JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2016, 67, 6, 919-932
www.jpp.krakow.pl

D. MOKRA[1], P. KOSUTOVA[1], S. BALENTOVA[2], M. ADAMKOV[2], P. MIKOLKA[1], J. MOKRY[3], M. ANTOSOVA[1], A. CALKOVSKA[1]

EFFECTS OF BUDESONIDE ON THE LUNG FUNCTIONS, INFLAMMATION AND APOPTOSIS IN A SALINE-LAVAGE MODEL OF ACUTE LUNG INJURY

[1]Biomedical Center Martin and Department of Physiology, [2]Department of Histology and Embryology, [3]Biomedical Center Martin and Department of Pharmacology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

Diffuse alveolar injury, edema, and inflammation are fundamental signs of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Whereas the systemic administration of corticosteroids previously led to controversial results, this study evaluated if corticosteroids given intratracheally may improve lung functions and reduce edema formation, migration of cells into the lung and their activation in experimentally-induced ALI. In oxygen-ventilated rabbits, ALI was induced by repetitive saline lung lavage, until PaO_2 decreased to < 26.7 kPa in FiO_2 1.0. Then, one group of animals was treated with corticosteroid budesonide (Pulmicort susp inh, AstraZeneca; 0.25 mg/kg) given intratracheally by means of inpulsion regime of high-frequency jet ventilation, while another group was non-treated, and both groups were oxygen-ventilated for following 5 hours. Another group of animals served as healthy controls. After sacrifice of animals, left lung was saline-lavaged and protein content was measured and cells in the lavage fluid were determined microscopically. Right lung tissue was used for estimation of edema formation (expressed as wet/dry weight ratio), for histomorphological investigation, immunohistochemical determination of apoptosis of lung cells, and for determination of markers of inflammation and lung injury (IL-1β, IL-6, IL-8, TNF-α, IFNγ, esRAGE, caspase-3) by ELISA methods. Levels of several cytokines were estimated also in plasma. Repetitive lung lavage worsened gas exchange, induced lung injury, inflammation and lung edema and increased apoptosis of lung epithelial cells. Budesonide reduced lung edema, cell infiltration into the lung and apoptosis of epithelial cells and decreased concentrations of proinflammatory markers in the lung and blood. These changes resulted in improved ventilation. Concluding, curative intratracheal treatment with budesonide alleviated lung injury, inflammation, apoptosis of lung epithelial cells and lung edema and improved lung functions in a lavage model of ALI. These findings suggest a potential of therapy with inhaled budesonide also for patients with ARDS.

Key words: *acute lung injury, acute respiratory distress syndrome, bronchoalveolar lavage, animal model, budesonide, corticosteroids, apoptosis*

INTRODUCTION

Acute respiratory distress syndrome (ARDS) may originate from various reasons - aspiration, near-drowning, sepsis etc. It is characterized by acute hypoxemia, finding of bilateral infiltrates on chest X-ray without increased wedge pressure in the pulmonary artery. ARDS Definition Task Force in 2012 divided ARDS into 3 degrees according to the severity of hypoxemia expressed by a ratio between arterial partial pressure of oxygen and fraction of inspired oxygen (PaO_2/FiO_2) to mild (PaO_2/FiO_2 200–300 mm Hg, or 26.7 – 40 kPa), moderate (PaO_2/FiO_2 100 – 200 mm Hg, or 13.3 – 26.7 kPa), and severe (PaO_2/FiO_2 < 100 mm Hg, or < 13.3 kPa) (1). For experimental studies where respiratory insufficiency is induced artificially and other clinically relevant signs except of hypoxemia cannot be determined, the term acute lung injury is preferred.

Irrespective of the triggering factor, injury to the lung cells leads to surfactant alterations caused by reduced production of surface-active compounds, changes in their composition, imbalance in surfactant subtype distribution, and/or by inhibition of surfactant function by plasma protein leakage and inflammatory mediators. The mentioned changes together with edema formation, ventilation-perfusion mismatch, and inflammation finally decrease the lung compliance and cause hypoxemia. Diffuse alveolar injury is associated with massive infiltration of polymorphonuclears (PMN), mainly neutrophils into the lung, alveolar hemorrhage, and generation of hyaline membranes. Cells migrating through the injured alveolo-capillary membrane and activated structural lung cells produce high amounts of pro-inflammatory substances, such as interleukins (IL)-1β, IL-6, IL-8, tumor necrosis factor alpha (TNF-α), proteases, reactive oxygen species, inducible NO synthase (iNOS), or matrix-metalloproteinases which further deteriorate the lung functions (2-4). Complex action of the mentioned factors results in a disbalance in apoptotic processes in the lung, as well. While the apoptosis of neutrophils is delayed what causes their prolonged survival and detrimental effects in the

lung tissue, apoptosis of epithelial and endothelial cells is increased. Both these processes significantly contribute to the pathogenesis of ALI/ARDS (5).

Within last decades, a considerable progress has been made in understanding the pathophysiology of ALI/ARDS. However, its appropriate therapy is still questionable as only the lung-protective ventilation and fluid-conservative management have reduced mortality and morbidity, but no pharmacologic intervention has been clearly shown to be commonly effective in reducing mortality (6). Nevertheless, there are several groups of drugs, such as corticosteroids (CS), pulmonary vasodilators, antioxidants, statins, beta2-adrenergic agonists, protease inhibitors, neutrophil elastase inhibitors, or anticoagulants, which were beneficial in neutrophil-mediated chronic obstructive pulmonary disease (7, 8) and in the subgroups of patients with ARDS (3, 9).

In ALI/ARDS, CS are expected to mitigate the lung edema formation and inflammation. CS stabilize the membranes, reduce microvascular permeability, and decrease production of the vasoactive substances. In addition, CS reduce the migration and activation of neutrophils, eosinophils, mononuclears, and other cells and modulate chemotaxis and action of mediators released from the activated cells (10). CS can also inhibit lung epithelial cell apoptosis and thereby reduce the lung injury (11). The pleiotropic effects of CS are mediated by both genomic and nongenomic mechanisms. Genomic mechanisms include activation of cytosolic glucocorticoid receptor what leads to activation or repression of synthesis of proteins including cytokines and chemokines (e.g., IL-1β, IL-6, IL-8, and TNF-α), pro-inflammatory enzymes (e.g., phospholipase A_2, cyclooxygenase-2, iNOS), adhesion molecules, and other biologically active substances, such as platelet activating factor or endothelin-1 (10). Moreover, action of CS is mediated through nongenomic mechanisms which are responsible for rapid processes in various cells within seconds or minutes until the effects mediated by genomic mechanisms appear (12, 13).

Despite its potential, systemic use of CS in ALI/ARDS patients led to controversial results (14-18). Therefore, the aim of this study was to evaluate whether a single intratracheal dose of CS budesonide may alleviate lung injury, inflammation, apoptosis, and lung edema formation and thereby improve the lung functions in a rabbit model of ALI. We have hypothesized that a local delivery of budesonide directly into the diseased lung may enhance its therapeutic effect in comparison to systemic delivery which has appeared controversial.

The experimental model of ALI was induced by repetitive lung lavage with saline until the sufficient amount of pulmonary surfactant was removed and lung compliance decreased. Extent of the lung injury was monitored during the course of experiment when the changes in the blood gases, respiratory indexes and counts of white blood cells were evaluated continuously. Additional data were obtained from samples taken at the end of experiment, such as markers of the lung injury, inflammation, apoptosis, and lung edema formation. The model of ALI was performed on adult rabbits, as the use of this animal species compared to smaller animals enables application of different modes of artificial ventilation and provides a possibility to take regularly the blood samples for measurement of blood gases and for estimation of cell and biochemical markers of inflammation during the experiment (19-22).

MATERIALS AND METHODS

General design of experiments

Experimental protocol was performed in accordance with the ethical guidelines and was authorized by the local Ethics Committee of Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava and by National Veterinary Board.

In the study, adult New Zealand white rabbits in a total number of eighteen (n = 18) of both genders and a mean body weight (b.w.) ± standard deviation (SD) of 2.5 ± 0.3 kg were used. Animals were anesthetized with intramuscular ketamine (20 mg/kg b.w.; Narketan, Vetoquinol, UK) and xylazine (5 mg/kg b.w.; Xylariem, Riemser, Germany), followed by an infusion of ketamine (20 mg/kg b.w./h). Tracheotomy was performed and catheters were inserted into the femoral artery and right atrium for sampling the blood, and into the femoral vein to administer anesthetics. One group of animals which served as healthy non-ventilated controls (Contr group, n = 6) was euthanized at this stage of experiment by an overdose of anesthetics. Other animals were paralyzed with pipecuronium bromide (0.3 mg/kg b.w./30 min; Arduan, Gedeon Richter, Hungary) and subjected to a pressure-controlled ventilator (Beat-2, Chirana, Slovakia) and were ventilated conventionally with following settings: frequency (f.) of 30/min, fraction of inspired oxygen (FiO_2) of 1.0, time of inspiration (Ti) 50%, peak inspiratory pressure (PIP)/positive end-expiratory pressure (PEEP) 1.5/0.3 kPa and tidal volume (V_T) of 6 – 8 ml/kg b.w. After 15 min of stabilization, respiratory parameters were recorded and blood samples taken for analysis of blood gases (RapidLab 348, Siemens, Germany) and estimations of total and differential white blood cell (WBC) counts were performed. Lung injury was induced by repetitive lung lavage with 0.9% saline (30 ml/kg b.w., 37°C), which was instilled into the endotracheal cannula in the semi-upright right and left lateral positions of the animal and was immediately suctioned by a suction device. Lavage was performed 6 – 10 times, until PaO_2 decreased to < 26.7 kPa in FiO_2 1.0 in 2 measurements at 5 and 15 min after the lavage. When the criteria for the ALI model were fulfilled, animals were treated with budesonide (Pulmicort susp inh, AstraZeneca, 0.25 mg/kg b.w.; ALI + Bud group, n = 6) or were left without therapy (ALI group, n = 6). Budesonide was given intratracheally during 1 min by means of inpulsion regime of high-frequency jet ventilation (HFJV; f. 300/min, Ti 20%) and this regime continued for additional 1 min to supply a homogenous distribution of the drug throughout the lung (23, 24). Then the ventilation was switched back to conventional ventilation (FiO_2 1.0, f. 30/min, PIP/PEEP 1.5/0.3 kPa, V_T 6 – 8 ml/kg b.w.). All animals with ALI (ALI group and ALI + Bud group) were ventilated with these ventilator settings for an additional 5 hours. Blood gases and respiratory parameters were measured at 0.5, 1, 2, 3, 4, and 5 hours after the treatment, WBC counts were estimated from samples taken at 0.5, 1, 3 and 5 hours after the treatment. At the end of experiment, animals were euthanized by an overdose of anesthetics.

Measurement and calculation of respiratory parameters

Tracheal airflow and V_T were measured by a heated Fleisch head connected to a pneumotachograph. Airway pressure was registered *via* a pneumatic catheter placed in the tracheal tube and connected to an electromanometer. Mean airway pressure was calculated as: MAP = (PIP + PEEP)/2. Oxygenation index (OI) was calculated as: OI = (MAP × FiO_2)/PaO_2. Ventilation efficiency index was calculated as VEI = 3800/[(PIP-PEEP) × frequency × $PaCO_2$].

Counting of cells in the arterial blood and in the BAL fluid

Samples of the arterial blood for counting WBC were taken before induction of ALI model and at 0.5, 1, 3, and 5 h after the treatment with budesonide. Total WBC count was determined microscopically in a counting chamber after staining by Turck. Differential WBC count was estimated microscopically after staining by May-Grunwald/Giemsa-Romanowski.

After euthanizing the animal at 5 hours time point, lung and trachea were excised. Left lung was lavaged 3-times with a pre-heated saline (0.9% NaCl, 37°C) at a dose of 10 ml/kg b.w., the bronchoalveolar lavage (BAL) fluid was then centrifuged at 1500 rpm for 10 min. Total number of cells in the BAL fluid was determined microscopically in a counting chamber. Differential count of cells in the BAL fluid sediment was evaluated microscopically after staining by May-Grunwald/Giemsa-Romanowski.

Estimation of lung edema formation

Strips of the right lung tissue were cut, weighed and dried at 60°C for 24 hours to determine the wet/dry weight ratio, where higher wet/dry ratio indicated higher fluid accumulation in the tissue.

Measurement of total protein content in the BAL fluid was performed by colorimetric method according to Bradford (25) with bovine serum albumin (BSA) as a standard. Analysis was performed in the sample of the lavage fluid taken from the first lung lavage used for induction of ALI model (initial value) and in the sample of the lavage fluid taken at the end of experiment (5 hours after the therapy), and results were expressed in μg/ml.

Histomorphological investigation of the lung injury and inflammation

For histological analysis, upper right lung lobe was fixed in buffered 4% formaldehyde. After paraffin embedding the sections of 4 μm were cut on microtome. The slides were further deparaffinized, rehydrated in descending grades of ethanol and stained with hematoxylin. After differentiation and washing, the slides were immersed in eosin dye, dehydrated and finally coverslipped with Entellan mounting medium (Merck Millipore, Germany). To score lung injury and inflammation, lung tissue samples were screened for the following histopathological signs: 1) atelectasis, 2) emphysema, 3) hemorrhagia, and 4) PMN infiltration. Samples were evaluated by an experienced histopathologist blinded to the grouping of animals, and results were scored of 0 – 3 with 0 as absent (normal), 1 as mild, 2 as moderate, and 3 as severe lung injury. The total injury score was calculated as a sum of these scores.

Immunohistochemical detection of apoptosis in the lung tissue

1. In situ labeling of DNA strand breaks by TUNEL methods

The presence of apoptotic cells in the lung tissue sections was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique. First, the lung samples were immersed in 4% formaldehyde solution. After paraffin embedding 4 μm thick slides were cut followed by deparaffinization and pretreatment with a proteinase K. The tissue sections were further processed by DeadEnd™ Colorimetric TUNEL System (Promega, USA) to label fragmented DNA of apoptotic cells. Biotinylated nucleotide is incorporated at 3'-OH DNA ends using the recombinant terminal deoxynucleotidyl transferase (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides. For detection of nucleotides and blocking endogenous peroxidases, the sections were incubated with 0.3% H_2O_2 solution. Color of sections was developed after incubation with diaminobenzidine (DAB)-chromogen solution (Dako, Denmark). The sections were then counterstained with Mayer´s hematoxylin and mounted with an Permount (Fisher, USA). The slides were viewed with an Olympus BX41 microscope (Olympus, Japan). The image capture was performed with Quick Photo Micro software, version 2.2 (Olympus, Japan). The apoptotic index of alveolar and bronchial epithelium was calculated as a percentage of TUNEL immunoreactive (TUNEL-IR) dark brown stained nuclei in a total 100 nuclei randomly counted from three sites within each section.

2. Detection of activated caspase-3

After deparaffinization, revitalisation and rehydratation, the tissue slides were treated with 3% H_2O_2 solution for 10 min for blocking endogenous peroxidases. Washing with Tris buffer was used after each handling step. The sections were incubated with the primary antibody rabbit anti-caspase 3 (1:500; Bioss, USA) for 30 min at room temperature. The specimen was then incubated by sequential 10 min incubation with biotinylated anti-rabbit secondary antibody and pexodidase-labelled streptavidin conjugated to HRP (DAKO LSAB®2 System-HRP; Dako). Color of sections was developed after incubation with DAB-chromogen solution (Dako). The sections were then counterstained with Mayer´s hematoxylin and mounted with an Entellan (Merck, USA). The slides were viewed with an Olympus BX41 microscope (Olympus). The image capture was performed with Quick Photo Micro software, version 2.2 (Olympus). The density of activated caspase-3 immunoreactive cells (dark-brown cytoplasm and plasma membrane; caspase 3-IR) in the alveolar and bronchial epithelium was measured randomly from three sites within each section and was calculated as the total numbers of caspase 3-IR cells in the field.

Detection of biochemical markers of lung injury, inflammation and apoptosis

1. Preparation of the blood plasma

Samples of the arterial blood taken at the end of experiment were centrifuged (3000 rpm for 15 min, 4°C) and plasma was stored at –70°C until the analysis was performed.

2. Preparation of the lung tissue homogenate

Strips of the right lung lobe were homogenized (5-times for 25 s, 1200 rpm) in an ice-cold phosphate buffer (pH 7.4). Homogenates were then 3-times freezed and centrifuged (12000 rpm for 15 min, 4°C). Final supernatants were then stored at –70°C until the analysis was performed. Protein concentrations in the lung homogenates were determined according to the methods described by Lowry *et al*. (26), using a bovine serum albumin as a standard.

3. Measurement of markers of inflammation and lung injury by enzyme-linked immunosorbent assay (ELISA).

Concentrations of cytokines (IL-1β, IL-6, and IL-8, TNF-α, and interferon gamma (IFNγ)) and markers of lung injury (endogenous secretory receptor for advanced glycation end-products (esRAGE) and caspase-3) were measured in the lung homogenates, wheras concentrations of IL-1β, -6, -8, and TNF-α were measured also in the blood plasma. The measurements were performed using commercially available rabbit-specific ELISA kits (USCN kits for cytokines, ABIN for esRAGE, Cusabio for caspase-3) according to the manufacturers' instructions. Results were analyzed by a spectrophotometer at 450 nm using an ELISA microplate reader.

Statistics

For analysis of the data, statistical package SYSTAT for Windows was used. Differences among 3 groups (Contr, ALI

and ALI + Bud) were analyzed by one-way ANOVA with post-hoc Fisher's LSD test, differences in respiratory parameters between ALI + Bud group and ALI group were evaluated by Kruskal-Wallis test. Within-group differences were evaluated by Wilcoxon test. A value of $P < 0.05$ was considered statistically significant. Data are expressed as means ± S.E.M.

RESULTS

At the beginning of experiments, i.e. before induction of ALI model, there were no statistically significant differences among the groups in the body weight of animals and initial values of the respiratory parameters (all $P > 0.05$). Induction of ALI model affected the respiratory parameters, however, before administration of budesonide treatment there were no statistically significant differences in the values of these parameters between the ALI and ALI + Bud group ($P > 0.05$).

Respiratory parameters

During the whole experiment, animals of both groups with ALI (ALI group and ALI + Bud group) were ventilated with ventilatory pressures PIP/PEEP of 1.5/0.3 kPa and a resulting value of MAP of 0.9 kPa ($P > 0.05$).

After induction of ALI, values of VEI decreased in ALI group to 34% and in ALI + Bud group to 43% of the initial values (both $P < 0.05$; *Table 1*). Reduced ventilation efficiency resulted in worsened oxygenation and oxygen saturation of hemoglobin compared to values before induction of ALI (all $P < 0.05$; *Table 1*). After induction of ALI in the ALI group and ALI + Bud group, PaO_2/FiO_2 decreased to 13 – 18% of the initial values, OI increased 7 – 8 times and oxygen saturation decreased to 84 – 88% of the initial values (all $P < 0.05$ versus initial values). In the ALI + Bud group, budesonide treatment increased VEI by 35% at 30 min after the treatment compared to the VEI value after induction of ALI model, and by 27% at 1 h, 29% at 2 h and 15% at 3 h, and this effect declined to 5 – 6% at 4 – 5 h. Despite, that the mentioned within-group differences were not significant compared to the value. After ALI (all $P > 0.05$ versus value After ALI), significant between-group differences in VEI were found between ALI + Bud group and ALI group at 30 min ($P < 0.01$), and at 2 and 3 h after the treatment delivery (both $P < 0.05$; *Table 1*). Parameters of oxygenation (PaO_2/FiO_2, OI and oxygen saturation) also improved after administration of budesonide. However, due to a big inter-individual variability the within-group differences (vs. values after ALI) as well as the between-group differences between the ALI + Bud group and ALI group were not statistically significant (*Table 1*).

Cells in the BAL fluid and in the arterial blood

Induction of ALI caused a migration of WBC from the circulation into the lung tissue. This process was accompanied with WBC slight decrease in the peripheral blood. Budesonide prevented the cell migration into the lung, as indicated by increased WBC in the blood at the end of experiment ($P < 0.01$ versus non-treated ALI group at 3 and 5 hours after the treatment; *Fig. 1A*). Differential counts of WBC at the end of experiment showed significant increase in percentages and absolute numbers of neutrophils and decrease in percentages of lymphocytes in both ALI and ALI + Bud groups in comparison with initial (before ALI) values (both $P < 0.001$, *Fig. 1B* and *Fig. 1C*). In budesonide-treated group (ALI + Bud), budesonide increased significantly the absolute numbers of neutrophils ($P < 0.05$) and monocytes ($P < 0.01$) compared to non-treated ALI group, whereas the percentage of these cells was not significantly different (*Fig. 1B* and *Fig. 1C*).

Analysis of BAL fluid showed an increase in the total number of cells migrating into the lung of non-treated ALI animals compared to healthy controls ($P < 0.01$; *Fig. 2A*). Determination of BAL cell types showed higher percentages of neutrophils ($P < 0.001$) and eosinophils ($P < 0.05$) and lower percentage of monocytes-macrophages ($P < 0.001$) in non-treated ALI group versus healthy control group (*Fig. 2B* and *Fig. 2C*). Treatment with budesonide decreased a total number of cells in the BAL fluid ($P < 0.05$; *Fig. 2A*), decreased percentages and absolute numbers of neutrophils (both $P < 0.05$) and absolute number of eosinophils ($P < 0.05$), and increased percentage of mononuclears ($P < 0.05$; *Fig. 2B* and *Fig. 2C*) versus non-treated ALI group.

Lung edema formation

Repetitive saline lung lavage for induction of the ALI model increased fluid accumulation in the lung tissue in the ALI group as

Table 1. Respiratory parameters before and after induction of ALI model (Before/After ALI) and 0.5, 1 – 5 hours after the therapy (Th) administration in the non-treated ALI group (ALI group, n = 6) and in the animals with ALI treated with budesonide (ALI + Bud group, n = 6).

	Before ALI	After ALI	0.5 h Th	1 h Th	2 h Th	3 h Th	4 h Th	5 h Th
Ventilation efficiency index (VEI)								
ALI	38.1±3.0	13.1±2.1§	14.2±1.5	14.3±3.9	11.1±1.9	9.4±1.5	9.3±1.5	9.4±1.5
ALI+Bud	40.3±1.4	17.5±1.9§	23.7±2.4$^{\#\#}$	22.3±1.9	22.6±3.5$^{\#}$	20.2±4.0$^{\#}$	18.6±4.6	18.4±4.9
PaO_2/FiO_2								
ALI	70.9±3.3	9.1±1.0§	10.4±1.3	9.1±1.1	9.3±0.9	9.1±0.9	9.1±1.1	9.0±1.0
ALI+Bud	62.9±6.5	11.5±2.5§	12.6±1.7	15.5±4.5	21.8±8.6	21.8±8.7	23.4±9.5	23.1±9.8
Oxygenation index (OI)								
ALI	1.3±0.1	10.4±1.1§	9.5±1.3	10.6±1.2	10.2±0.9	10.3±0.8	10.6±1.2	10.7±1.2
ALI+Bud	1.5±0.2	10.7±2.2§	7.8±1.2	7.5±1.6	7.4±2.2	7.9±2.5	7.4±2.3	7.6±2.3
Oxygen saturation of hemoglobin (%)								
ALI	99.9±0.0	83.2±4.3§	88.1±4.0	84.9±4.1	84.0±3.8	81.0±4.4	78.4±6.2	77.9±6.7
ALI+Bud	99.9±0.0	87.9±3.5§	91.3±3.7	91.4±3.6	89.5±3.9	85.6±5.5	86.2±5.7	84.4±6.6

For differences within ALI and ALI + Bud groups between the values After ALI versus Before ALI : $^{\S}P < 0.05$. For differences between ALI + Bud versus ALI groups at actual time points: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Data are expressed as means ± S.E.M.

indicated by increase of wet-dry weight ratio by 30% compared with Contr group ($P < 0.001$). Budesonide treatment (ALI + Bud group) reduced wet-dry weight ratio by 60%, ($P < 0.01$ for ALI + Bud versus ALI group) so after budesonide treatment this ratio was increased only by 12% compared with Contr group (*Fig. 3A*). In the ALI group, increased permeability through alveolocapillary membrane was confirmed also by 2-fold increased protein content in the samples of BAL fluid taken at the end of experiment compared to initial values (i.e. before ALI) ($P < 0.05$) and compared to Contr group ($P < 0.001$). Treatment with budesonide decreased protein content in BAL fluid by 28% compared to non-treated ALI group ($P < 0.01$; *Fig. 3B*).

Histomorphological analysis of the lung injury and inflammation

Histological investigation of the lung sections in the non-treated ALI group showed 5-fold and statistically significant increase for occurance of atelectasis as compared to Contr group ($P < 0.01$), 4-fold and statistically significant increase for PMN

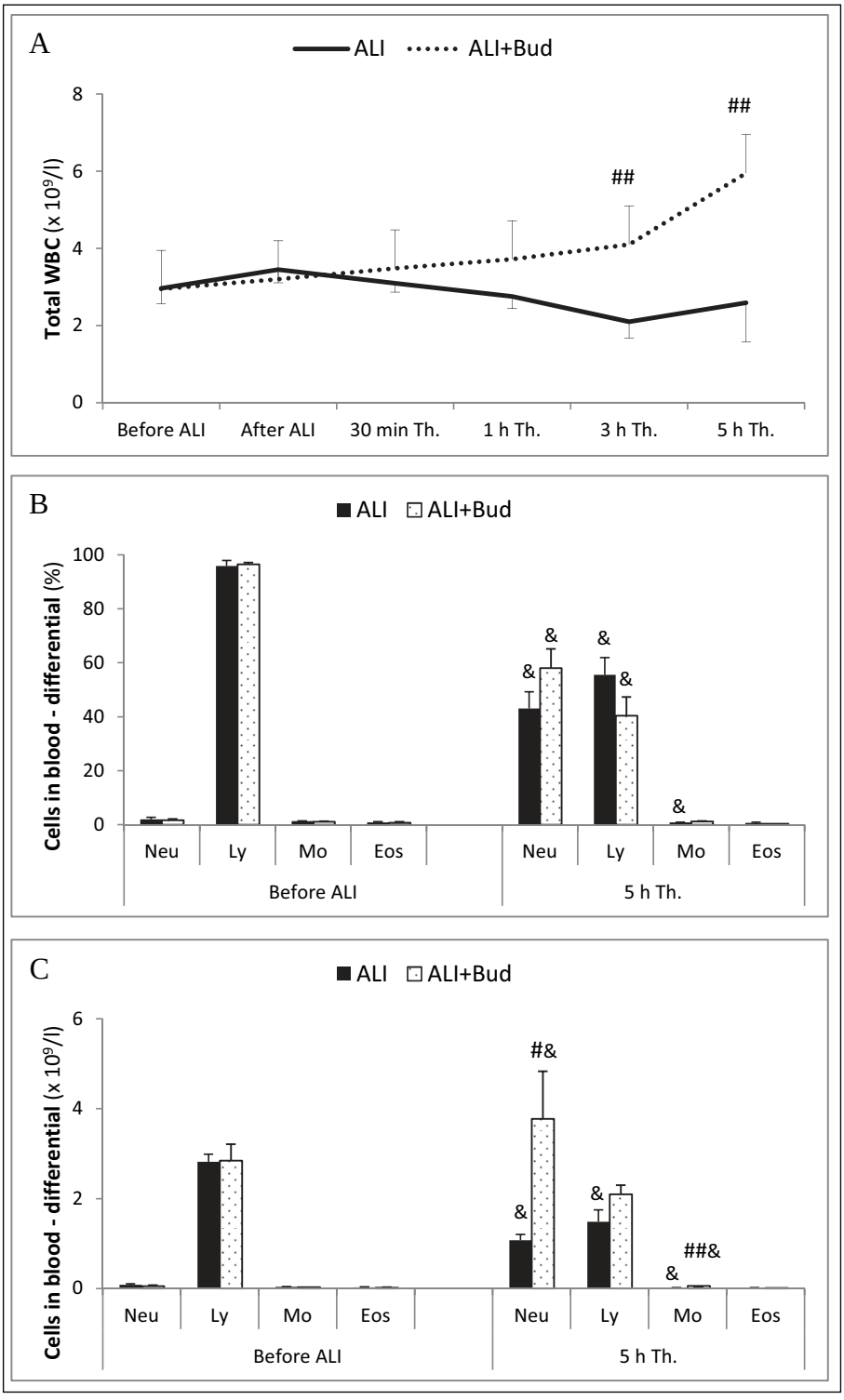

Fig. 1. Total and differential counts of white blood cells (WBC) in the arterial blood. (A) Total count of (WBC) in the arterial blood during experiment. (B) Differential counts of WBC (expressed in %) in the arterial blood at the end of experiment. (C) Differential counts of WBC (expressed in absolute numbers, count $\times 10^9$/l) in the arterial blood at the end of experiment. For differences between ALI + Bud and ALI groups: $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$; $^{\&}P < 0.05$ for within-group comparison of values ALI at 5 h after the therapy versus Before ALI. Data are expressed as means ± S.E.M., number of animals (n = 6) in each group. *Abbreviations*: Neu, neutrophils; Ly, lymphocytes; Mo, monocytes; Eos, eosinophils.

infiltration (P < 0.01), and more than 3-fold but non-significant increases for occurance of emphysema and hemorrhagia (both P > 0.05; *Table 2)*. In the non-treated ALI group, also the 4-fold higher total injury score was found compared to Contr group (P < 0.001; *Fig. 3C*). Budesonide treatment significantly reduced PMN infiltration by about 56% compared to ALI non-treated group (P < 0.01), reduced emphysema to nearly control levels and decreased total lung injury score by about 42% although these two later effects did not reach statistical significances (P > 0.05; *Table 2* and *Fig. 3C*).

Detection of apoptosis in the lung tissue by TUNEL methods

In situ labeling of DNA strand breaks using the TUNEL technique indicated higher number of apoptotic alveolar and bronchial cells in the lung tissue sections in the non-treated ALI group versus controls where increase in apoptotic index in alveolar cells was about 2-fold (P < 0.05) and increase in apoptotic index in bronchial cells was 25-fold (P < 0.001; *Figs. 4A* and *5A-5F*). Treatment with budesonide showed a tendency to decrease the number of apoptotic alveolar cells (P > 0.05)

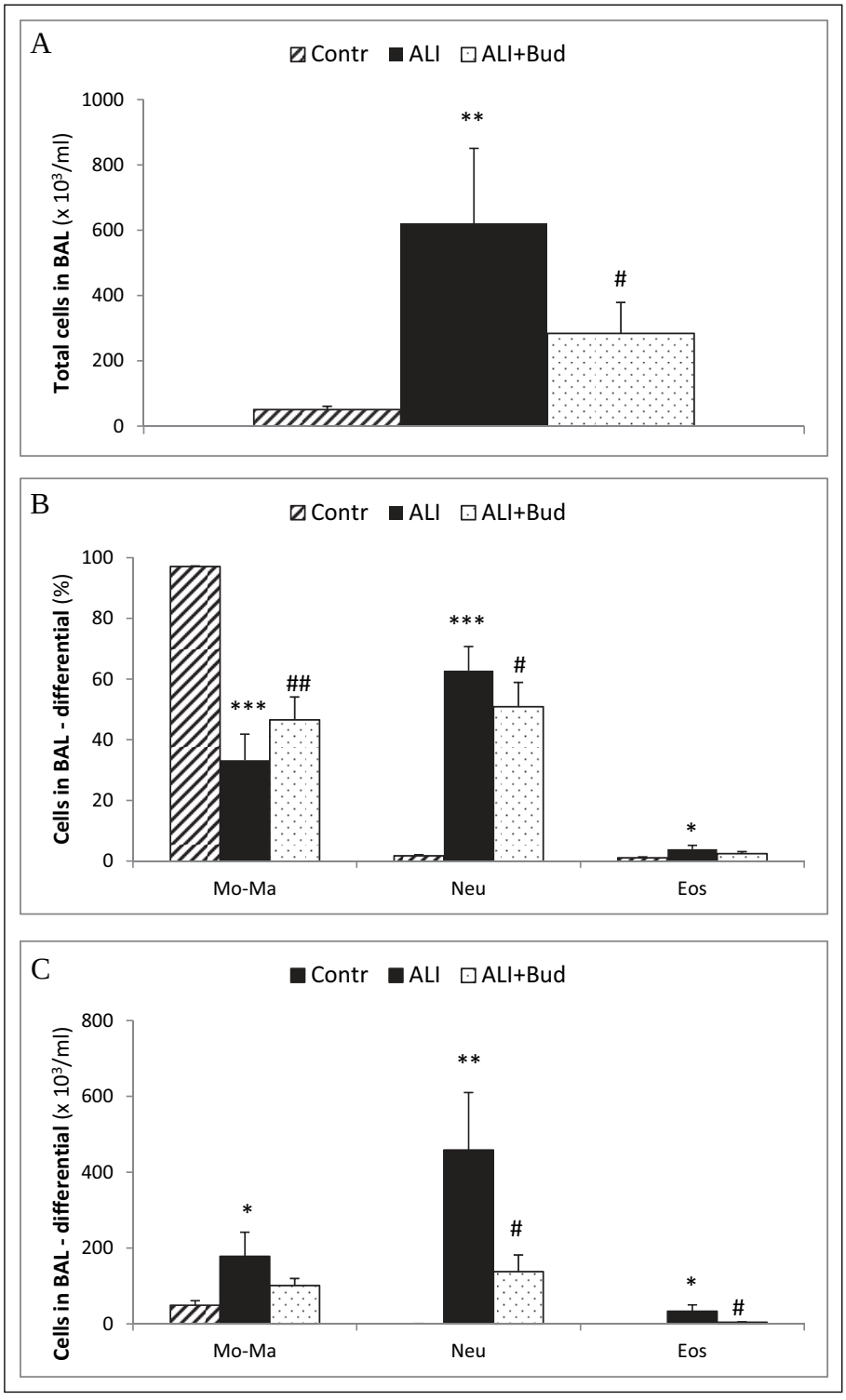

Fig. 2. Total and differential counts of cells in the BAL fluid. (A) Total count of cells in the bronchoalveolar (BAL) fluid at the end of experiment. (B) Differential counts of cells (expressed in %) in the BAL fluid at the end of experiment. (C) Differential counts of cells (expressed in absolute numbers, count × 10^3/ml) in the BAL fluid at the end of experiment. For between-group differences: *P < 0.05, **P < 0.01, and ***P < 0.001 for ALI group versus healthy controls (Contr group); #P < 0.05 for ALI + Bud versus ALI groups. Data are expressed as means ± S.E.M., number of animals (n = 6) in each group.
Abbreviations: Mo-Ma, monocytes-macrophages; Neu, neutrophils; Eos, eosinophils.

and exerted huge decrease in number of apoptotic bronchial cells ($P < 0.001$; *Figs. 4A* and *5A-5F*) compared to untreated ALI group, lowering the apoptotic index to nearly control levels.

Detection of activated caspase-3 in the lung

Number of activated caspase-3 immunoreactive cells (caspase 3-IR) in the lung epithelium increased in the non-treated ALI group compared to healthy controls; about 2-fold for alveolar cells ($P < 0.01$) and about 8-fold for bronchial cells ($P < 0.001$; *Figs. 4B* and *6A-6F*). Budesonide treatment lowered the increased number of caspase 3-IR alveolar cells by about 70% ($P < 0.05$) and the increased number of bronchial cells by about 60% ($P < 0.01$; *Figs. 4B* and *6A-6F*).

Biochemical markers of the lung injury, inflammation, and apoptosis

After induction of ALI, migrating leukocytes and structural lung cells have been activated, as indicated by elevated concentrations of pro-inflammatory cytokines in the lung

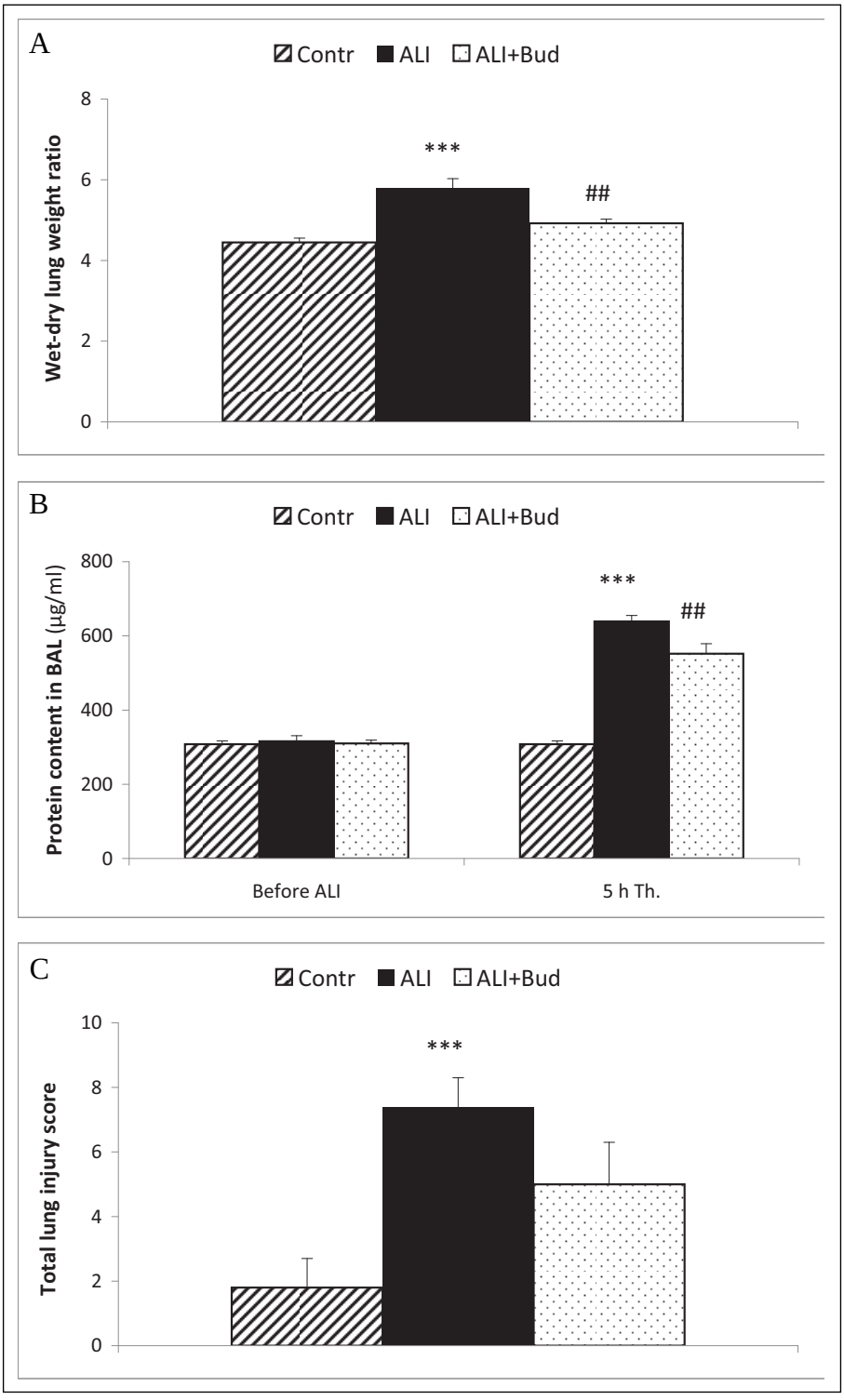

Fig. 3. Markers od lung edema formation and lung injury. (A) Lung edema formation expressed as wet-dry lung weight ratio. (B) Protein content in the BAL fluid (in µg/ml). (C) Total lung injury score. For between-group differences: ***$P < 0.001$ for ALI versus Contr groups; ##$P < 0.01$ for ALI + Bud versus ALI groups; &$P < 0.05$ for within-group comparison of values Before ALI versus values at 5 h after the therapy. Data are expressed as means ± S.E.M., number of animals (n = 6) in each group.

homogenates and in the blood plasma (*Table 3*). Increases in concentrations of some cytokines were huge, for example IL-1β and IL-8 were increased approximately 10-fold in plasma and about 2-fold in lung homogenates, and these effects were statistically significant ($P < 0.01$ in plasma and $P < 0.001$ in lung homogenates). Injury to the lung cells is expressed by increased concentration of esRAGE by 19% compared to controls ($P < 0.001$). Increased lung cell apoptosis is expressed by 2-fold increase of caspase-3 in the lung homogenate compared to controls ($P < 0.001$). Treatment with budesonide significantly lowered the increase of plasma concentrations of IL-1β by about 40% and IL-8 by about 30% compared to non-treated ALI group (both $P < 0.05$). In the lung tissue homogenates, budesonide lowered the increase of concentrations of IL-1β ($P < 0.05$) and TNF-α ($P \alpha 0.001$), both by about 30%, caspase-3 by about 65% ($P < 0.01$), and completely inhibited increase of esRAGE ($P < 0.001$) in comparison with non-treated ALI group. Almost all other markers (except of IFNγ) in plasma and lung tissue were also decreased by budesonide compared to ALI non-treated group although these effects did not reach statistical significance (*Table 3*).

DISCUSSION

Considering the role of inflammation and lung edema formation in the pathogenesis of ALI/ARDS (6), our study was carried out to evaluate if and to what extent the treatment with intratracheal corticosteroid can alleviate the inflammatory response, epithelial cell injury, apoptosis, and edema formation in an early phase of experimentally-induced ALI. Repetitive saline lung lavage triggered migration of PMN (particularly

Table 2. Histopathological signs (expressed as a score) in the lungs of healthy controls (Contr group, n = 6), in the non-treated ALI group (ALI group, n = 6) and in the animals with ALI treated with budesonide (ALI + Bud group, n = 6).

	Contr group	ALI group	ALI + Bud group
Atelectasis	0.4 ± 0.3	2.1 ± 0.3**	1.8 ± 0.6
Emphysema	0.4 ± 0.2	1.4 ± 0.5	0.6 ± 0.6
Hemorrhagia	0.4 ± 0.2	1.3 ± 0.5	1.2 ± 0.7
PMN infiltration	0.6 ± 0.2	2.4 ± 0.2**	1.4 ± 0.2$^{\#\#}$

For between-group comparisons: ALI versus Contr: $^{**}P < 0.01$; ALI + Bud versus ALI: $^{\#\#}P < 0.01$. Data are expressed as means ± S.E.M.

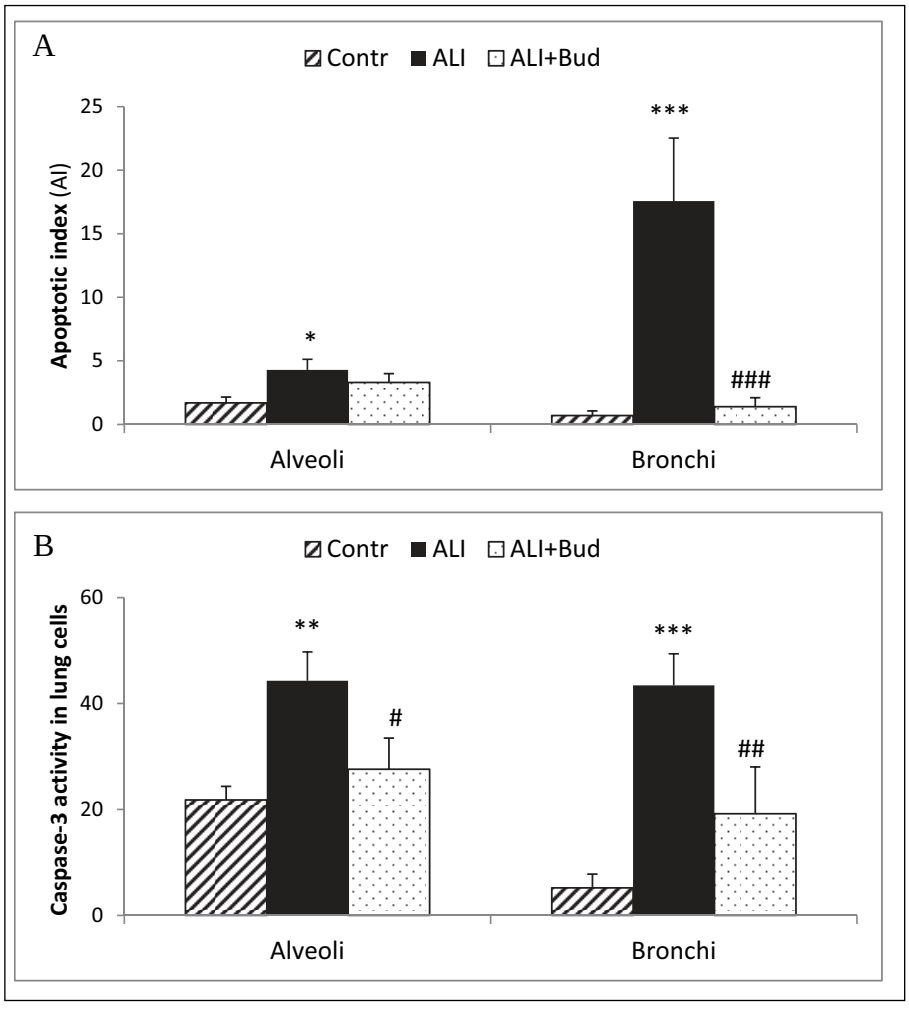

Fig. 4. Markers of apoptosis of lung epithelial cells. (A) Apoptosis of the alveolar and bronchial cells in the lung tissue sections detected by the TUNEL technique, expressed as apoptotic index. (B) Activated caspase-3 immunoreactive alveolar and bronchial cells detected immunohistochemically. For between-group differences: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for ALI versus Contr groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ for ALI + Bud versus ALI groups. Data are expressed as means ± S.E.M., number of animals (n = 6) in each group.

Table 3. Concentrations of several biochemical markers of inflammation and lung injury in the plasma and lung homogenates (expressed in pg/ml) at the end of experiments in the healthy controls (Contr group, n = 6), in the non-treated ALI group (ALI group, n = 6) and in the animals with ALI treated with budesonide (ALI + Bud group, n = 6).

	Contr group	ALI group	ALI+Bud group
Plasma			
IL-1β	15.2±2.8	132.8±32.0**	84.3±18.2$^{\#}$
IL-6	25.2±1.5	29.5±2.8	24.8±2.8
IL-8	19.4±4.1	207.1±43.6**	148.3±37.6$^{\#}$
TNFα	286.8±1.0	291.6±1.0*	289.1±1.1
Lung homogenates			
IL-1β	380.0±46.7	688.1±16.1***	591.1±24.9$^{\#}$
IL-6	3.3±0.4	4.1±0.3	3.3±0.4
IL-8	438.5±62.4	1144.7±29.9***	1031.8±55.1
TNF-α	293.1±0.4	307.6±0.7***	303.6±0.7$^{\#\#\#}$
IFNγ	688.4±15.6	715.0±4.2	743.2±2.6
esRAGE	5.4±0.2	6.4±0.2***	5.2±0.1$^{\#\#\#}$
caspase-3	1.8±0.2	4.1±0.4***	2.6±0.4$^{\#\#}$

Abbreviations: IL, interleukin; TNF, tumor necrosis factor; IFNγ, interferon gamma; esRAGE, endogenous soluble receptor for advanced glycation end-products. For between-group comparisons: ALI versus Contr: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ALI + Bud versus ALI: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Data are expressed as means ± S.E.M.

neutrophils) into the alveolar spaces, increased production of pro-inflammatory cytokines, and caused accumulation of liquid in the lung tissue which finally worsened the lung functions. Curative treatment with budesonide mitigated infiltration of the inflammatory cells, decreased concentrations of pro-inflammatory cytokines in the plasma and lung tissue, decreased epithelial cell injury and apoptosis, reduced lung edema, and enhanced respiratory parameters in the animals with ALI.

Diffuse alveolar injury is associated with an intensive migration of PMN from circulation into the lung interstitium and alveolar spaces. In our study, higher total counts of inflammatory cells were found in the BAL fluid at 5 h after induction of ALI model, but according to the changes in WBC count at 1 h and 3 h we can presume that the changes in the BAL fluid could occur earlier. Similar results were reported in various models of ALI (19, 20, 27, 28), and in patients with ARDS (29) in an early phase of the disorder. Infiltration of PMN into the airspaces is shown by elevated percentages of neutrophils and eosinophils in the BAL fluid at the end of experiment. In agreement with our results, other researchers detected higher percentage of PMN in the BAL fluid 4 hours after induction of ALI in animals (19, 20, 27). Transmigration of PMN into the lung was linked with their decrease in the peripheral blood and was observed by other authors (30), as well.

Activated neutrophils, eosinophils, alveolar macrophages, and structural lung cells produce vast quantities of bioactive substances which concentrations in plasma and BAL fluid are time-dependent and serve as markers of inflammation in an early phase of ARDS (4, 31). In the lung tissue, the cytokines were produced in rather high amounts already within 5 h after induction of the ALI model. Elevated levels of cytokines in the lung tissue within several hours after induction of ALI were previously measured also in other studies (19, 20, 27, 28, 32). However, we have found differences in elevation of the individual cytokines probably due to different dynamics of their synthesis. As demonstrated in sepsis, TNF-α and IL-1β are released within the first 30 – 90 min after exposure to lipopolysaccharide and in turn activate a second level of inflammatory cascade including cytokines and other biologically active substances (33). This could be the reason why in our study some cytokines increased significantly while the others showed just a tendency to elevate at the end of experiment. Early increase of TNF-α with earlier peak of concentration may explain why at 5 h we have seen only slight increase of TNF-α but greater increase of IL-1β.

To estimate an impact of the lung injury on the systemic level, some cytokines were measured also in the plasma. Concentrations of IL-1β, IL-8 and TNF-α in the plasma of ALI animals significantly elevated compared to healthy controls, whereas the level of IL-6, likely due to larger S.E.M., increased non-significantly. However, production of IL-6 reaches its maximum later then the 'first line' cytokines/chemokines (34) and possibly later than the 5 h time point. This might explain why we were not able to detect a significant increase in plasma IL-6 in our experiment. Higher plasma concentrations of pro-inflammatory cytokines in the models of ALI were also published by other authors (30, 35). Finding of higher concentrations of pro-inflammatory cytokines in the plasma at 5 h after induction of ALI model is of importance as it indicates that these cytokines may be released into the blood stream very early and may influence the function of distant organs shortly after the impact to the lung.

Lung injury and inflammation in ALI/ARDS are closely associated with an apoptosis, a process of programed death of lung cells and PMN. Apoptosis can be initiated by two alternative pathways: an extrinsic pathway triggered by binding of a death ligand (e.g., Fas/FasL ligand or TNF-α) to cell surface death receptors, and an intrinsic pathway induced in response to cytokines, action of hypoxia or oxidants. Both pathways converge into activating effector caspase-3, -6 or -7, which are responsible for cell alterations and execution of cell death (36). In ALI/ARDS, both pathways are activated and delay apoptosis of neutrophils and increase apoptosis of epithelial cells (5, 37). Delayed apoptosis of PMN mediated by macrophages, possibly *via* the Fas/FasL pathway, leads to prolonged survival of PMN at the site of injury and progressing inflammation. Several cytokines released in the early phase of ARDS, e.g. GM-CSF, IL-8 and IL-2, contribute

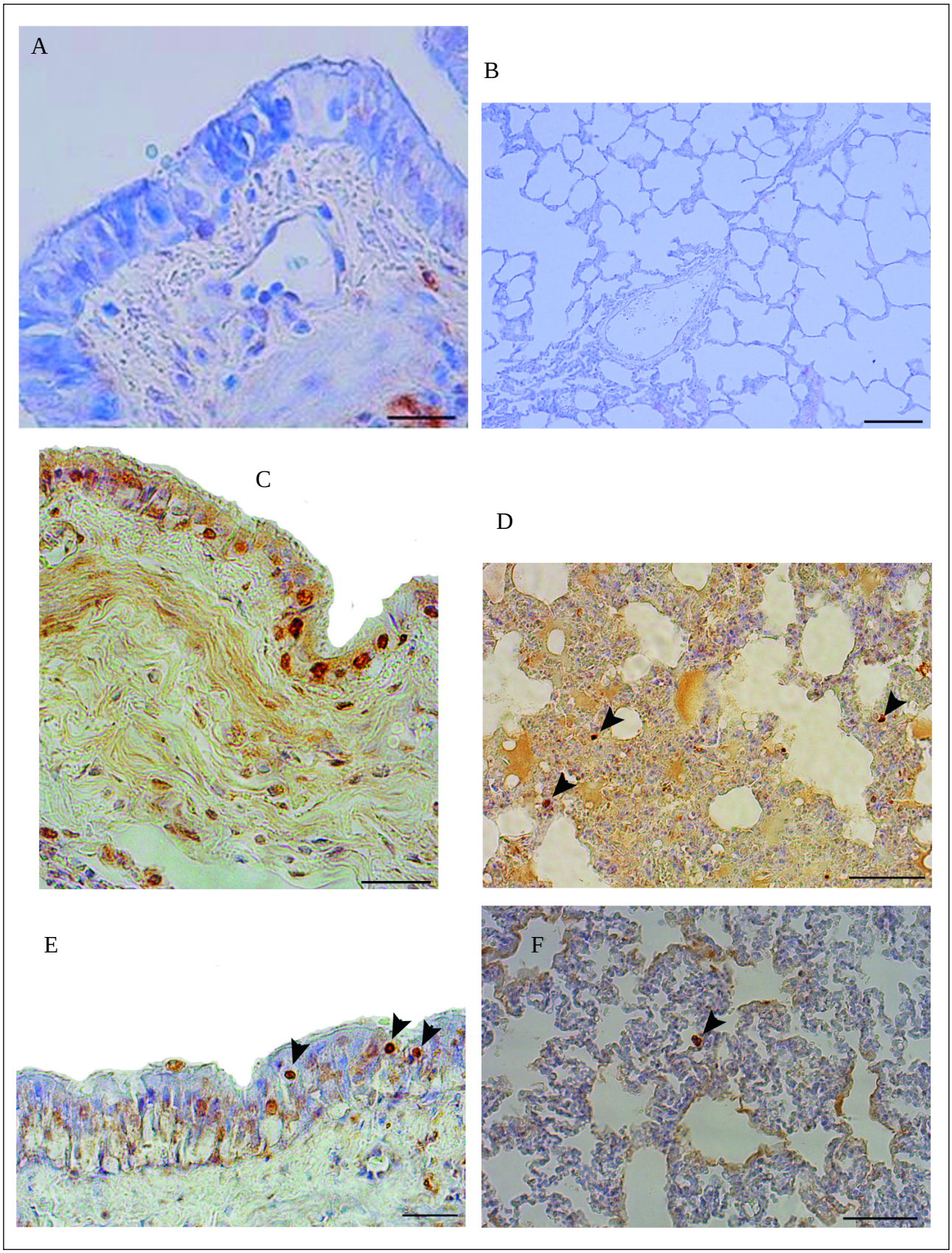

Fig. 5. Apoptosis of lung epithelial cells visualized by TUNEL methods. Representative microphotographs of the lungs of healthy controls (Contr group; Bronchi (A); Alveoli (B)), animals with non-treated ALI (ALI group; Bronchi (C); Alveoli (D)) and animals with ALI treated with budesonide (ALI + Bud group; Bronchi (E); Alveoli (F)) using the TUNEL method for detection of apoptosis. Arrowheads indicate higher number of apoptotic (dark brown) nuclei of epithelial cells in the Bronchi and Alveoli (C), (D). After treatment with budesonide, decreased number of apoptotic bronchial and alveolar cells was found (E), (F). Scale bars: (A), (C), (E) = 50 μm; B = 200 μm; D, F = 100 μm.

to this process (38). On the other hand, enhanced phagocytosis of apoptotic neutrophils by alveolar macrophages leads to faster resolution of inflammation and repair in the late phase of ARDS (5).

Activation of the Fas/FasL pathway participates in the epithelial injury, as well. In our study, number of apoptotic cells in the alveolar and bronchial epithelium increased in rabbits with ALI compared to healthy controls. Intensive apoptosis was

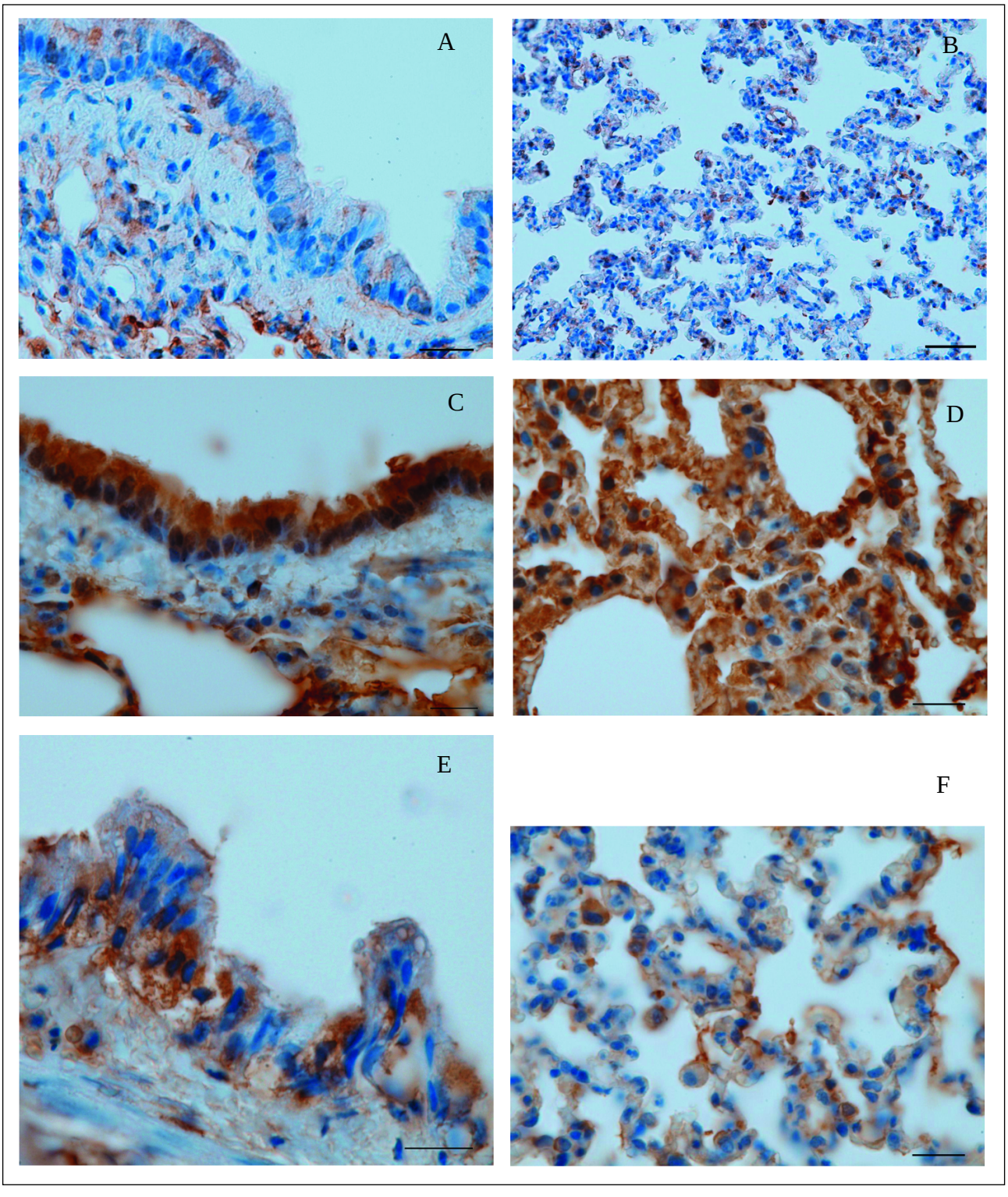

Fig. 6. Apoptosis of lung epithelial cells visualized by caspase-3 immunohistochemical staining. Representative microphotographs of the lungs of healthy controls (Contr group; Bronchi (A); Alveoli (B)), animals with non-treated ALI (ALI group; Bronchi (C); Alveoli (D)) and animals with ALI treated with budesonide (ALI + Bud group; Bronchi (E), Alveoli (F)) using activated caspase-3 immunohistochemical staining. Strong cytoplasmic caspase-3 immunoreactivity of bronchial and alveolar epithelium was observed in ALI group (C), (D), while caspase-3 immunoreactivity decreased after treatment with budesonide (E), (F). Scale bars: (A) – (F) = 50 μm.

confirmed by two immunohistochemical methods: by detection of DNA strand breaks using TUNEL method and by detection of activated caspase-3 immunoreactive cells in the lung tissue. Additionally, concentration of caspase-3 determined in the lung tissue by ELISA methods was significantly higher in the ALI group compared to controls. These results confirm a rapid activation of pro-apoptotic processes in the lung epithelial cells already within the first hours after induction of ALI. Similarly to our study, DNA damage in the lung tissue determined by a comet assay was observed 4 hours after saline lavage-induced ALI in rabbits (22), and DNA strand breaks detected by TUNEL methods and increased caspase-3 and -8 within 24 hours after induction were reported also in a model of sepsis (39). In human ARDS, decreased size, condensation of chromatin, and DNA fragmentation in pneumocytes were observed in an early disease phase (40, 41). Moreover, elevated levels of markers of apoptosis, including TUNEL-labeled DNA strand breaks and caspase-3, were found in the lung tissue from patients who died from ALI/ARDS (42). Interestingly, in this study apoptotic changes were more pronounced in the bronchial cells than in the alveolar cells. These findings are in contradiction to results of Nakamura *et al*. (43) who reported higher sensitivity to inflammation-induced apoptosis in distal (small airway) epithelial cells than in proximal (bronchial) epithelial cells in cell cultures. This discrepancy can be caused by different study designs (cell cultures in the Nakamura's study versus *in vivo* model in our study). Nevertheless, we may speculate that the process of induction of the ALI model, i.e., repetitive lung lavage procedure in our study can be partially responsible for some injury to the bronchial cells and therefore for their higher susceptibility to apoptosis.

Morphological changes of epithelial cells, particularly of type I cells, disturb the removal of fluid from the alveolar space, decrease production of surfactant, and contribute to the development of septic shock (5). In this study, histological investigation of the lung tissue samples taken at the end of experiments showed an increase in atelectasis, emphysema, hemorrhagia, and PMN infiltration in the lungs of animals with ALI compared to controls. In agreement to our results, histopathological changes in the lung within several hours after induction of ALI were observed also by other authors (21, 32, 44, 45). Furthermore, in this study injury to alveolar cells type I was proven by elevated concentrations of esRAGE which is responsible for propagation of inflammatory response *via* nuclear factor-kappa B, thus stimulating production of pro-inflammatory cytokines, reactive oxygen species and proteases in ALI/ARDS (46). Finally, damage to alveolocapillary membrane and increased leakage of plasma into the interstitium and alveolar space was in the present study expressed by increased wet-dry lung weight ratio and protein content in the BAL fluid in non-treated ALI animals compared to controls. Similar findings were reported in different models of ALI (30, 35).

Complex action of the lung injury, inflammation, and edema formation in this study resulted in serious worsening in the respiratory parameters. After induction of ALI model, decreased oxygenation and efficacy of ventilation were observed in comparison to the initial values (i.e., before induction of ALI), whereas these parameters remained relatively unchanged until the end of experiment in the non-treated ALI group. Deterioration in the gas exchange after induction of ALI model was presented also by other researchers (21, 22, 44, 47).

Considering the pathogenesis of ALI/ARDS we have presumed that CS have a potential to alleviate inflammation, lung injury, and edema and thereby can improve the lung functions in animals with ALI. However, intravenous delivery of CS previously led to controversial results (14-17), probably due to different study designs and heterogeneity on mortality endpoints and etiologies of ARDS as well as due to different dosage and timing of CS therapy in different studies (18). In this study, budesonide was administered intratracheally after ALI induction as a curative treatment. By this local way of delivery, we have expected more pronounced local effect in the injured lung and lower side effects (7, 48, 49). To avoid any potential complications resulting from maldistribution of CS powder throughout the lungs and to provide homogenous lung distribution, budesonide in the form of nebulization suspension was administered through the jet of ventilator during application of inpulsion regime of HFJV ventilation. After delivery, budesonide prevented migration of PMN into the lung and likely modulated their activation as suggested by decreased concentrations of almost all pro-inflammatory cytokines in the lung homogenate and in the plasma. Contrary, concentration of IFNγ in the lung tissue after budesonide slightly increased which might be related to lower efficacy of CS in T-lymphocytes, a main source of IFNγ, than in granulocytes (50). Anyway, effect of CS on production of cytokines by inflammatory cells is complex and dependent on CS concentration, timing and concomittant factors. Generally, CS suppress production of many pro-inflammatory cytokines and induce production of anti-inflammatory cytokines (e.g., IL-10). However, in an acute phase of inflammation, CS can provide not completely elucidated divergent actions where they both inhibit cytokine release while enhance cytokine receptor expression (51, 52). In addition to anti-inflammatory action, budesonide in our study reduced apoptosis of epithelial cells, as indicated by lower numbers of apoptotic cells detected by TUNEL methods. Budesonide decreased activation of caspase-3 in the epithelial cells and diminished concentration of caspase-3 in the lung tissue homogenate, as well. Similarly to our results, other CS dexamethasone alleviated inflammation and suppressed Fas ligand in the lung of mice with ALI (53) and inhibited caspase-3 and -7 activation in the lung epithelial cells in *in vitro* study (11). Furthermore, budesonide in the present study reduced histopathological signs of lung injury and concentrations of esRAGE in the lung homogenate, and decreased lung edema formation. Finally, we could observe rapid improvement in oxygenation and ventilation indexes. These favorable findings could be explained i) by administration of budesonide early after induction of ALI which might be more effective than the late treatment (15), ii) by local homogenous delivery by means of HFJV, and iii) by both genomic and nongenomic CS mechanisms since effects of budesonide were observed as early as 30 min after administration (12, 13). The findings on enhanced lung mechanics and reduced inflammation after budesonide treatment in various ALI models were recently published also by other authors (54-56) and also by us in meconium-injured rabbits (23, 24), while budesonide was not effective in animal models where lung injury was induced by phosgene (57, 58). Importantly, improved oxygenation and ventilatory parameters were observed in the subgroups of patients with ALI/ARDS after early CS therapy (14, 15), as well.

Concluding, curative intratracheal administration of budesonide reduced PMN migration into the lung, mitigated lung injury, decreased concentrations of pro-inflammatory cytokines, reduced apoptosis of lung epithelial cells, decreased lung edema formation, and improved ventilation in a rabbit model of ALI induced by repetitive saline lung lavage. The results from this experimental study suggest that inhaled budesonide may be of benefit also for patients with ARDS especially in early disease stage.

Acknowledgements: Authors thank D. Kuliskova, Z. Remisova, M. Petraskova, M. Hutko, M. Kondekova, M. Letrichova, and A. Resetarova for technical assistance.

This work was supported by the project Biomedical 'Center Martin', ITMS code: 26220220187, the project is co-financed from EU sources; and by projects APVV-0435-11 and APVV-15-0075, and VEGA 1/0305/14.

Conflicts of interest: None declared.

REFERENCES

1. ARDS Definition Task Force: Ranieri VM, Rubenfeld GD, Thompson BT, *et al*. Acute respiratory distress syndrome: the Berlin Definition. *JAMA* 2012; 307: 2526-2533.
2. Verbrugge SJ, Sorm V, Lachmann B. Mechanisms of acute respiratory distress syndrome: role of surfactant changes and mechanical ventilation. *J Physiol Pharmacol* 1997; 48: 537-557.
3. Pierrakos C, Karanikolas M, Scolletta S, Karamouzos V, Velissaris D. Acute respiratory distress syndrome: pathophysiology and therapeutic options. *J Clin Med Res* 2012; 4: 7-16.
4. Bhargava M, Wendt CH. Biomarkers in acute lung injury. *Transl Res* 2012; 159: 205-217.
5. Galani V, Tatsaki E, Bai M, *et al*. The role of apoptosis in the pathophysiology of acute respiratory distress syndrome (ARDS): an up-to-date cell-specific review. *Pathol Res Pract* 2010; 206: 145-150.
6. Matthay MA, Zemans RL. The acute respiratory distress syndrome: pathogenesis and treatment. *Annu Rev Pathol* 2011; 6: 147-163.
7. van Overveld FJ, Demkow U, Gorecka D, de Backer WA, Zielinski J. New developments in the treatment of COPD: comparing the effects of inhaled corticosteroids and N-acetylcysteine. *J Physiol Pharmacol* 2005; 56 (Suppl. 4): 135-142.
8. Mroz RM, Lisowski P, Tycinska A, *et al*. Anti-inflammatory effects of atorvastatin treatment in chronic obstructive pulmonary disease. A controlled pilot study. *J Physiol Pharmacol* 2015; 66: 111-128.
9. Boyle AJ, Mac Sweeney R, McAuley DF. Pharmacological treatments in ARDS; a state-of-the-art update. *BMC Med* 2013; 11: 166. doi: 10.1186/1741-7015-11-166
10. Czock D, Keller F, Rasche FM, Haussler U. Pharmacokinetics and pharmacodynamics of systemically administered glucocorticoids. *Clin Pharmacokinet* 2005; 44: 61-98.
11. Wen LP, Madani K, Fahrni JA, Duncan SR, Rosen GD. Dexamethasone inhibits lung epithelial cell apoptosis induced by IFN-gamma and Fas. *Am J Physiol* 1997; 273: L921-L929.
12. Stahn C, Buttgereit F. Genomic and nongenomic effects of glucocorticoids. *Nat Clin Pract Rheumatol* 2008; 4: 525-533.
13. Ayroldi E, Cannarile L, Migliorati G, Nocentini G, Delfino DV, Riccardi C. Mechanisms of the anti-inflammatory effects of glucocorticoids: genomic and nongenomic interference with MAPK signaling pathways. *FASEB J* 2012; 26: 4805-4820.
14. Steinberg KP, Hudson LD, Goodman RB, *et al*. Efficacy and safety of corticosteroids for persistent acute respiratory distress syndrome. *N Engl J Med* 2006; 354: 1671-1684.
15. Meduri GU, Golden E, Freire AX, *et al*. Methylprednisolone infusion in early severe ARDS: results of a randomized controlled trial. *Chest* 2007; 131: 954-963.
16. Deal EN, Hollands JM, Schramm GE, Micek ST. Role of corticosteroids in the management of acute respiratory distress syndrome. *Clin Ther* 2008; 30: 787-799.
17. Tang BM, Craig JC, Eslick GD, Seppelt I, McLean AS. Use of corticosteroids in acute lung injury and acute respiratory distress syndrome: a systematic review and meta-analysis. *Crit Care Med* 2009; 37: 1594-1603.
18. Ruan SY, Lin HH, Huang CT, Kuo PH, Wu HD, Yu CJ. Exploring the heterogeneity of effects of corticosteroids on acute respiratory distress syndrome: a systematic review and meta-analysis. *Crit Care* 2014; 18: R63. doi: 10.1186/cc13819
19. Noda E, Hoshina H, Watanabe H, Kawano T. Production of TNF-alpha by polymorphonuclear leukocytes during mechanical ventilation in the surfactant-depleted rabbit lung. *Pediatr Pulmonol* 2003; 36: 475-481.
20. Waragai A, Yamashita H, Hosoi K, *et al*. High-frequency oscillation (HFO) prevents activation of NF-kappaB found with conventional mechanical ventilation (CMV) in surfactant-depleted rabbit lung. *Pediatr Pulmonol* 2007; 42: 440-445.
21. Ronchi CF, dos Anjos Ferreira AL, Campos FJ, *et al*. High-frequency oscillatory ventilation attenuates oxidative lung injury in a rabbit model of acute lung injury. *Exp Biol Med (Maywood)* 2011; 236: 1188-1196.
22. Ronchi CF, Fioretto JR, Ferreira AL, *et al*. Biomarkers for oxidative stress in acute lung injury induced in rabbits submitted to different strategies of mechanical ventilation. *J Appl Physiol* (*1985*) 2012; 112: 1184-1190.
23. Mokra D, Mokry J, Drgova A, Petraskova M, Bulikova J, Calkovska A. Intratracheally administered corticosteroids improve lung function in meconium-instilled rabbits. *J Physiol Pharmacol* 2007; 58 (Suppl. 5): 389-398.
24. Mikolka P, Kopincova J, Tomcikova Mikusiakova L, *et al.* Effects of surfactant/budesonide therapy on oxidative modifications in the lung in experimental meconium-induced lung injury. *J Physiol Pharmacol* 2016; 67: 57-65.
25. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-254.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-275.
27. Lee BH, Lee TJ, Jung JW, *et al*. The role of keratinocyte-derived chemokine in hemorrhage-induced acute lung injury in mice. *J Korean Med Sci* 2009; 24: 775-781.
28. Menk M, Graw JA, Steinkraus H, *et al*. Characterization of inflammation in a rat model of acute lung injury after repeated pulmonary lavage. *Exp Lung Res* 2015; 41: 466-476.
29. Nakos G, Kitsiouli EI, Tsangaris I, Lekka ME. Bronchoalveolar lavage fluid characteristics of early intermediate and late phases of ARDS. Alterations in leukocytes, proteins, PAF and surfactant components. *Intensive Care Med* 1998; 24: 296-303.
30. Miniati M, Cocci F, Monti S, *et al*. Lazaroid U-7489F attenuates phorbol ester-induced lung injury in rabbits. *Eur Respir J* 1996; 9: 758-764.
31. Park WY, Goodman RB, Steinberg KP, *et al*. Cytokine balance in the lungs of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2001; 164: 1896-1903.
32. Takano K, Yamamoto S, Tomita K, *et al*. Successful treatment of acute lung injury with pitavastatin in septic mice: potential role of glucocorticoid receptor expression in alveolar macrophages. *J Pharmacol Exp Ther* 2011; 336: 381-390.
33. Cohen J. The immunopathogenesis of sepsis. *Nature* 2002; 420: 885-891.

34. Bhargava R, Janssen W, Altmann C, *et al*. Intratracheal IL-6 protects against lung inflammation in direct, but not indirect, causes of acute lung injury in mice. *PLoS One* 2013; 8: e61405.
35. Li G, Zhou C, Zhou Q, Zou H. Galantamine protects against lipopolysaccharide-induced acute lung injury in rats. *Braz J Med Biol Res* 2016; 49: e5008. doi: 10.1590/1414-431X20155008.
36. Lu Q, Harrington EO, Rounds S. Apoptosis and lung injury. *Keio J Med* 2005; 54: 184-189.
37. Martin TR, Hagimoto N, Nakamura M, Matute-Bello G. Apoptosis and epithelial injury in the lungs. *Proc Am Thorac Soc* 2005; 2: 214-220.
38. Martin TR, Nakamura M, Matute-Bello G. The role of apoptosis in acute lung injury. *Crit Care Med* 2003; 31 (Suppl. 4): S184-S188.
39. Chopra M, Reuben JS, Sharma AC. Acute lung injury: apoptosis and signaling mechanisms. *Exp Biol Med (Maywood)* 2009; 234: 361-371.
40. Bardales RH, Xie SS, Schaefer RF, Hsu SM. Apoptosis is a major pathway responsible for the resolution of type II pneumocytes in acute lung injury. *Am J Pathol* 1996; 149: 845-852.
41. Bem RA, Bos AP, Matute-Bello G, van Tuyl M, van Woensel JB. Lung epithelial cell apoptosis during acute lung injury in infancy. *Pediatr Crit Care Med* 2007; 8: 132-137.
42. Albertine KH, Soulier MF, Wang Z, *et al*. Fas and fas ligand are up-regulated in pulmonary edema fluid and lung tissue of patients with acute lung injury and the acute respiratory distress syndrome. *Am J Pathol* 2002; 161: 1783-1796.
43. Nakamura M, Matute-Bello G, Liles WC, *et al*. Differential response of human lung epithelial cells to fas-induced apoptosis. *Am J Pathol* 2004; 164: 1949-1958.
44. Wang SG, Guo GH, Fu ZH, Zhou SF. Comparison of conventional mandatory ventilation and high frequency oscillatory ventilation for treatment of acute lung injury induced by steam inhalation injury. *Burns* 2006; 32: 951-956.
45. Kamiyama J, Jesmin S, Sakuramoto H, *et al*. Assessment of circulatory and pulmonary endothelin-1 levels in a lavage-induced surfactant-depleted lung injury rabbit model with repeated open endotracheal suctioning and hyperinflation. *Life Sci* 2014; 118: 370-378.
46. Uchida T, Shirasawa M, Ware LB, *et al*. Receptor for advanced glycation end-products is a marker of type I cell injury in acute lung injury. *Am J Respir Crit Care Med* 2006; 173: 1008-1015.
47. Bang JO, Ha SI, Choi IC. The effect of positive-end expiratory pressure on oxygenation during high frequency jet ventilation and conventional mechanical ventilation in the rabbit model of acute lung injury. *Korean J Anesthesiol* 2012; 63: 346-352.
48. Poetker DM, Reh DD. A comprehensive review of the adverse effects of systemic corticosteroids. *Otolaryngol Clin North Am* 2010; 43: 753-768.
49. Brozmanova M, Calkovsky V, Plevkova J, Tatar M. Effects of inhaled corticosteroids on cough in awake guinea pigs with experimental allergic rhinitis - the first experience. *J Physiol Pharmacol* 2004; 55 (Suppl. 3): 23-30.
50. Umland SP, Nahrebne DK, Razac S, *et al*. The inhibitory effects of topically active glucocorticoids on IL-4, IL-5, and interferon-gamma production by cultured primary CD4+ T cells. *J Allergy Clin Immunol* 1997; 100: 511-519.
51. Wiegers GJ, Reul JM. Induction of cytokine receptors by glucocorticoids: functional and pathological significance. *Trends Pharmacol Sci* 1998; 19: 317-321.
52. Yeager MP, Guyre PM, Munck AU. Glucocorticoid regulation of the inflammatory response to injury. *Acta Anaesthesiol Scand* 2004; 48: 799-813.
53. Beck JM, Preston AM, Wilcoxen SE, Morris SB, Sturrock A, Paine R. Critical roles of inflammation and apoptosis in improved survival in a model of hyperoxia-induced acute lung injury in Pneumocystis murina-infected mice. *Infect Immun* 2009; 77: 1053-1060.
54. Gao W, Ju N. Budesonide inhalation ameliorates endotoxin-induced lung injury in rabbits. *Exp Biol Med* (*Maywood*) 2015; 240: 1708-1716.
55. Gao W, Ju YN. Budesonide attenuates ventilator-induced lung injury in a rat model of inflammatory acute respiratory distress syndrome. *Arch Med Res* 2016; 47: 275-284.
56. Ju YN, Yu KJ, Wang GN. Budesonide ameliorates lung injury induced by large volume ventilation. *BMC Pulm Med* 2016; 16: 90. doi: 10.1186/s12890-016-0251-z
57. Smith A, Brown R, Jugg B, *et al*. The effect of steroid treatment with inhaled budesonide or intravenous methylprednisolone on phosgene-induced acute lung injury in a porcine model. *Military Med* 2009; 174: 1287-1294.
58. Luo S, Pauluhn J, Trubel H, Wang C. Corticosteroids found ineffective for phosgene-induced acute lung injury in rats. *Toxicol Lett* 2014; 229: 85-92.

Received: June 1, 2016
Accepted: December 28, 2016

Author's address: Dr. Daniela Mokra, Biomedical Center Martin and Department of Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, 4C Mala Hora Street, SK-03601 Martin, Slovakia.
E-mail: mokra@jfmed.uniba.sk