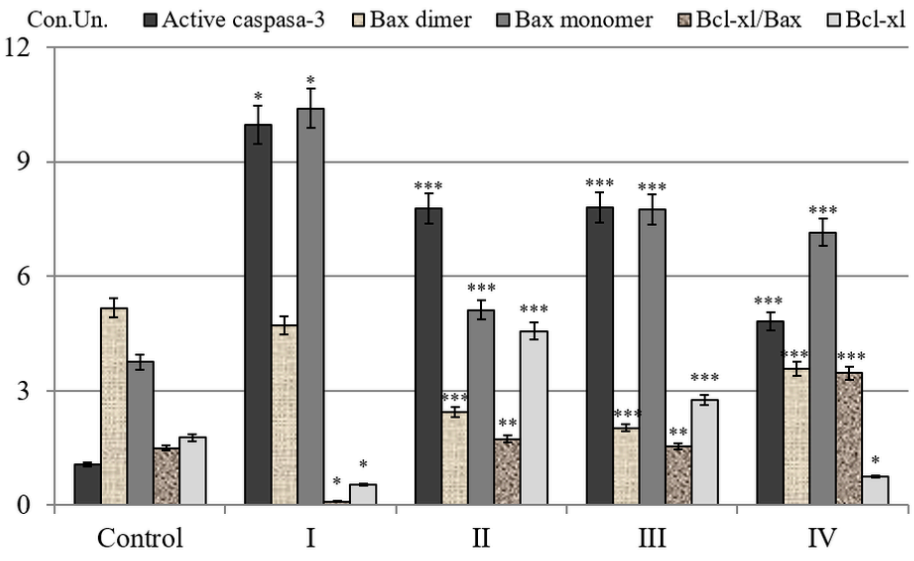
Lung tissue from rats with acute lung injury. Representative IHC staining for caspase-3, counterstained with hematoxylin; ×400;

a – Inhalation of saline (placebo control). Note thickened bronchial wall, exudate, and numerous positive cells;

b – Inhalation of methylprednisolone. Preserved tissue with hyperplasia of bronchial epithelium and fewer positive cells.



a



b

Figure 4

Content of pro-caspase-3, active caspase-3, Bax monomer and dimer, Bcl-xL/Bax complex, and Bcl-xL protein in lung tissue;

a – Examples of representative immunoblots for studied proteins;

b – Densitometry results (normalized to β-actin; conventional units);

*– statistically significant difference from control ($p<0.05$);

** – statistically significant difference from placebo-control ($p<0.05$);

Groups: Control (intact), I – placebo (saline), II – camostat mesylate, III – methylprednisolone, IV – enoxaparin sodium.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Uncroppedgelsandblots.png