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LUNG PROTECTION IN CARDIO-PULMONARY BYPASS

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Since the invention of the heart-lung machine paediatric cardiac surgery developed rapidly. For correction of complex cardiac malformations the application of a cardio-pulmonary bypass (CPB) has become indispensable but possible negative effects of this technique should not be neglected. Especially, both bypassed organs i.e. heart and lung are not perfused during the procedure and therefore are threatened by ischemia and reperfusion injury. Additionally, CPB was developed with a non-pulsatile flow but there are clinical observations that pulsatile flow might be superior with improved patient outcomes. Thus, the aim of our study was to evaluate the effect of CPB on lung structure and to assess whether different flow modalities (pulsatile vs. non-pulsatile flow) or application of the antibiotic minocycline might be advantageous. Thirty five piglets of four weeks age were examined and divided into five experimental groups: control (no CPB) without or with minocycline, CPB (non-pulsatile flow) without or with minocycline and CPB with pulsatile flow. CPB was performed for 90 min followed by a 120 min reperfusion and recovery phase. Thereafter, adenosine triphosphate-content of lung biopsies and histology was carried out. We found that CPB was associated with a significant thickening of alveolar wall accompanied by an infiltration of neutrophil leucocytes. Moreover, markers for hypoxia, apoptosis, nitrosative stress, inflammation and DNA damage were significantly elevated after CPB. These cellular damages could be partially inhibited by minocycline or pulsatile flow. Both, minocycline and pulsatile flow attenuate lung damage after CPB.

Key words: *minocycline, cardio-pulmonary bypass, lung injury, apoptosis, adenosine triphosphate, tumor necrosis factor-alpha, poly-ADP-ribose, bronchial epithelium*

INTRODUCTION

The development of a heart-lung machine, also called cardio-pulmonary bypass (CPB), in the fifties was a milestone for cardiac surgery. The extracorporeal circulation technique allowed very complex heart operations like congenital heart defects (1). However, possible negative effects of CPB on the bypassed organs (heart and lung) should not be neglected. Heart and lung are not or insufficiently perfused during the whole time of bypass (which could be up to 2 hours or more depending on the kind of heart operation) and thus are subjected to ischemia and reperfusion injury. Although blood supply of the lung is maintained *via* the Vasa privata, i.e. the bronchial arteries, which are supplied with blood by the heart-lung machine, it has been demonstrated that during CPB blood flow is low in these vessels and not sufficient to avoid ischemia (2). Moreover, typically lung ventilation is reduced or even discontinued during bypass which might promote the occurrence of atelectasis, pulmonary vasoconstriction (*via* Euler-Liljestrand-reflex) and interstitial pulmonary oedema (3). Thus, postoperative lung dysfunction is not uncommon and has serious implications on patient recovery. Furthermore, the flow profile of CPB has to be taken into consideration: traditionally CPB was developed with a non-pulsatile flow and this flow regimen is the most common

procedure today. However, a more physiological pulsatile flow might be of advantage for organ integration. Thus, hypothetically pulsatile flow may lead to a better mixture of blood components and might enable each red blood cell to come into contact with the vessel wall thereby improving gas exchange in peripheral tissue so that finally this might reduce organ damage during pulsatile CPB. However, in a recent study of 37 patients beneficial effects of pulsatile CPB on lung function could not be detected (4)

At the same period of time when CPB was introduced in heart surgery a new class of antibiotics were detected: the tetracyclines with aureomycin (which is chlortetracycline) being the first of this class (5). Tetracyclines belong to the broad spectrum antibiotics and up to now several synthetic and semi-synthetic derivatives have been found. Minocycline, which has a long half-life was introduced in 1966 and still is in use nowadays (6). In addition to its anti-microbial activity it has also some interesting 'side-effects': neuroprotective properties in neurodegenerative diseases like Morbus Huntington or Morbus Parkinson and anti-inflammatory as well as anti-apoptotic effects *via* attenuation of pro-inflammatory cytokines like TNF- α and inhibition of caspase-dependent or caspase independent pathways (7, 8). These anti-inflammatory properties might be advantageous in CPB since it is known that CPB induces significant systemic inflammatory

responses (9). This inflammatory reaction is presumably triggered by the contact of blood components with external surfaces of the CPB (oxygenator, tubes, coupling pieces etc.) (10). Moreover, besides inflammation a transient decline of blood pressure during CPB (that might occur during manipulation of the large vessels) results in reduced organ perfusion and might cause organ malfunction which impairs patient outcome.

In summary, lung protection during cardiac operations is still a matter of debate and protective measures have to be found to better preserve lung function.

Thus, the aim of our study was to evaluate in a clinical setting of CPB the influence of the heart-lung machine on the lung and to assess histologically which kind of injury might occur during this procedure. Moreover, the anti-apoptotic and anti-inflammatory drug minocycline or a pulsatile flow during CPB was investigated. As an experimental model piglets of four weeks age were used.

MATERIAL AND METHODS

Animals

The following procedures were reviewed and approved by the Animal Care Committee of the German Regional Council Leipzig which ensured humane treatment of all animals as indicated by the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

For our experiments we used 35 Angeln Saddleback piglets of 4 weeks age weighting between 8 and 12 kg. The piglets were divided in 5 groups a 7 animals:

- 1) control group without minocycline;
- 2) control group with minocycline;
- 3) non-pulsatile CPB group without minocycline;
- 4) non-pulsatile CPB group with minocycline;
- 5) pulsatile CPB (without minocycline).

Experimental procedure

The operation procedure was carried out as described earlier by our group (11). In brief, all animals received the same pre-

medication (atropine, midazolam, ketamine), they were intubated and mechanically ventilated using a Cato anesthesia apparatus (Drägerwerk, Lubeck, Germany). Settings of the mechanical ventilator, for all piglets, were as follows: 50% air and 50% O₂ = FiO₂ 50; tidal volume 10 ml/kg body weight, respiratory rate 20 – 30/minute, PEEP (positive end-expiratory pressure) 2 mbar, maximum pressure 25 mbar, ratio inspiration/expiration 1:2. During CPB the piglets were continuously ventilated with a tidal volume of 5 ml/kg body weight, a respiratory rate of 10/minute and PEEP 2 mbar in order to avoid formation of atelectasis (12). In contrast, for the whole experimental procedure control piglets were ventilated with the ventilator settings described above (this means no reduction of tidal volume or respiratory rate).

Anaesthesia was carried out using isoflurane and sufentanil-dihydrogenecitrate. All piglets were thoracotomized but only animals of the three CPB groups were connected to the heart lung machine, which consisted of a roller pump (SIII Stoeckert, Freiburg, Germany), a membrane oxygenator (Dideco, Mirandola, Italy) and a pediatric tubing set (Sorin, Munchen, Germany). Priming volume was 200 ml whole blood supplemented with heparin to achieve an activated clotting time of > 400 s. Arterial line was inserted in the ascending aorta. Venous line was connected to both superior and inferior caval vein *via* the right atrial auriculum. For the duration of pulsatile and non-pulsatile bypass piglets were perfused with a flow rate of 100 ml/kg/min using a roller pump (SIII Stoeckert, Freiburg, Germany), which can be switched between a laminar flow and a pulsatile flow. For the pulsatile CPB pulsation was set to 100 beats/min, pump base flow was 30%, pulsatile flow was 70% and pump runtime was 50% according to Alka-Bozkaya *et al.* and as published earlier (13, 14). Resulting peak pressure in the femoral artery was 78 ± 6 mmHg and diastolic pressure was 59 ± 5 mmHg.

Cardioplegia was induced by application of cold Custodiol solution (Dr. Franz Kohler Chemie GmbH, Bensheim, Germany), the aorta was clamped, CPB was started and the piglets were cooled to 28°C for 60 minutes. Thereafter, the piglets, still connected to the CPB, were re-warmed again for another 30 minutes. Subsequently, after reaching, for that species, a normal core body temperature of 38 – 39°C the aortic clamp was opened and the CPB flow was gradually reduced

Table 1. Functional parameters.

	paO ₂ /FiO ₂ (mmHg)	compliance (ml/mbar)	MAP (mmHg)	serum lactate (mmol/L)	tissue ATP (µg/g tissue)
<u>control</u>					
time 0	394 ± 28.4	8.95 ± 0.85	66 ± 3.75	1.76 ± 0.29	/
time 210	322 ± 29.6*	6.42 ± 0.79*	70 ± 2.58	1.65 ± 0.17	0.70 ± 0.19§
<u>Control + mino</u>					
time 0	446 ± 2.2.8	8.49 ± 0.69	65 ± 2.44	1.59 ± 0.12	/
time 210	270 ± 19.0*	6.84 ± 0.42*	69 ± 4.15	2.11 ± 0.28	0.75 ± 0.06§
<u>CPB</u>					
time 0	357 ± 36.4	8.73 ± 0.20	62 ± 4.71	1.39 ± 0.17	/
time 210	270 ± 41.9*	6.07 ± 0.94*	59 ± 1.76	6.43 ± 0.92*§	0.06 ± 0.03
<u>CPB+mino</u>					
time 0	456 ± 20.8	8.13 ± 0.48	78 ± 4.69	1.32 ± 0.22	/
time 210	389 ± 63.9*	5.91 ± 0.19*	84 ± 4.76	4.92 ± 0.94*§	0.69 ± 0.069§
<u>CPB pulsatile</u>					
time 0	441 ± 20.6	10.39 ± 0.59	58 ± 3.74	1.92 ± 0.43	/
time 210	266 ± 35.0*	6.80 ± 0.46*	58 ± 2.00	6.72 ± 1.63*§	0.96 ± 0.14§

ATP, adenosine triphosphate; CPB, cardio-pulmonary bypass; FiO₂ fraction of inspired oxygen; MAP, mean arterial pressure; mino, minocycline; paO₂, arterial oxygen pressure.

*P < 0.05 versus time 0, §P < 0.05 versus CPB, §P < 0.05 versus control.

thereby allowing the heart to take over body circulation. At time 120 minutes the animals were disconnected from CPB, but not de-cannulated, if necessary circulation was supported with catecholamines to maintain an arterial mean pressure above 50 mmHg (cumulative dose was 0.004 mg/kg norepinephrine and 0.03 mg/kg epinephrine, with no difference among the CPB groups). Moreover, oxygen saturation, pH, lactate, blood gases

as well as central venous pressure were measured and if necessary therapeutic interventions were performed. After disconnection from CPB the piglets recovered for additional 90 minutes. Post-CPB ventilation was carried out with the following settings: FiO_2 50; tidal volume 10 ml/kg body weight, respiratory rate 20 – 30/minute, PEEP 2 mbar, maximum pressure 25 mbar and ratio inspiration/expiration 1:2.

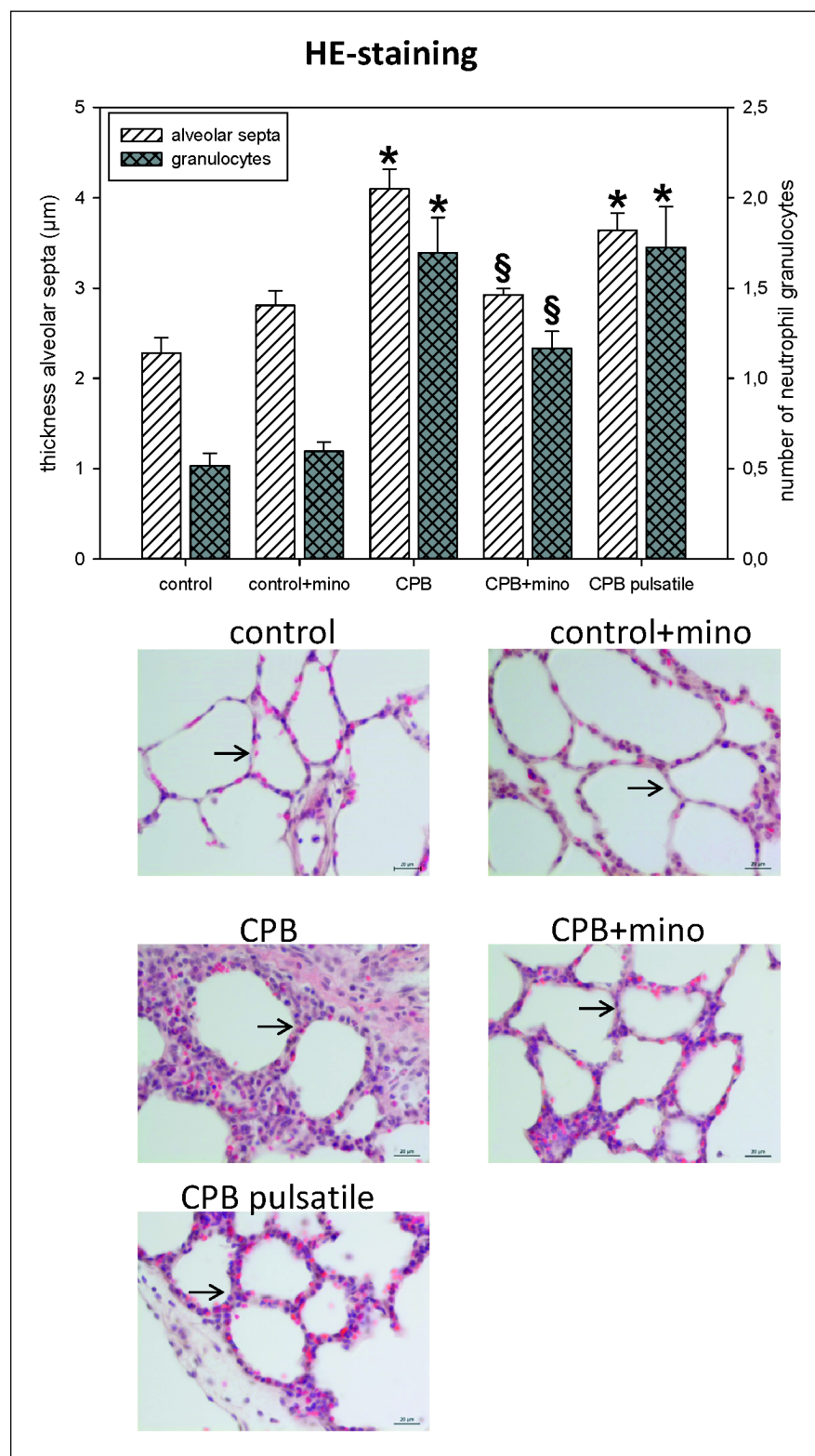


Fig. 1. HE-staining of alveoli. Bar graphs depict alveolar septa thickness (white bars, left y-axis) and number of neutrophil granulocytes (grey bars, right y-axis). Original images below show thickness of alveolar septa marked with a black arrow.

In the experimental groups receiving drug 4 mg/kg body weight minocycline was administered 30 minutes before start of CPB or start of control and a second dose of 2 mg/kg body weight after disconnection from CPB or for the controls at the

appropriate time points. Minocycline was dissolved in 10 ml aqua ad injectionem which was also used as solvent control in the experimental groups without minocycline.

The following time scale was used:

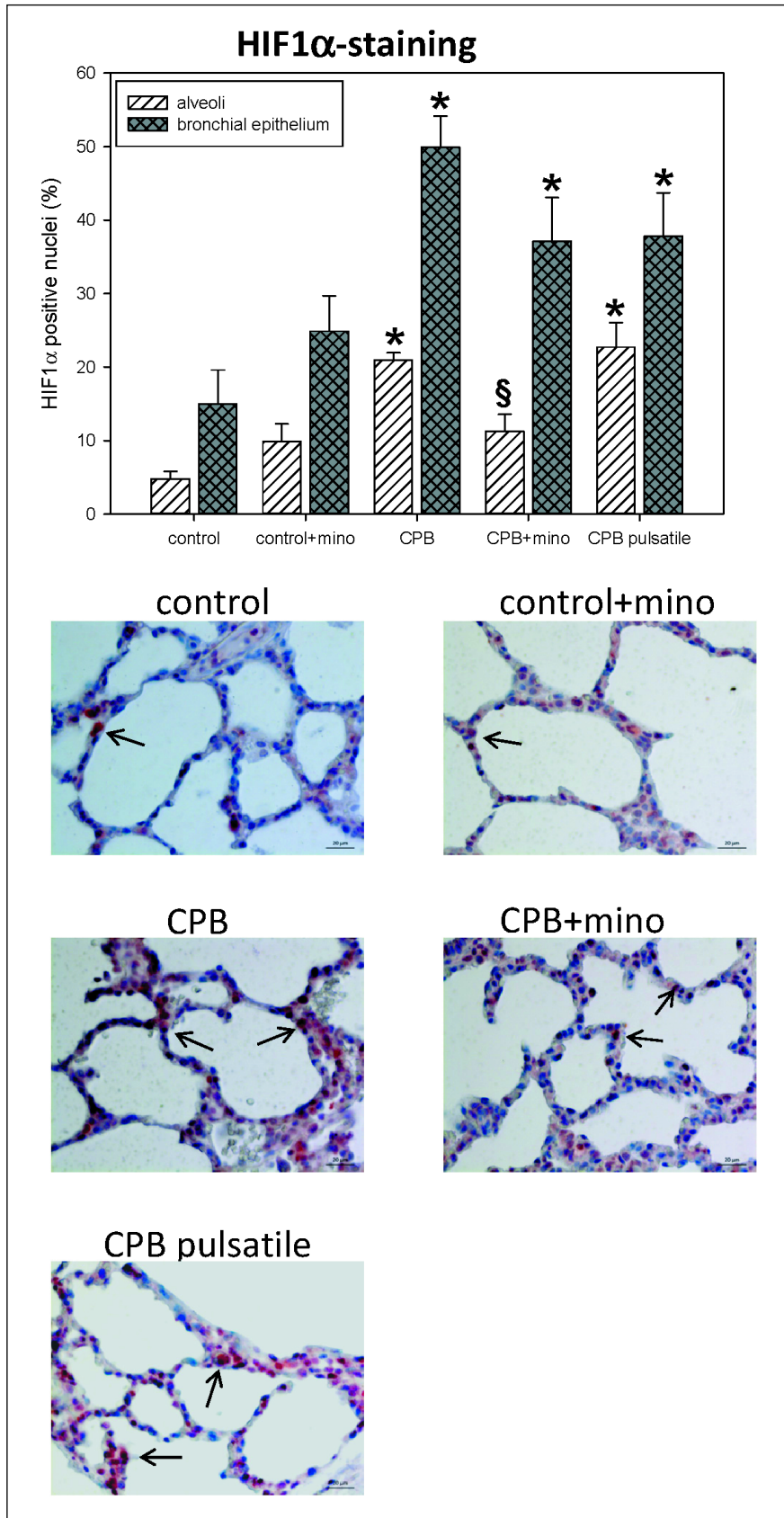


Fig. 2A. HIF1 α -staining of alveoli and bronchial epithelium. Bar graphs depict the number of positive nuclei (in %). Original images of alveoli are depicted below; black arrows indicate HIF1 α positive cells, stained in red. Significant differences versus control are indicated by asterisks, significant differences versus non-pulsatile CPB by a section sign ($P < 0.05$).

- time -30: administration of minocycline 4 mg/kg and solvent respectively;

- time 0: clamping of the aorta and start of CPB or start of controls. The animals of CPB-groups were cooled to 28°C, the control animals remained normothermic.

After 60 minutes the cooled piglets, still connected to the CPB, were rewarmed to 38 – 39°C.

- time 90: opening of aortic cross clamp and reperfusion. During the 30 minutes timeframe piglets were weaned from the CPB;

- time 120: disconnection of CPB and application of minocycline 2 mg/kg and solvent respectively;

- time 210: end of recovery and also end of the controls.

During CPB volatile anaesthesia with isoflurane was switched to propofol and was re-administered during reperfusion. The same anaesthesia pattern was performed in both groups of control piglets.

At the end of experiments lung biopsies were taken and were either fixed in neutral buffered 4% formalin solution for histological examination or snap frozen in liquid nitrogen for adenosine triphosphate (ATP) analysis.

Adenosine triphosphate-measurement by high-pressure liquid chromatography (HPLC)

Tissue ATP levels were determined by HPLC (high-pressure liquid chromatography) as previously described (15). In brief, lung samples were mixed with cold perchloric acid and precipitated with KOH. Subsequently, probes were centrifuged and 20 µL of each supernatant was injected at a flow of 1 mL/min onto a pre-equilibrated RP18 column (Lichrocart, Merck, Darmstadt, Germany). ATP was detected using an UV-detector (PDA Detector 2800) and a HPLC-apparatus (both Knauer Berlin, Germany). ATP-peaks were measured at 254 nm. For calibration ATP-standards were injected during each run.

Each sample (standard and probe) was injected three times, and the concentration was determined as the mean of these three injections.

Lung function

Lung function was measured *in vivo* by assessing paO_2 (arterial oxygen pressure), FiO_2 (fraction of inspired oxygen), compliance, $paCO_2$ (arterial carbon dioxide pressure) and blood pH.

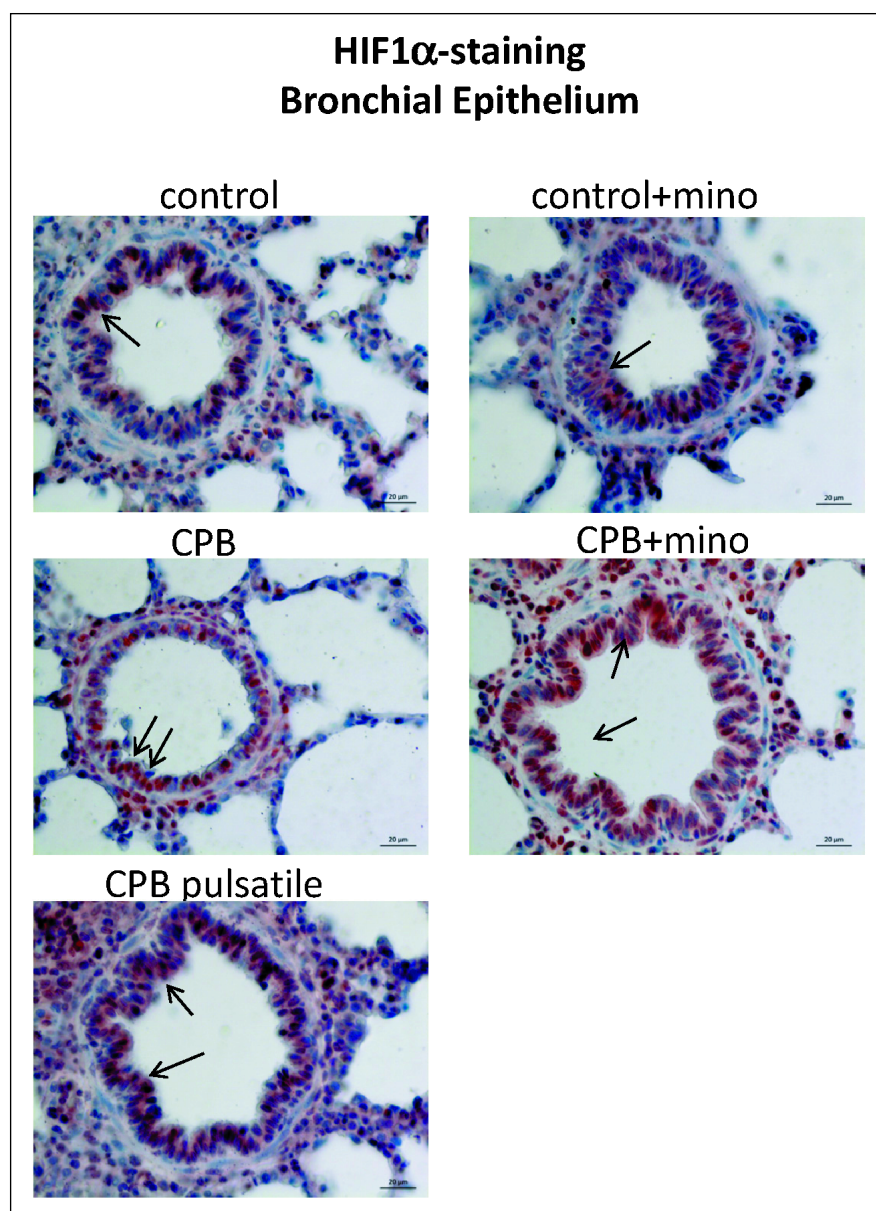


Fig. 2B. HIF1 α -staining of bronchial epithelium. Black arrows indicate HIF1 α positive cells.

Dynamic lung compliance was calculated according to Slottoch *et al.* (16), using the following formula:

$$\text{Compliance} = \frac{\text{tidal volume}}{\text{PIP} - \text{PEEP}}$$

where PIP - peak inspiratory pressure, PEEP - positive end-expiratory pressure.

Histology

Preparation and staining of the samples were done as previously published (15). Briefly, lung samples embedded in paraffin were sectioned in 2 μm slices. Thereafter, the slices

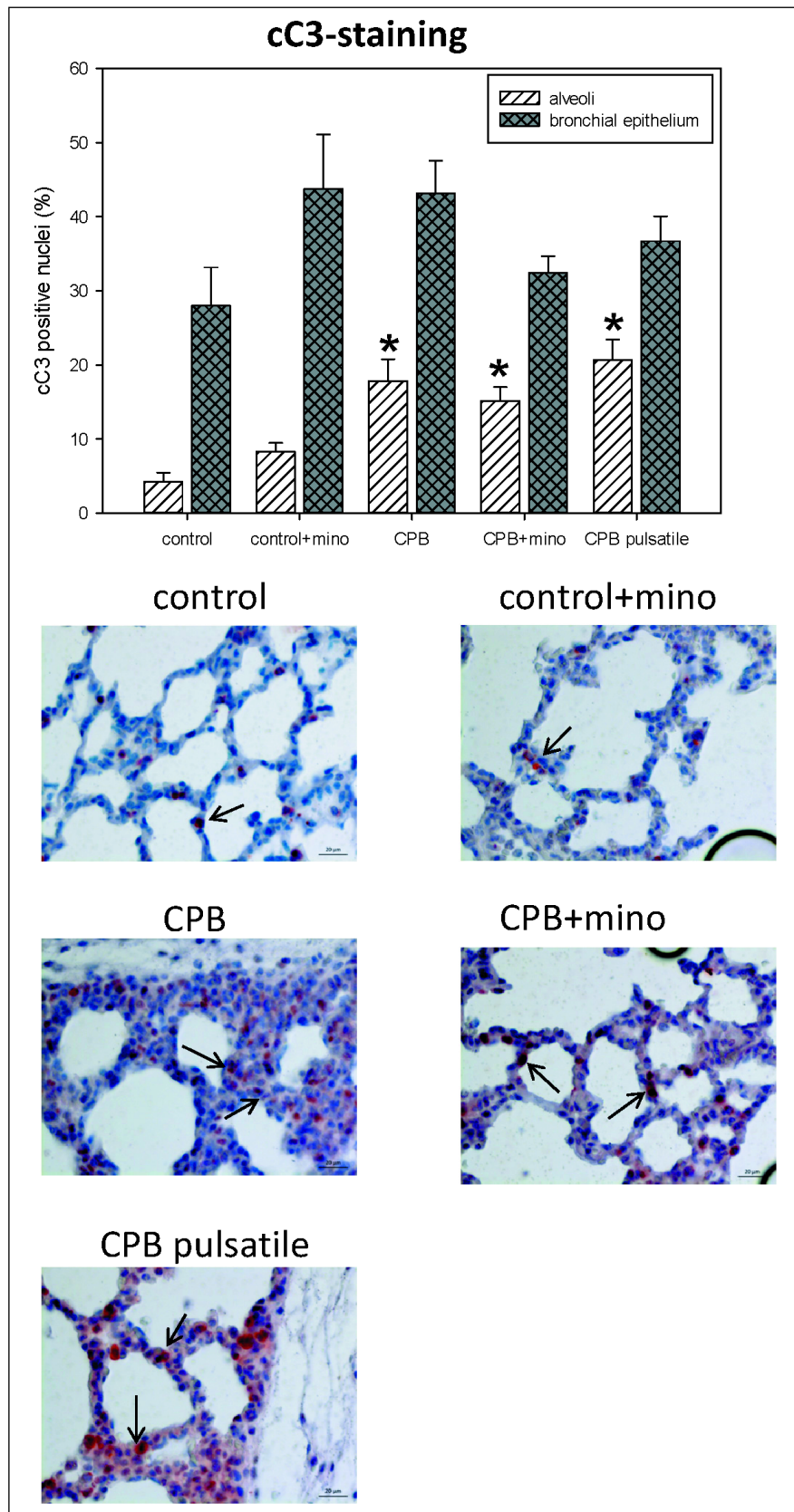


Fig. 3A. cC3-staining of alveoli and bronchial epithelium. Bar graphs depict the number of positive nuclei (in %). Original images of alveoli are depicted below; black arrows indicate cC3 positive cells, stained in red. Significant differences versus control are indicated by asterisks (P < 0.05).

were de-waxed and hematoxylin-eosin (HE) staining or immunostainings were carried out.

All specimens were viewed and analysed using the Axioimager M1 from Zeiss and the Zen Pro 2012 software (Jena, Germany). Pictures were randomly taken at 400 × magnification and 15 pictures per piglet were evaluated by a blinded observer.

Hematoxylin-eosin (HE) staining

HE staining was carried out following standard protocols. For each piglet more than 60 alveoli were analysed and the thickness of alveolar wall was measured as described earlier (11). Moreover, neutrophil granulocytes were counted and related to the number of alveoli.

Immunohistology

Lung specimens were stained for markers of hypoxia, inflammation, apoptosis, and nitrosative stress. Moreover, the amount of apoptotic cells was also evaluated.

Hypoxia-inducible factor 1alpha (HIF1 α)

It is known that the transcription factor HIF1 α translocates from cell cytoplasm into the nucleus as a response to decreases in ambient oxygen (17). We evaluated the amount of cell nuclei positive for HIF1 α in the alveoli and bronchial epithelium. Therefore, lung specimen were treated with 2% bovine serum albumin (BSA) to reduce unspecific background and stained with rabbit anti-HIF1 α primary antibody (1:100, Santa Cruz, Heidelberg, Germany) at 4°C over night. After several washing steps secondary horseradish peroxidase-labelled antibody was applied (1:200, 1 hour, room temperature) and visualized using the red chromogen AEC (3-amino-9-ethylcarbazol, DAKO, Hamburg, Germany). Nuclei were counterstained with hematoxylin. The number of HIF1 α positive nuclei (red) were counted and related to the number of negative nuclei (blue).

Apoptosis-inducing factor (AIF) and poly-ADP-ribose (PAR)

PAR stimulates the mitochondrial release of AIF, a transcription factor which triggers the caspase-independent pathway of apoptosis

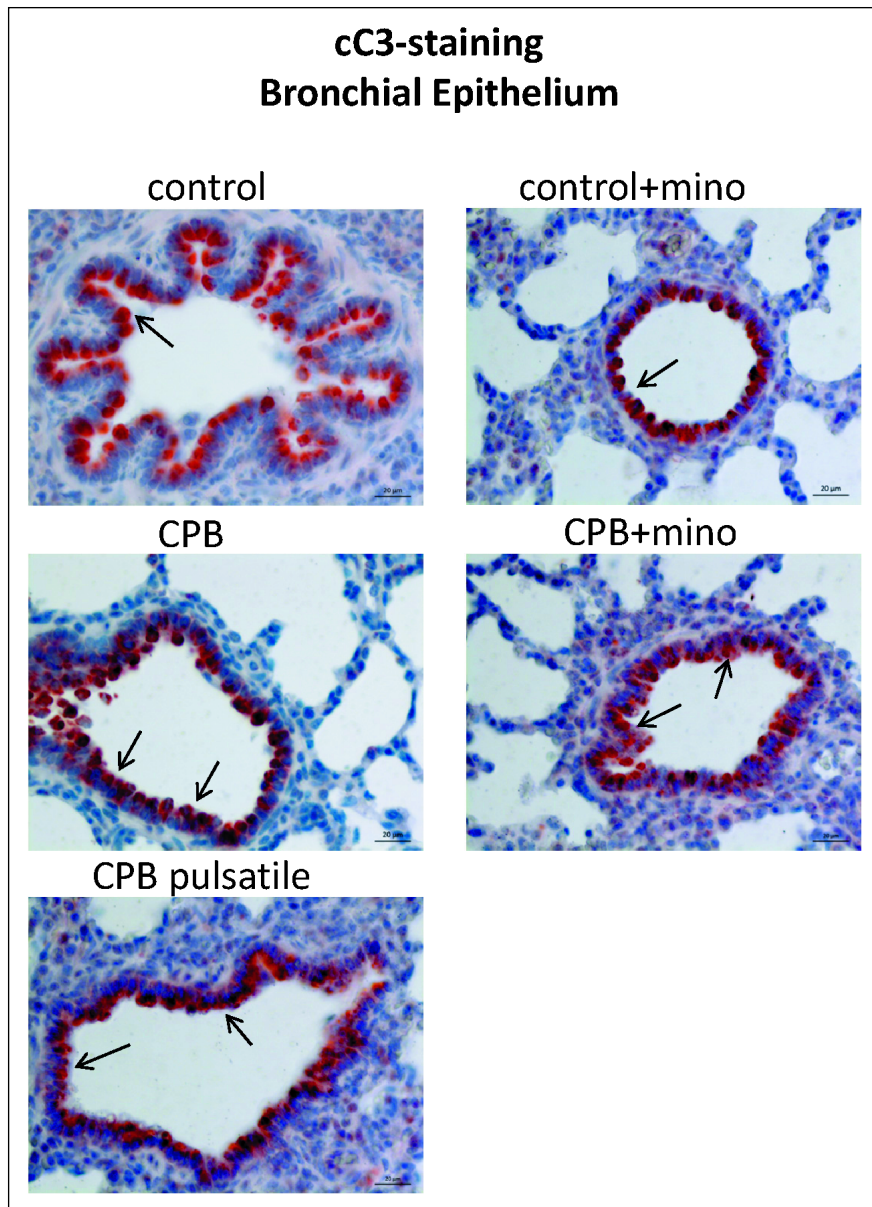


Fig. 3B. cC3-staining of bronchial epithelium. Black arrows indicate cC3 positive cells.

(18, 19). PAR itself is formed during DNA repair processes by PAR-polymerase (PARP) and might serve as a marker for DNA strand breaks. Thus, in our specimen AIF was stained using rabbit anti-AIF primary antibody (1:50, Santa Cruz, Heidelberg, Germany) and

PAR using mouse anti-PAR primary antibody (1:300, Bio-Rad, Munchen, Germany) according to a previously published protocol (15). After washing the appropriate secondary antibodies (1:200) were applied and detection was carried out using AEC. Again

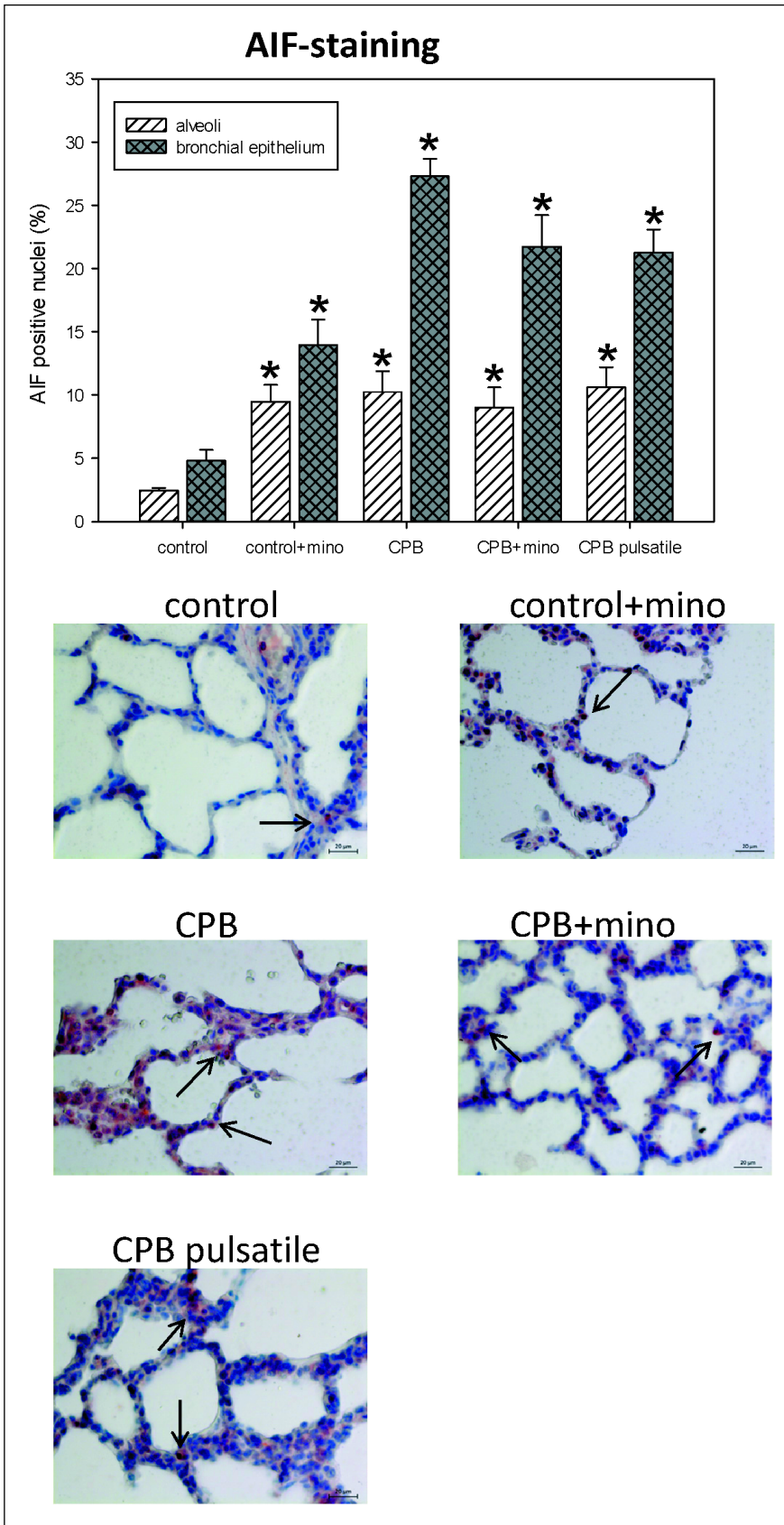


Fig. 4A. AIF-staining of alveoli and bronchial epithelium. Bar graphs depict the number of positive nuclei (in %). Original images of alveoli are depicted below; black arrows indicate AIF positive cells, stained in red. Significant differences versus control are indicated by asterisks ($P < 0.05$).

counterstaining of nuclei was done with hematoxylin. Evaluation was performed as described for HIF1 α .

Cleaved caspase-3 (cC3)

Caspases are also involved in the programmed cell death. The caspase cascade is initiated by various intrinsic and extrinsic stimuli. Once activated, executioner caspases like caspase-3 start the process of apoptosis (20). As the cleaved form of caspase-3 is the activated form we used rabbit anti cleaved caspase-3 antibody (1:200, New England Biolabs, Frankfurt, Germany) in our lung samples. Detection of positive cell nuclei was carried out as described for HIF1 α .

Nitrotyrosine staining

Reactive nitrogen species like peroxynitrit which occur during reperfusion are able to nitrosylate tyrosine residues of proteins. The resulting nitrotyrosines might be used as a biomarker for nitrosative stress (21). Thus, we determined the amount of nitrotyrosine-positive cells in our samples using a

rabbit anti-nitrotyrosine antibody (1:500, Millipore, Darmstadt, Germany). Detection was carried out as described above and the number of nitrotyrosine positive cells (red) was evaluated in relation to the unstained cells.

Tumor necrosis factor alpha (TNF- α)

CPB may initiate an inflammatory reaction and enhance TNF- α release from neutrophil granulocytes (22). Thus, we also investigated TNF- α in our lung probes. We used a rabbit anti-TNF- α antibody (1:100, Santa Cruz, Heidelberg, Germany). Detection was carried out as described for the nitrotyrosine staining.

In situ detection of apoptosis

In addition to the investigation of AIF as an early step in apoptosis, we also aimed to detect apoptotic cells using the 'In situ Apoptosis Detection Kit' from Abcam (Berlin, Germany). Conduction of the experimental work was carried out according to the manufacturer's instruction. Briefly, specimen were permeabilised with proteinase K, labelled with biotin-labelled deoxynucleotides by

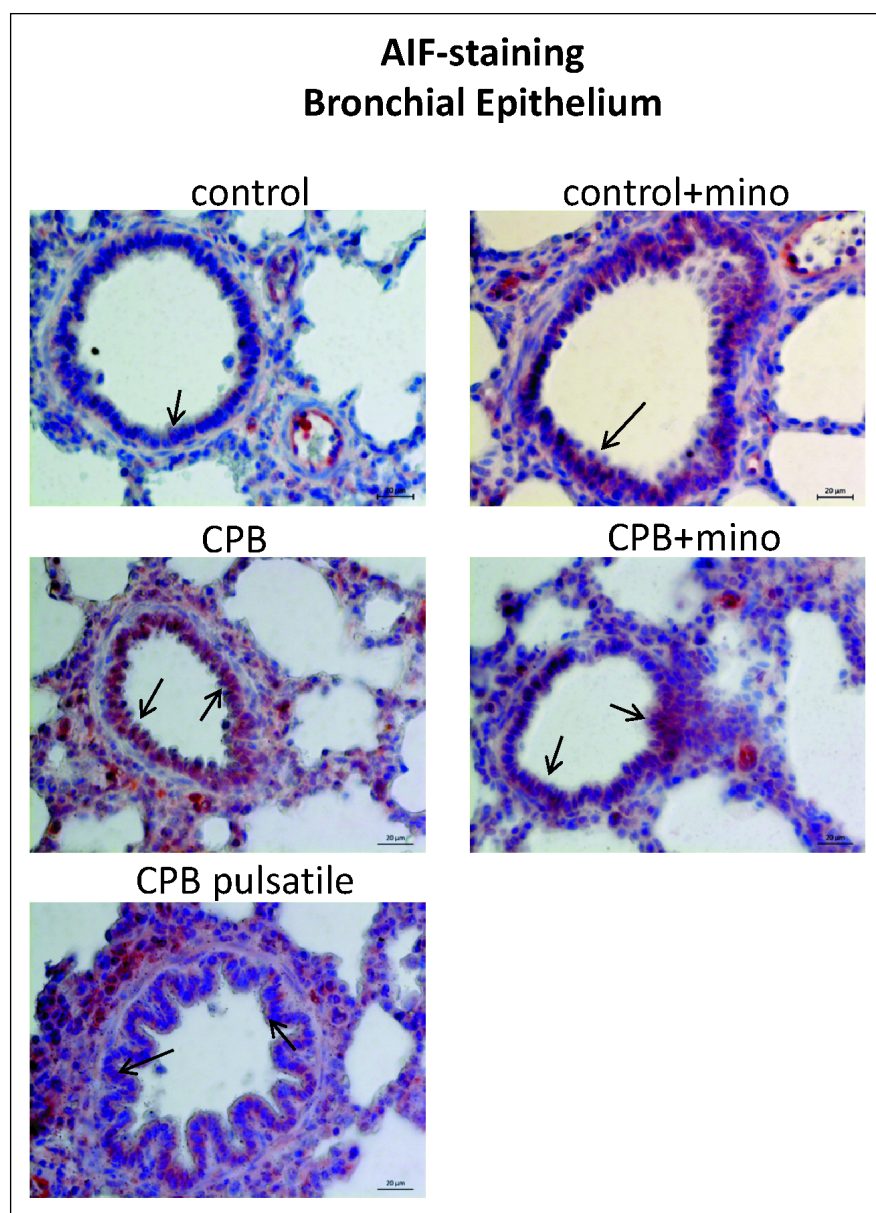


Fig. 4B. AIF-staining of bronchial epithelium. Black arrows indicate AIF positive cells.

TdT (terminal deoxynucleotidyl transferase) and finally detected with DAB (diaminobenzimide). Counterstaining was done with methyl green. For evaluation positive that is apoptotic cell nuclei (brown) were related to negative nuclei (green).

Statistical analysis

For statistical analysis, analysis of variance was performed using the software Systat for Windows, version 11

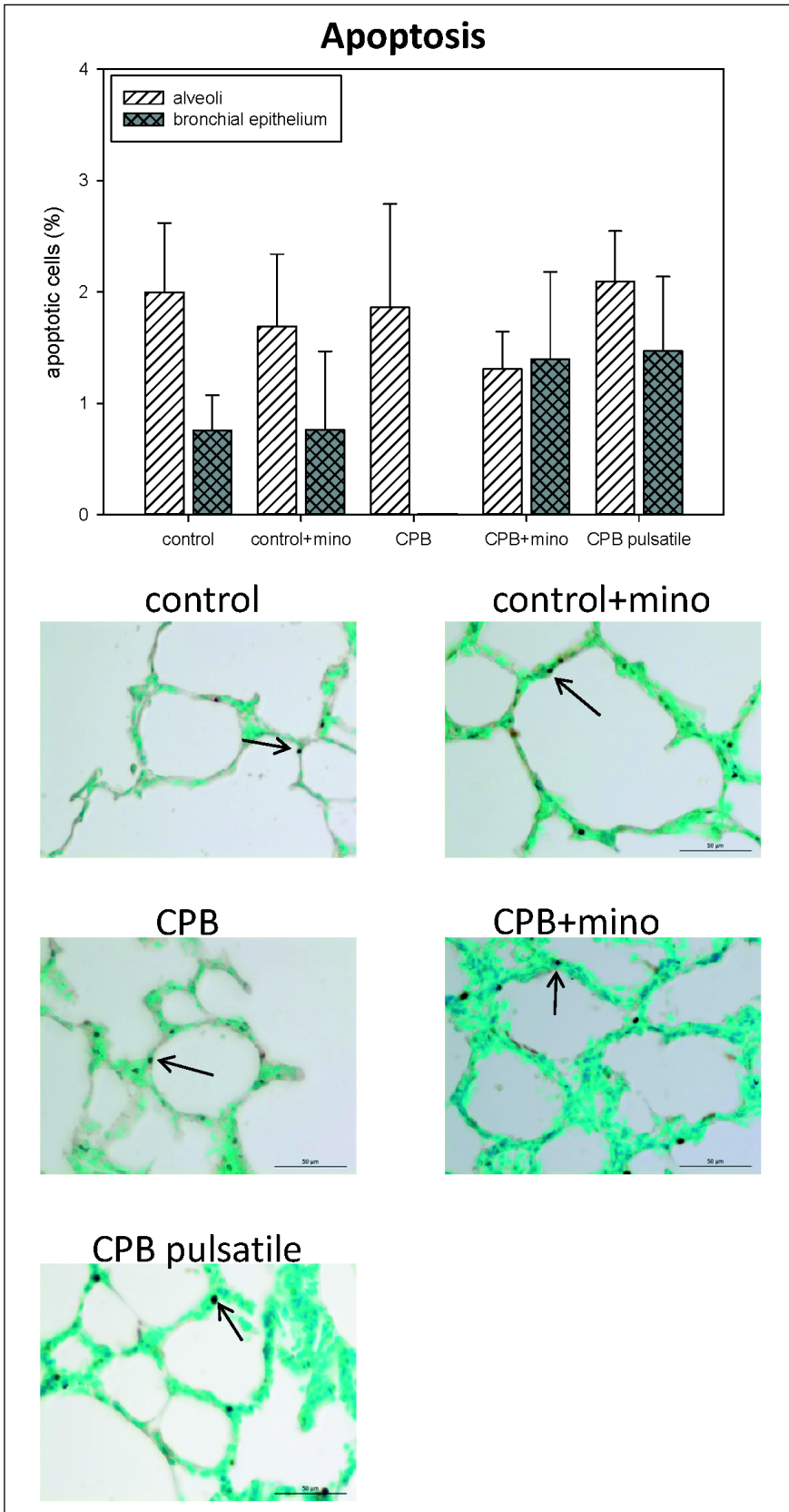


Fig. 5. Apoptosis. Bar graphs depict the number of apoptotic cells (in %). Original images of alveoli are displayed below, black arrows indicate apoptotic cells, stained in brown.

(Systat Inc., Evanston, IL, USA), and if ANOVA indicated significant differences ($P < 0.05$), post-hoc Tukey's HSD test was carried out.

All data are given as means \pm S.E.M. of $n = 7$ experiments.

RESULTS

At the beginning of the experiments all piglets had the same functional status (heart rate, ejection fraction, blood pressure,

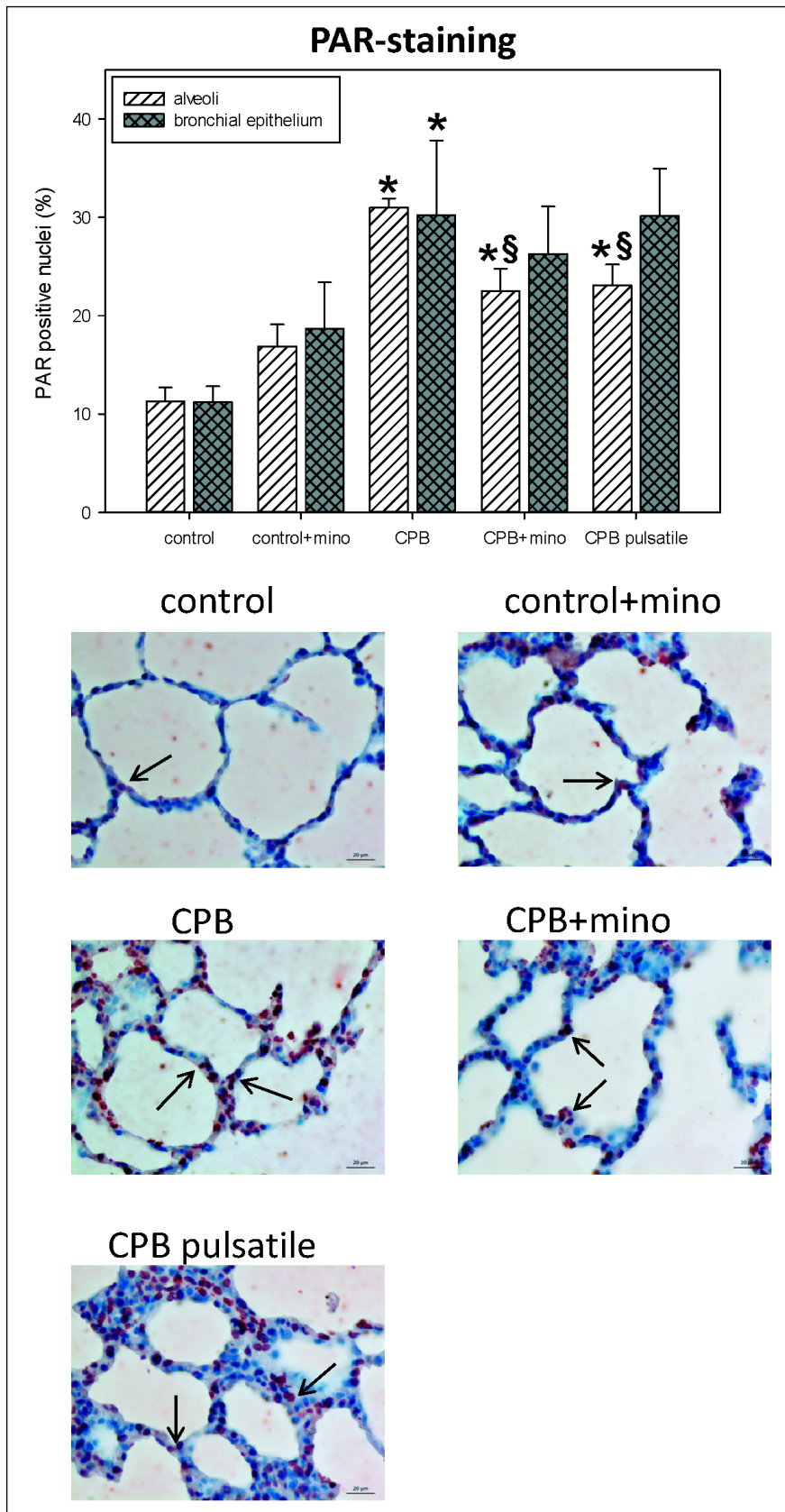


Fig. 6A. PAR-staining of alveoli and bronchial epithelium. Bar graphs depict the number of positive nuclei (in %). Original images of alveoli are depicted below; black arrows indicate PAR positive cells, stained in red. Significant differences versus control are indicated by asterisks, significant differences versus non-pulsatile CPB by a section sign ($P < 0.05$).

central venous pressure etc.) and these parameters were controlled and kept within the physiological range during the experiment.

In our clinical setting of CPB all piglets were successfully weaned from CPB. During recovery some of them transiently needed inotropic support. However, at the end of the recovery period inotropic support could be terminated due to stable cardiac function. Mean arterial pressure was not different between all experimental groups (Table 1). Lactate levels were highest in the CPB groups with no differences among them i.e. pulsatile flow or minocycline had no influence on blood lactate concentration. Lung ATP content significantly decreased in the non-pulsatile CPB group, whereas supplementation of minocycline during CPB or pulsatile flow significantly prevented from ATP decline (Table 1). PaO₂/FiO₂ as well as lung compliance significantly decreased during the entire experiment (i.e. PaO₂/FiO₂ and compliance at time 210 was lower compared to time 0) without differences between the 5 experimental groups. paCO₂ also was not significantly altered.

Hematoxylin-eosin staining

HE staining of alveoli revealed a significant increase in alveolar wall thickness in the CPB group. Pulsatile flow had no

influence on alveolar septa width whereas addition of minocycline led to a reduction in wall thickness to control levels (Fig. 1, white bars). Additionally, CPB enhanced the number of alveolar neutrophil granulocytes, which could be reduced by minocycline but not by pulsatile flow (Fig. 1, grey bars).

Immunohistology

Alveolar translocation of HIF1α was significantly increased during non-pulsatile CPB as could be estimated from the enhanced number of HIF1α positive cell nuclei. Minocycline but not pulsatile flow significantly prevented nuclear HIF1α accumulation in alveolar epithelium (Fig. 2A, white bars). As alveoli, bronchial epithelium also showed significant increases in HIF1α within all CPB groups when compared to the control group (Fig. 2A, grey bars). In contrast to alveoli, in bronchial epithelium enhanced nuclear HIF1α could not be decreased neither by minocycline nor by pulsatile flow (Fig. 2A, gray bars and Fig. 2B).

Examination of cC3 showed a rise in the number of positive cell nuclei within the alveolar wall in all three CPB-groups without differences among them i.e. minocycline or pulsatile

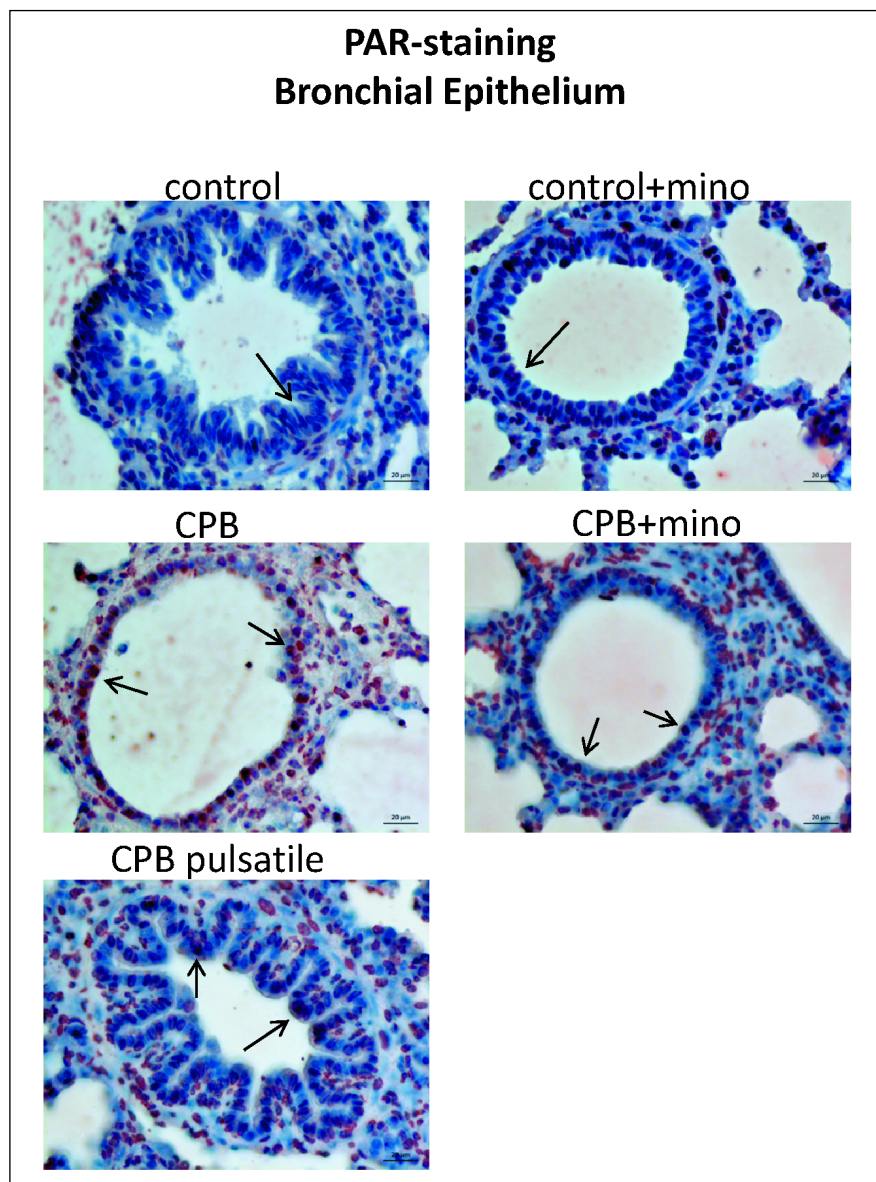


Fig. 6B. PAR-staining of bronchial epithelium. Black arrows indicate PAR positive cells.

flow did not reduce elevated cC3 (Fig. 3A, white bars). In bronchial epithelium the amount of cC3 positive cell nuclei was generally higher compared to alveolar epithelium. In contrast to

alveoli, in bronchial epithelium CPB did not enhance cC3 and no differences in cC3 within the 5 experimental groups could be detected (Fig. 3A, grey bars and Fig. 3B).

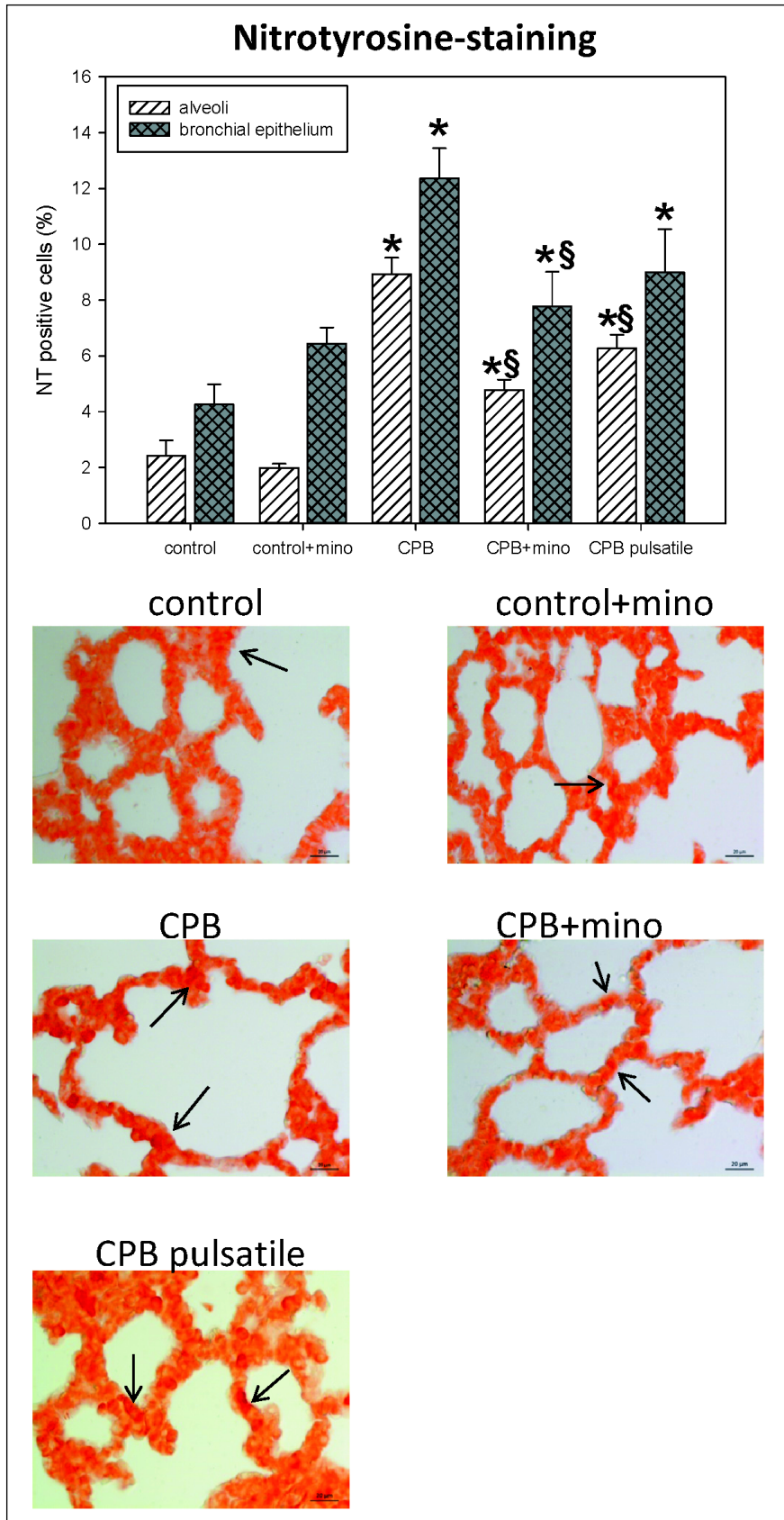


Fig. 7A. Nitrotyrosine-staining of alveoli and bronchial epithelium. Bar graphs depict the number of positive cells (in %). Original images of alveoli are depicted below; black arrows indicate nitrotyrosine (NT) positive cells, stained in red. Significant differences versus control are indicated by asterisks, significant differences versus non-pulsatile CPB by a section sign ($P < 0.05$).

AIF evaluation revealed that CPB increased nuclear AIF. However, neither minocycline nor pulsatile flow during CPB were able to decrease enhanced nuclear AIF in the alveoli and bronchial epithelium (*Fig. 4A and 4B*).

Furthermore, we also examined the number of apoptotic cells using the In Situ Apoptosis Detection Kit from Abcam. Interestingly, the number of cells undergoing apoptosis was about 1-2% and was not different among our 5 experimental groups (*Fig. 5*).

CPB led to a significant formation of PAR in both alveolar and bronchial epithelium. It is known from cell culture experiments that minocycline can inhibit PARP thereby inhibiting PAR formation (23). Accordingly, we saw a lowering effect of minocycline on elevated PAR production during non-pulsatile CPB in alveolar cells (*Fig. 6A*, white bars). However, in bronchial cells this effect of minocycline was almost absent. Similarly, pulsatile flow diminished the number of PAR positive cells in the alveoli but not in bronchial epithelium (*Fig. 6A* grey bars and *Fig. 6B*). However, in bronchial epithelium the preventive effect of pulsatile flow was at the border of significance.

Non-pulsatile CPB significantly increased nitrotyrosine formation in both alveoli and bronchial epithelium. This raise in

nitrotyrosine positive cells was diminished by minocycline and by pulsatile flow in both cell types (*Fig. 7A and Fig. 7B*).

TNF- α levels are enhanced during CPB which was also shown in our study: we could detect significantly increased TNF- α positivity in cells of the alveoli and in bronchial epithelium. Minocycline and pulsatile flow both reduced the number of cells with elevated TNF- α within the alveoli (*Fig. 8A*, white bars). Bronchial epithelium showed the highest increases of TNF- α during CPB which was significantly attenuated by minocycline application and only slightly by pulsatile flow (*Fig. 8A* grey bars and *Fig. 8B*).

DISCUSSION

In our study we could demonstrate that non-pulsatile CPB leads to impaired lung parameters such as decreased tissue ATP-levels, structural changes and inflammation (as became evident by enhanced alveolar wall thickening, by the elevated number of neutrophil granulocytes and elevated TNF- α). Moreover, it was shown that markers of hypoxia, nitrosative stress and apoptosis were increased in both the alveolar wall and bronchial

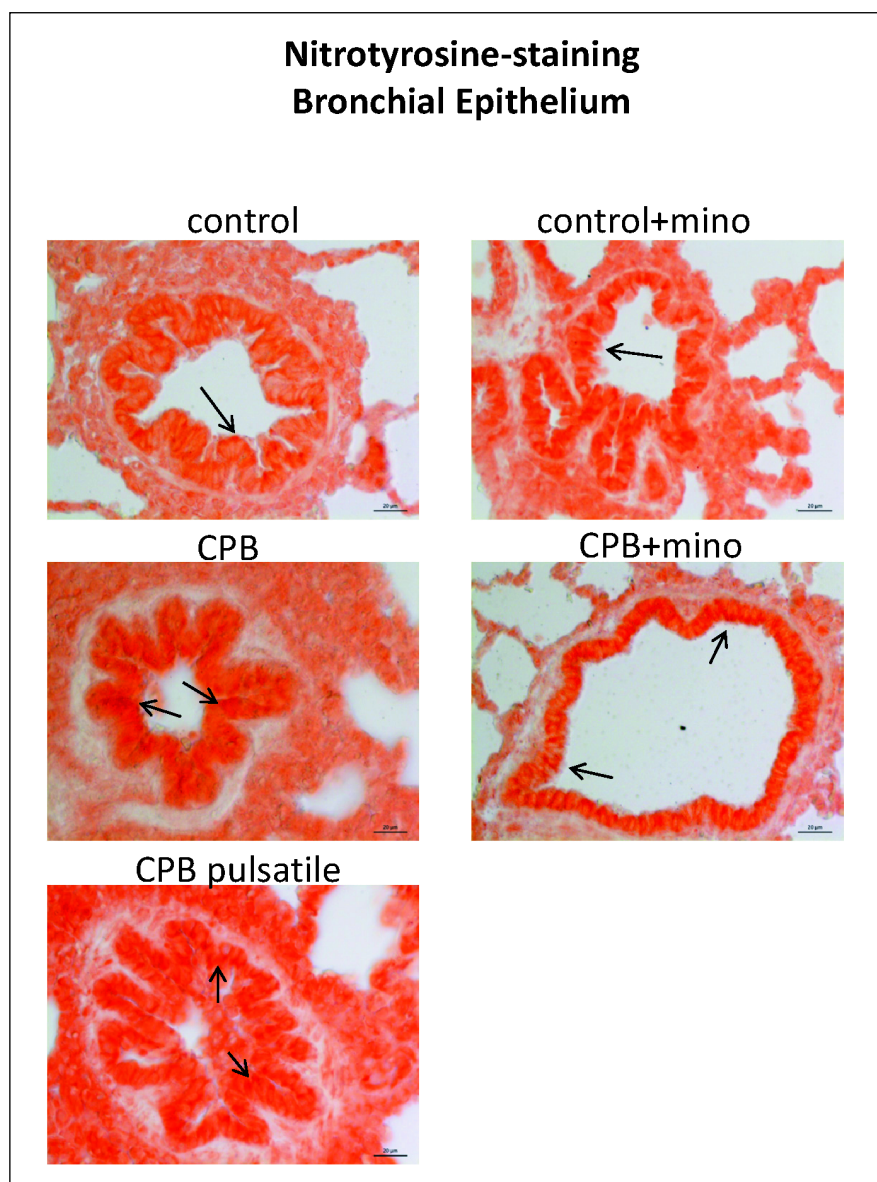


Fig. 7B. Nitrotyrosine-staining of bronchial epithelium. Black arrows indicate nitrotyrosine positive cells.

epithelium. Minocycline and pulsatile flow significantly attenuated this lung injury in our experimental model. Interestingly, independent of CPB, lung compliance as well as paO_2/FiO_2 were lower at the end of experiment compared to

baseline, which might be due to surgical traumata and/or mechanical ventilation. However, there was no CO_2 retention. Thus at the end of the experiment we found a discrepancy between only slight functional changes but significant and large

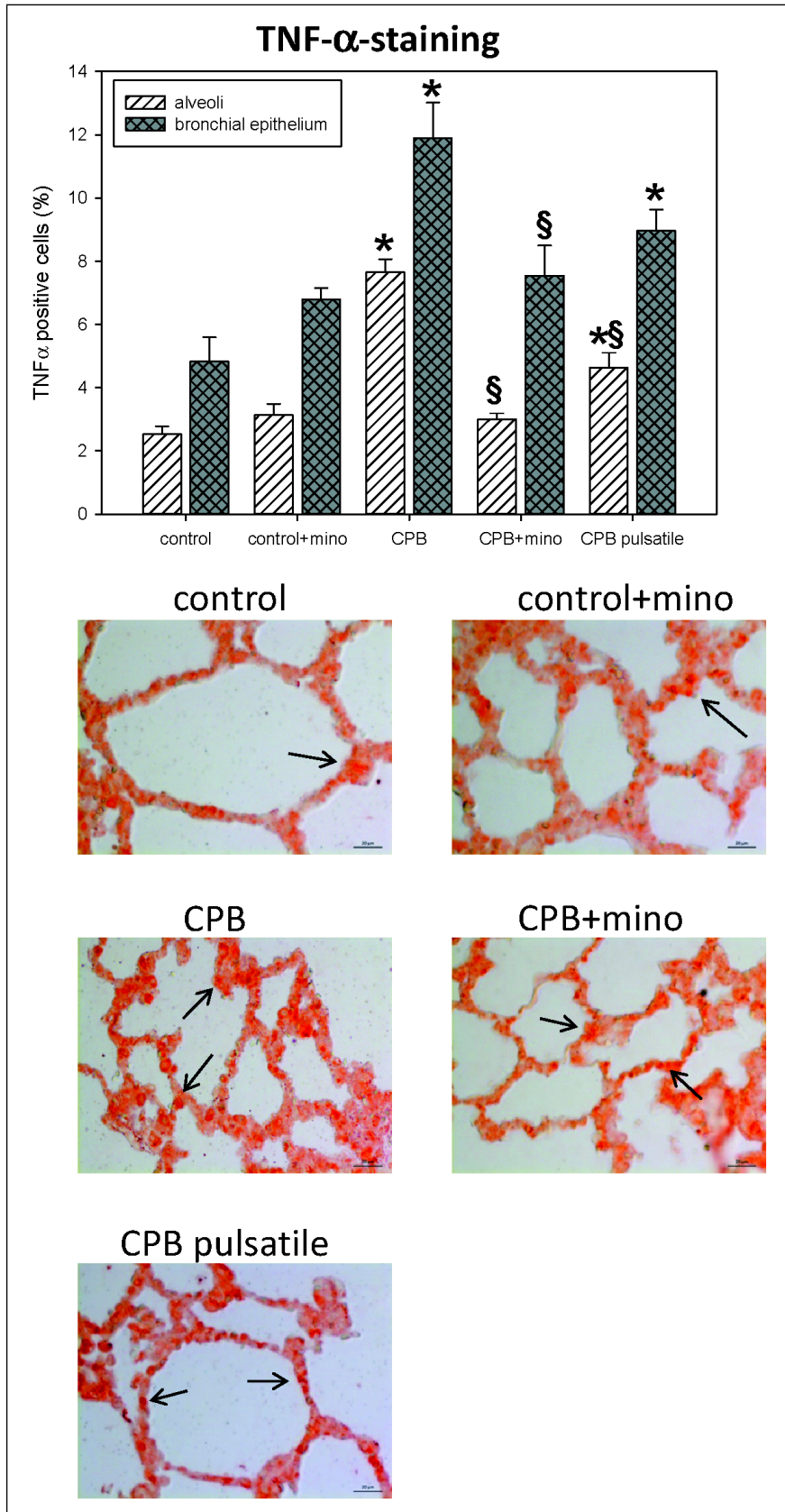


Fig. 8A. TNF- α -staining of alveoli and bronchial epithelium. Bar graphs depict the number of positive cells (in %). Original images of alveoli are depicted below; black arrows indicate TNF- α positive cells, stained in red. Significant differences versus control are indicated by asterisks, significant differences versus non-pulsatile CPB by a section sign ($P < 0.05$).

biochemical alterations. This is however very typical for the clinical situation, when the patient normally leaves the operation theatre with good blood gases but subsequently runs into pulmonary dysfunction within the following 48 hours. Thus, the histochemical data may indicate what functionally may occur later.

Several aspects need to be discussed: a) the impairing effect of CPB on the lung b) the protective effects of minocycline and pulsatile flow and c) the differences in the effects on alveolar versus bronchial epithelium.

a) During CPB the lung is not perfused (except a rest flow *via* Vasa privata) and as a consequence ATP content decreased nearly to zero, thus demonstrating the existence of ischemia despite ventilation. Interestingly, HIF1 α was elevated, which may indicate that intracellularly metabolism was hypoxic although minimal ventilation was maintained and bronchial arteries were perfused *via* the CPB. Pathophysiologically, this situation with continued ventilation but reduced blood flow should lead to the production of free radicals which might impair DNA integrity. According to this theory, nitrotyrosine was elevated which means that increased levels of nitric oxide have

been released (as a consequence of ischemia, presumably) and reacted with cellular proteins. Free radicals also can cause strand breaks of DNA which activate PARP repair mechanism (24). This also could be demonstrated in our data as the product of PARP, i.e. PAR was elevated in CPB. PAR production together with lack of ATP induces AIF release from mitochondria (25) and AIF translocation into the nucleus, which clearly could be seen in our study. Furthermore, the process leading to AIF release from mitochondria together with DNA-damage will finally activate caspase 3 and accordingly we also observed increased cleavage of caspase 3. The time course of our experiments, however, was probably too short to allow complete development of apoptosis (*Fig. 8*); this can be explained by the well-known fact that the development of apoptosis following caspase 3 activation and AIF translocation needs about > 12 hours (26). Taken together, our data show an ischemic damage in the lung during CPB leading to the initiation of apoptosis. However, only the early first steps of apoptosis induction (cC3, AIF, PAR) could be observed due to the 4 hours time frame of the experiments, so that it is not possible to estimate the number of cells finally undergoing apoptosis.

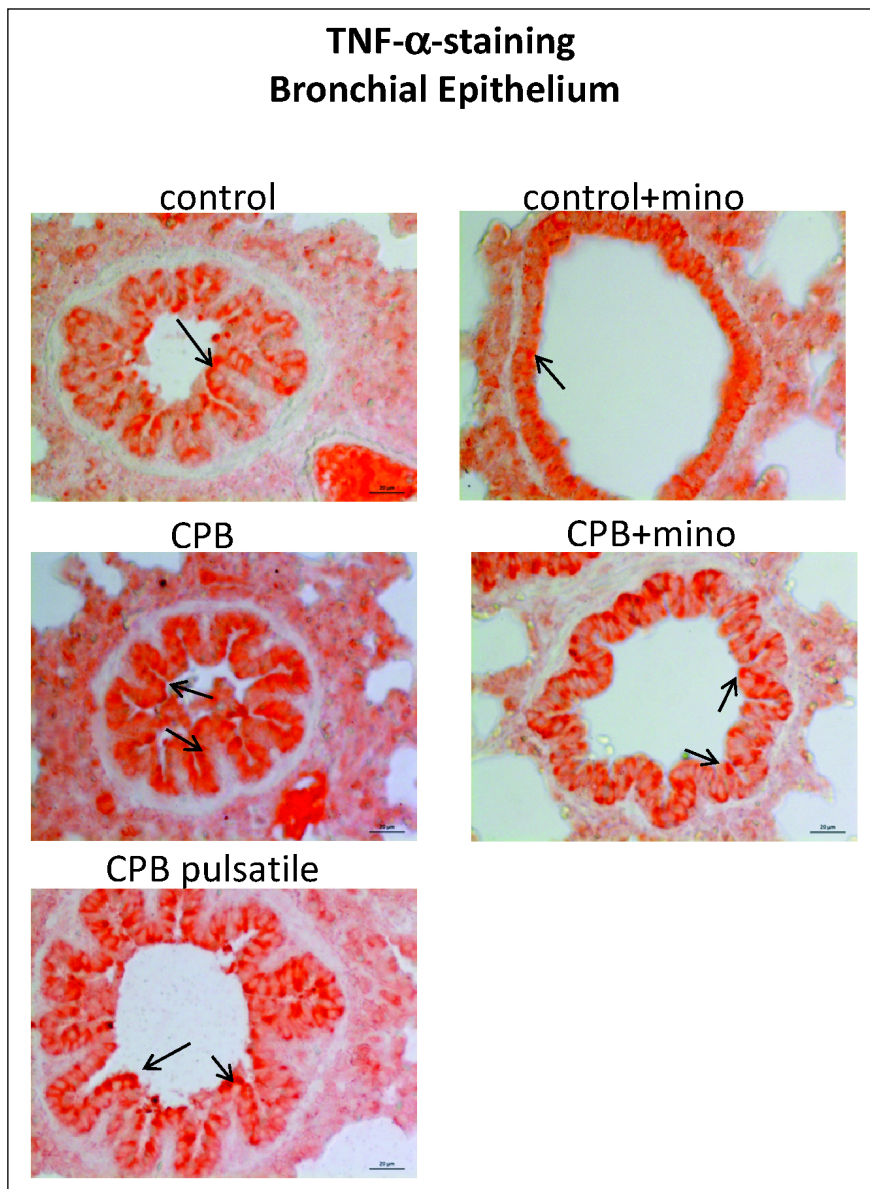


Fig. 8B. TNF- α -staining of bronchial epithelium. Black arrows indicate TNF- α positive cells.

b) Minocycline according to the data presented here prevented from loss of ATP, production of PAR, thickening of alveolar membrane, granulocyte accumulation, nitrotyrosine formation and increased TNF- α expression. It is known that minocycline can act as a radical scavenger (27) and can reduce nitrosylation (28). Thus, it should lead to less 3-nitrotyrosine formation as was demonstrated in our study and to reduced DNA damage, which should result in diminished PAR-formation, which also could be shown in our study. The reduced PARP activity should theoretically lead to less ATP consumption, which is in good accordance with our data demonstrating preservation of ATP in CPB with minocycline. Besides this, minocycline reduced TNF- α release and alveolar granulocyte accumulation, which is in good accordance with previous observations of anti-inflammatory effects of the drug (7, 29). TNF- α release and granulocyte accumulation may be part of an inflammatory lung reaction, which has been observed in several animal models and which has been described as ventilator induced lung injury (30, 31).

Pulsatile flow also protected against CPB-associated impairment, preserving ATP, reducing PAR and nitrotyrosine formation and -to a lower extent than minocycline- attenuating TNF- α release. However, granulocyte accumulation and alveolar thickening were not prevented. Thus, the protective effects of pulsatile flow are present but do not reach the effects of minocycline. It may be assumed that pulsatile flow leads to better mixing of the cellular and non-cellular phase of the blood so that oxygen delivery to the cells may be improved, thus reducing ischemic impairment.

c) An interesting aspect of the study is that alveolar epithelium was less reactive to CPB-associated injury than bronchial epithelium as far as HIF1 α and AIF are concerned. It might be that due to ciliary activity and secretory action bronchial epithelium consumes more oxygen and thus is more prone to ischemic damage. On the other hand effects of minocycline or pulsatile flow treatment were more prominent in the alveoli which might be due to the fact, that the distance between alveolar cells and erythrocytes/blood is lower than in bronchiolar epithelium. In contrast, the inflammatory response with regard to TNF- α was more prominent in the bronchial epithelium, which may indicate that bronchial epithelium is more prone to inflammation, i.e. has a higher inflammatory responsiveness. This effect, which should be independent from circulation, accordingly could not be influenced by pulsatility of flow but by the anti-inflammatory action of the drug minocycline.

Taken together, this study shows that CPB can damage the lung, which helps to explain why patients often suffer from pulmonary dysfunction after CPB. Moreover, two strategies could be identified which have the potential to prevent from lung injury in CPB. Clinical studies are now needed to clarify if this holds also true in humans.

Abbreviations: AEC, 3-amino-9-ethylcarbazol; AIF, apoptosis-inducing factor; ATP, adenosine triphosphate; BSA, bovine serum albumin; cC3, cleaved caspase-3; DAB, diaminobenzimide; CPB, cardio-pulmonary bypass; HE, hematoxylin-eosin; HIF1 α , hypoxia-inducible factor-1alpha; HPLC, high-pressure liquid chromatography; PAR, poly-ADP-ribose; paO₂, arterial oxygen pressure; paCO₂, arterial carbon dioxide pressure; PEEP, positive end-expiratory pressure; PIP, peak inspiratory pressure; TdT, terminal deoxynucleotidyl transferase; TNF- α , tumor necrosis factor alpha

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