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Pulmonary effects of remote ischemic preconditioning in a porcine model of ventilation-induced lung injury

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ABSTRACT

Background: : One-lung ventilation (OLV) may result in lung injury due to increased mechanical stress and tidal recruitment. As a result, a pulmonary inflammatory response is induced. The present randomized, controlled, animal experiment was undertaken to assess the effects of remote ischemic preconditioning (RIP) on diffuse alveolar damage and immune response after OLV.

Methods: : Fourteen piglets (26 ± 2 kg) were randomized to control (n = 7) and RIP group (n = 7). For RIP, a blood pressure cuff at hind limb was inflated up to 200 mmHg for 5 min and deflated for another 5 min, this being done four times before OLV. Mechanical ventilation settings were constant throughout the experiment: V_T = 10 ml/kg, F_IO_2 = 0.40, PEEP = 5cmH_2O. OLV was performed by left-sided bronchial blockade. Number of cells was counted from BAL fluid; cytokines were assessed by immunoassays in lung tissue and serum samples. Lung tissue samples were obtained for histological analysis and assessment of diffuse alveolar damage (DAD) score.

Results: : Hemodynamic and respiratory data were similar in both groups. Likewise, no differences in pulmonary tissue TNF-α and protein content were found, but fewer leukocytes were counted in the ventilated lung after RIP. DAD scores were high without any differences between controls and RIP. On the other hand, alveolar edema and microhemorrhage were significantly increased after RIP.

Conclusions: : OLV results in alveolar injury, possibly enhanced by RIP. On the other hand, RIP attenuates the immunological response and decreased alveolar leukocyte recruitment in a porcine model of OLV.

1. Introduction

Mechanical ventilation induces lung damage as a result of elevated airway pressures, stretch and shear forces secondary to cyclic opening and collapsing of the alveoli (Tremblay and Slutsky, 2005). The pathophysiological changes in the lungs are further increased by one-lung-ventilation (OLV), *e.g.* during thoracic surgery (Schilling et al., 2005), where the whole tidal volume is delivered to only the ventilated lung. In addition, the non-ventilated lung is damaged by temporary collapse and later by re-expansion and ischemia-reperfusion-injury (Heerdt et al., 2007).

Previous experimental studies have demonstrated that OLV induces protein-rich alveolar edema, increases the numbers of activated granulocytes and the release of pro-inflammatory cytokines indicating an acute pulmonary inflammatory response (Schilling et al., 2013).

In addition, persistent hyperperfusion and increased gas content in the ventilated lung with a concomitant hypoperfusion in the non-ventilated lung have been demonstrated in a porcine model (Kozian et al., 2008; Kozian Anesthesiology 2009 and 2011). Moreover, post-mortem histological analysis revealed an increased diffuse alveolar damage score in the ventilated lung as a result of injurious OLV (Kozian et al., 2008, 2010).

As a clinical consequence, patients undergoing thoracic surgery and OLV have a higher risk of postoperative pulmonary complications (Lohser and Slinger, 2015; Yin et al., 2007). Therefore, different approaches have been applied to attenuate the pulmonary injury.

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Mechanical ventilation with reduced tidal volumes (Licker et al., 2003) and positive end-expiratory pressure (Fernandez-Perez et al., 2006; Theroux et al., 2010) did not completely inhibit pro-inflammatory responses in rats and rabbits (Crespo et al., 2006), even though reduced tidal volumes and decreased peak airway pressures had a significant protective effect on the alveolar inflammatory response after OLV in patients (Schilling et al., 2005).

In the last decade, remote ischemic preconditioning (RIP) has evolved as a clinical tool to prevent organs from ischemia-reperfusion injury. RIP is generally performed by alternating, repetitive periods of ischemia and reperfusion of remote tissue that might protect target organs such as the heart, the kidneys or the brain from subsequent transient episodes of hypoxemia or ischemia (Przyklenk et al., 1993). The underlying mechanisms are not completely understood but may involve activation of the NF-KB pathway with production and release of inflammatory mediators like TNF-α, the interleukins (IL) 1, 6, 10 including their receptors as well as activation of neuronal mechanisms (de la Gala et al., 2015).

However, the effects of RIP on the lungs have only been assessed in very few studies. Li et al demonstrated that RIP applied before OLV and thoracic surgery in patients operated on for pulmonary cancer had a positive effect on oxygenation and reduced the pulmonary damage (Zheng et al., 2016). RIP decreased oxidative stress markers in pulmonary exhalation and thus attenuated lung injury (Garcia-de-la-Asuncion et al., 2017). These results seem promising but do not shed light on the protective mechanisms of RIP. In addition, tissue samples representing different regions of both lungs cannot be obtained in thoracic surgical patients. Thus, potential effects of RIP on the ventilated, non-operated lung and on the non-ventilated, collapsed lung have not been studied yet.

The objective of this prospective, randomized, controlled experimental study was therefore to assess the effects of RIP on respiratory function and pulmonary tissue integrity as well as on alveolar injury characterized by leukocyte recruitment and cytokine release after injurious OLV in a porcine model.

2. Materials and methods

The experiment was planned as a prospective, randomized, controlled animal study in a single cohort of Yorkshire / Norwegian land pigs. The experiment was conducted in accordance with the National Institutes of Health guidelines for ethical animal treatment. The Animal Ethics Committee of Uppsala (Sweden) approved the experimental protocol.

2.1. Animals

Fourteen male 2.5 month-old piglets, mean weight 26 ± 2 kg, obtained from a local breeder, were used in the study. The pigs fasted overnight with free access to water. All piglets underwent the same preparatory algorithm (induction and maintenance of anesthesia and monitoring, Fig. 1). Seven animals were randomly assigned to the control group and seven to the RIP group by a list of random numbers generated by Microsoft EXCEL® (Microsoft Corp, Redmond, WA, USA).

2.2. Anesthesia

After randomization, general anesthesia was induced by an *i. m.* injection of xylazine (2.2 mg kg^{-1}, Rompun®; Bayer, Leverkusen, Germany) and tiletamine / zolazepam (6 mg kg^{-1}, Zoletil®; Virbac, Carros, France). After testing for the absence of corneal reflexes and hind limb flexion reflex response, ear veins were cannulated and fentanyl (up to 8 µg kg^{-1}) was administered i.v. prior to the tracheostomy (see below). Anesthesia was then maintained by continuous *i. v.* infusion of fentanyl (0.4 µg kg^{-1} min^{-1}, Leptanal®; Janssen-Cilag AB, Sweden), midazolam (0.12 mg kg^{-1} h^{-1}, midazolam Actavis, Actavis Group, Hafnersfjordur, Iceland) and propofol (Diprivan®; Astra, Södertälje, Sweden). Muscle relaxation was achieved with a continuous intravenous infusion of 2.5 mg kg^{-1} h^{-1} rocuronium bromide (Esmeron®, N.V. Organon, Oss, Netherlands).

At the end of the experiment, the pigs were killed by an intravenous bolus injection of potassium chloride (150 meq) under deep anesthesia.

2.3. Airway management and mechanical ventilation

The settings have been already described in detail (Kozian et al.). The present experiment was closely related to this protocol, and the ventilator settings remained unchanged in all pigs throughout the study (Fig. 1).

Briefly, the animals were placed supine and tracheostomy with insertion of an ID 8.5 mm endotracheal tube (Mallinckrodt, Athlone, Ireland) was performed. Pressure-controlled ventilation was applied throughout the procedure with F_IO_2 of 0.4 in air and positive end-expiratory pressure (PEEP) of 5 cmH_2O, provided by a KION® anesthesia ventilator (Maquet Critical Care, Solna, Sweden). The tidal volume was set to 10 ml kg^{-1}, and respiratory rate was adjusted to maintain a $paCO_2$ of 40 mmHg. Fresh gas flow, airway pressures and gas concentrations were measured at the proximal end of the endotracheal tube with a standard monitor for ventilation and hemodynamic measurements (SC 9000 XL, Siemens; Erlangen, Germany).

A left-sided endobronchial blocker (9.0 French Arndt Endobronchial Blocker Set, COOK®; Bjaeverskov, Denmark) was inserted and secured in the left main bronchus. The animals were kept in supine position throughout the experiment.

2.4. Instrumentation and monitoring

A flow-directed pulmonary artery catheter (PAC) (7.0 French, Swan-Ganz thermodilution catheter, Baxter, Irvine, CA, USA) and a central venous catheter (4.0 French, Becton-Dickinson Critical Care Systems; Singapore) were inserted *via* the right external jugular vein. The PAC was used for cardiac output (CO) measurements and mixed venous blood sampling. It was repositioned before each experimental step to ensure that the tip was always located in regions with high pulmonary blood flow.

In addition, the pigs received a right carotid arterial catheter for continuous arterial blood pressure measurements and for blood sampling (20 G; Becton-Dickinson Critical Care Systems). Blood gas analysis was performed immediately after bubble free blood sampling with standard blood gas electrodes specifically set up to analyze pig blood (ABL 500 and OSM3; Radiometer, Copenhagen, Denmark). Finally, a suprapubic urinary catheter (Sympakath®; Ruesch AG, St. Gallen, Switzerland) was placed to monitor urine output.

2.5. RIP procedure

The animals that had previously been randomized to the RIP group (n = 7) had a blood pressure cuff at the left hind limb inflated up to 200 mmHg for five minutes followed by five minutes of reperfusion after deflating the cuff, this being repeated three times.

2.6. OLV

After RIP or at similar time point in the controls, the left-sided endobronchial blocker was inflated under fiber-optic control. The ventilation of the left lung was discontinued for 120 min (OLV period). After two hours of OLV, the bronchial blocker was removed and the lungs were re-inflated by a constant airway pressure of 30 cm H_2O, applied to the whole lung for 10 s (alveolar recruitment maneuver, ARM). Thereafter, two-lung ventilation (TLV) was resumed, with the ventilator settings remaining the same as described before.

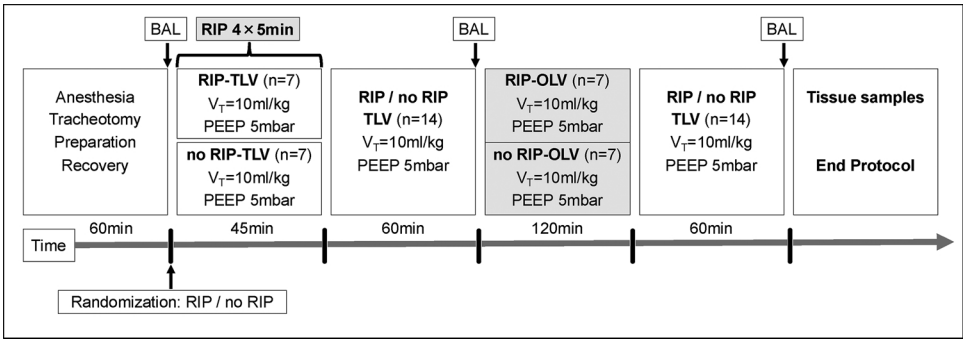

Fig. 1. Experimental protocol and timing.
(Abbreviations: BAL Broncho-alveolar lavage, OLV one-lung ventilation, PEEP positive end-expiratory pressure, RIP remote ischemic preconditioning, TLV two-lung ventilation, V_T tidal volume)

2.7. Bronchoalveolar lavage

Fiber-optic bronchoscopy and bronchoalveolar lavage (bronchoscope EF-B 14/L, Xion Ltd., Garching, Germany) were performed at defined time points (Fig. 1). Before and after each manipulation, both lungs were recruited by ARM, and blood samples were taken.

Bronchoalveolar lavage (BAL) was based on a standardized procedure: The tip of the bronchoscope was brought into wedge position in a segmental bronchus of the left and right lower lung lobes, respectively. For each BAL, a different segmental bronchus was randomly chosen and 30 ml of sterile, isotonic saline solution (Fresenius Kabi AB; Halden, Norway) was sequentially instilled (10 ml portions) and gently suctioned; ~50% of the fluid was recovered. Bronchoalveolar lavage fluid was collected in sterile tubes and immediately placed on crushed ice.

2.8. Measurements

Ventilation and hemodynamic variables were recorded, and arterial and mixed-venous blood samples were taken during baseline TLV, after the RIP-procedure before OLV, after 60 min and after 120 min of OLV as well as during TLV, 60 min after OLV (Fig. 1).

2.9. Tissue sampling

The entire lungs were excised *via* median sternotomy immediately after the pigs were killed. Blocks of lung tissue of approximately 1 cm^3 were harvested from both lungs largest diameter at three locations: from subpleural, intermediate and parahilar regions. Three samples from each location were obtained; either immediately frozen in liquid nitrogen and stored at −80 °C until analysis or fixed in 4% neutral-buffered *p*-formaldehyde for at least 72 h and finally embedded in paraffin.

Tissue pieces were homogenized in lysis buffer (15 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% Triton X-100), with addition of protease inhibitor (Thermo Scientific, Waltham, MA, USA) using an Ultra-Turrax T8 homogenizer. The homogenates were centrifuged at 300 *g* at 4 °C for 10 min.

2.10. Preparation of cell counting

The lavage fluid was filtered through sterile gauze filters and collected on ice in siliconized containers. For staining and cell counting, 50 µl of BAL fluid was used.

2.11. Measurement of cytokine and protein concentrations

The total protein content of tissue homogenates was measured by Coomassie Plus Assay (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Cytokine concentrations were assessed in serum samples and in tissue homogenates using Quantikine ELISA (R&D systems, Minneapolis, MN, USA), for porcine TNF-α, IL-8, IL-10 and IL-1β according to the manufacturer's instructions. The detection ranges of the assays were: 23.4 −1500 pg/ml for TNF-α, 62.5–4000 pg/ml for IL-8, 31.3 −2000 pg/ml for IL-10 and 39.1 −2500 pg/ml for IL-1β. The cytokine concentrations of the lung tissue homogenates were normalized against total protein content.

2.12. Staining procedures

Paraffin-embedded lung tissue samples were sectioned (2–3 µm slices) and stained with hematoxylin and eosin (H&E) for light-microscopic analysis (microscope Model CHK; Olympus, Taiwan). The sections were randomly selected by an assisting technician blinded to the experimental protocol, and the slides were evaluated by a blinded pathologist. The extent of histomorphological changes was scored by the diffuse alveolar damage (DAD) score.

2.13. DAD score

The alveolar injury was characterized by the following features (Ferguson et al., 2005; Spieth et al., 2007): Alveolar edema, interstitial edema, microhemorrhage, neutrophil infiltration, microatelectasis and alveolar overdistension. Four isolated non-overlapping fields of view of the different samples were analyzed separately. The values of all sectors per lung (n = 12) were averaged.

The severity of the DAD features was characterized as follows: 0 = normal appearance, 1 = slight effect, 2 = intermediate effect and 3 = severe effect. The extent of alveolar damage in each sector was described as follows: 0 = no damage, 1 = up to 25%, 2 = 25–50%, 3 = 50–75%, 4 = 75% to almost complete and 5 = complete. Each property was given by its severity multiplied by the extent. The DAD score was calculated by summarizing the products of severity and extent of each DAD quality (Yokoyama et al., 2001).

2.14. Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS, v. 23, IBM Corporation, Armonk, New York, USA) and SigmaPlot® v. 13 (Systat Software Inc., San Jose, CA, USA). The estimation of sample size was based on previous experimental studies (Kozian et al., 2010; Schilling et al., 2013), which used a similar experimental setup. Power calculation using a two-sided design at a significance level of 5% ($\alpha = 0.05$) and a power of 80% ($\beta = 0.20$) revealed that at least five animals per group were needed to detect a difference of more than 25% in alveolar cytokine concentrations in piglets prior to and after OLV.

All data were tested for normal distribution with the Shapiro-Wilk W test and are presented as means and standard deviations in the case

of normal distribution (cardiopulmonary and ventilation variables). In case of non-normal distribution, results are displayed as box plots (median and interquartile range (IQR, P_{25}-P_{75}). The analysis of normally distributed data was performed by repeated measures one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction.

A repeated measures general linear model (type III sums of squares) assessed the sequential changes of cytokine concentrations in each group. Subsequent between-group comparisons were performed by two-way ANOVA using the independent variables "group" and "time". Post-hoc multiple comparisons were performed by the Bonferroni procedure.

The concentrations of TNF-α and IL-8 remained heteroscedastic even after logarithmic transformation. Therefore, non-parametric Friedman´s test and subsequent Kruskal-Wallis H-Test with adjustment of α-levels for repeated measurements were used to analyze these data sets.

No data were lost during the experiment or were missed in the statistical analysis. In some cases, mediator concentrations were below the detection limits of the immunoassays. These data were included into the statistical analysis with a value of 0.1.

The differences were considered to be statistically significant for all procedures if $p < 0.05$.

3. Results

3.1. Hemodynamic and ventilation variables

The mean values of hemodynamic and respiration data are presented in the Tables 1 and 2. Before RIP, all animals underwent the identical setup resulting in similar hemodynamics and ventilation mechanics. After the RIP procedure, mean arterial pressure (MAP) and SvO_2 were slightly but significantly increased in RIP pigs as compared to controls. However, in both groups, MAP was elevated when compared to baseline.

OLV with delivery of the entire tidal volume to the right lung increased MPAP and venous admixture, and it resulted in higher peak and mean airway pressures. After restoration of TLV after 120 min of OLV, data returned to the initial values.

All animals had a similar decrease in oxygenation as compared to the pre-OLV data, and both groups showed a trend back to baseline paO_2 values during the succeeding TLV. Nevertheless, oxygenation index ($F_IO_2 * P_{AW}$ mean / paO_2) was lower after RIP intervention, and it remained decreased in those pigs that underwent the RIP procedure (Fig. 2). The oxygenation index highlights the fraction of inspired oxygen (F_IO_2) and how it is used in the organism (paO_2). If oxygenation deteriorates higher F_IO_2 results in lower PaO_2.

3.2. DAD score

Mechanical ventilation including a period of OLV for 120 min resulted in a significant increase of global DAD score values in all pigs without any differences between the experimental groups (Fig. 3A). The median DAD score was 29.8 (range 28.3–32.1) in control piglets and 30.4 (range 26.5–32.6) in animals with RIP intervention. However, in the ventilated lungs of RIP pigs, the items "alveolar edema" and "microhemorrhage" were more pronounced than in control animals (Fig. 3B, Table 1 in Ref. Bergmann et al., 2018). The mean values for alveolar edema were 5.4 (range 4.5–5.7) in controls *vs.* 6.0 (range 5.5–6.5) in RIP pigs ($p < 0.05$), and for microhemorrhage (controls *vs.* RIP) 3.5 (range 2.4–4.4) *vs.* 4.8 (range 4.5–5.4; $p < 0.05$).

3.3. Alveolar cell numbers

Mechanical ventilation itself increased the number of cells in the broncho-alveolar lavage fluids, which represents the recruitment of leukocytes in the lung periphery (distal airways and alveoli) (Fig. 4A, B). However, cell recruitment was significantly higher in the lungs of control animals in comparison with the RIP group, at the end of the study period. In addition, these differences were more pronounced in the right, ventilated lung.

3.4. Cytokine concentrations in lung tissue and serum samples

In the lysates of lung tissue samples, the concentration of TNF-α was more increased in controls as compared to RIP pigs (Fig. 5A), and the ventilated part of the lungs was more affected. The total protein content was lower in lung tissue of the RIP group [controls *vs.* RIP: 11.6 mg/ml (IQR 8.5–19.9) *vs.* 7.2 mg/ml (IQR 6.3–7.7), $p < 0.05$]. This was accompanied with highest IL-8 concentrations in the non-ventilated lungs of RIP pigs (Fig. 5B). Accordingly, serum IL-8 and IL-1β but not TNF-α increased after OLV; however, there was no statistical difference between controls and RIP pigs (Table 3).

4. Discussion

The main results of the present experiment comprise that (I) RIP improves oxygenation after OLV, (II) RIP may accelerate alveolar edema and microhemorrhage in the ventilated lung and (III) RIP attenuates alveolar recruitment of leukocytes after OLV and TNF-α concentrations in the ventilated lung. Thus, RIP has no direct protective effects on lung structure and integrity but may attenuate alveolar inflammation after injurious ventilation.

In patients undergoing lung resection, pulmonary complications represent a major risk for increased postoperative morbidity and

Table 1
Hemodynamic variables in controls (n = 7) and in the RIP group (n = 7) at baseline and immediately after the RIP procedure, during OLV (60 min, 120 min) and at the end of the experiment (TLV, 60 min). The data are presented as mean ± standard deviation. * indicates differences ($p < 0.05$) in comparison of controls with RIP pigs; # $p < 0.05$ as compared to baseline.

Variable	Baseline		After RIP		60 min OLV		120 min OLV		End (TLV)	
	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)
MAP (mmHg)	89 ± 9	88 ± 12	62 ± 5 #	76 ± 11 *#	66 ± 4 #	78 ± 11 *#	71 ± 3 #	80 ± 10 *#	73 ± 6 #	82 ± 12 *
MPAP (mmHg)	15 ± 2	17 ± 1	15 ± 2	15 ± 1	20 ± 3 #	20 ± 1 #	21 ± 2 #	20 ± 1 #	19 ± 3	19 ± 2
CVP (mmHg)	4 ± 1	4 ± 1	6 ± 1	5 ± 1	7 ± 1	6 ± 1	7 ± 1	6 ± 1	7 ± 2	6 ± 1
PAOP (mmHg)	7 ± 1	6 ± 1	7 ± 1	6 ± 1	7 ± 1	6 ± 2	7 ± 1	7 ± 1	8 ± 1	8 ± 1
CO ($l \cdot min^{-1}$)	2.8 ± 0.5	2.7 ± 0.6	2.5 ± 0.5	2.2 ± 0.1	2.5 ± 0.5	2.2 ± 0.2	2.6 ± 0.5	2.4 ± 0.4	2.6 ± 0.5	2.3 ± 0.3
HR (s^{-1})	101 ± 8	100 ± 11	79 ± 10 #	84 ± 3 #	86 ± 13 #	84 ± 3 #	88 ± 14 #	83 ± 5 #	87 ± 14 #	82 ± 6 #
SvO2 (%)	53.9 ± 6.4	59 ± 3.1	49.2 ± 5.5	57.4 ± 7 *	43.5 ± 7.2	49.4 ± 5	43.3 ± 4.6	50.5 ± 4 *	44.6 ± 9	52.5 ± 4.2
Venous admixture (%)	3.4 ± 0.7	2.9 ± 0.7	3.2 ± 0.7	3.0 ± 0.4	5.4 ± 1.2 #	5.4 ± 1.7 #	4.5 ± 0.9 #	4.3 ± 1.3 #	7.1 ± 2.3 #	8.5 ± 1.9 #

RIP: remote ischemic preconditioning; OLV: one-lung ventilation; TLV: two-lung ventilation; MAP: mean arterial pressure; MPAP: mean pulmonary artery pressure; CVP: central venous pressure; CO: cardiac output; HR: heart rate; SvO_2: mixed venous oxygen saturation.

Table 2
I. Respiratory and gas exchange data in controls (n = 7) and in the RIP group (n = 7) at baseline, immediately after the RIP procedure, during OLV (60 min, 120 min) and at the end of the experiment. The data are presented as mean ± standard deviation. * Indicates differences ($p < 0.05$) in comparison of RIP pigs with controls; # $p < 0.05$ as compared to baseline.

Variable	Baseline		After RIP		60 min OLV		120 min OLV		End	
	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)
paO_2 (mmHg)	187 ± 9	194 ± 6	175 ± 13	184 ± 6	121 ± 18 #	132 ± 28 #	140 ± 16 #	155 ± 21#	155 ± 10 #	181 ± 10 *
$paCO_2$ (mmHg)	37 ± 1	37 ± 2	38 ± 1	38 ± 3	40 ± 1 #	38 ± 1 *	40 ± 1 #	38 ± 1 *	39 ± 1	38 ± 3
P_{AW} peak (mbar)	18 ± 2	17 ± 0	19 ± 1	19 ± 2	26 ± 2 #	25 ± 2 #	24 ± 2 #	24 ± 2 #	20 ± 1	19 ± 2
P_{AW} mean (mbar)	9 ± 1	9 ± 1	9 ± 1	9 ± 1	11 ± 1 #	11 ± 0 #	10 ± 1	10 ± 1	9 ± 0	8 ± 0
PEEP (mbar)	5 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0
MV ($l \cdot min^{-1}$)	6.3 ± 0.6	5.9 ± 0.4	6.2 ± 0.6	6.0 ± 0.4	6.1 ± 0.7	5.9 ± 0.4	6.0 ± 0.6	5.9 ± 0.4	6.1 ± 0.6	5.9 ± 0.4
V_T (ml)	262 ± 12	254 ± 12	260 ± 9	258 ± 13	259 ± 9	259 ± 13	256 ± 8	257 ± 14	258 ± 9	258 ± 14

RIP: remote ischemic preconditioning; OLV: one-lung ventilation; paO_2: arterial partial pressure of oxygen; $paCO_2$: arterial partial pressure of carbon dioxide; P_{AW}: airway pressure; PEEP: positive end-expiratory pressure; MV: minute ventilation; V_T: tidal volume.

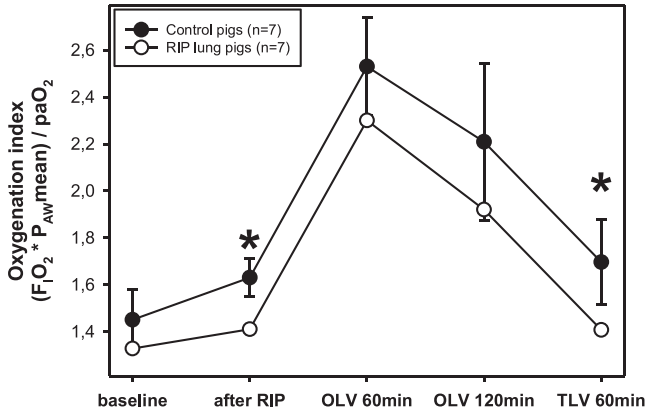

Fig. 2. Time course of oxygenation index (F_IO_2 * P_{AW} mean / paO_2) at baseline, after RIP prior to OLV, during OLV (60 min and 120 min) and during TLV (60 min) after OLV; * indicates the differences ($p < 0.05$) between controls (n = 7) and RIP lung pigs (n = 7). Please note that as shown by the equation itself, a lower index is better than a high one. In the equation, the numerator will decrease and / or the denominator will increase, thus lowering the oxygenation index.
(Abbreviations: OLV one-lung ventilation, P_{AW} mean airway pressure, RIP remote ischemic preconditioning, TLV two-lung ventilation)

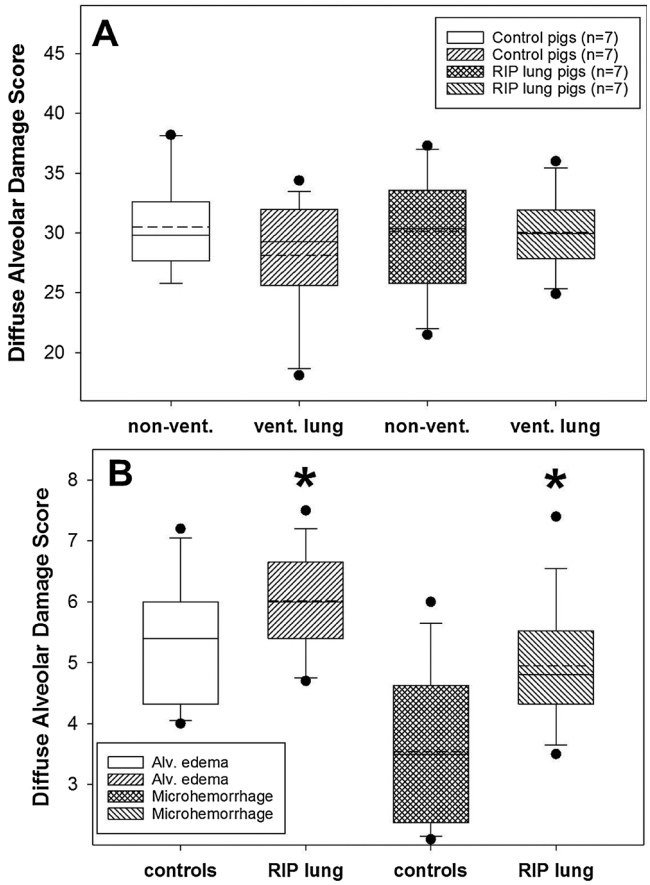

Fig. 3. A, B: Box plots of global diffuse alveolar damage scores (A), separately displayed for the non-ventilated and ventilated lungs of controls (n = 7) and RIP pigs (n = 7). Figure (B) presents box plots of the DAD features "alveolar edema" and "microhemorrhage" in the ventilated lungs.
* = $p < 0.05$ in comparison between controls and RIP lung pigs.
(Abbreviations: RIP remote ischemic preconditioning)

mortality (Rosen et al., 2014). Pre-existing cardiopulmonary diseases and the extent and invasiveness of the surgical procedure have an important impact on the postoperative outcome. In addition, intraoperative OLV may also contribute to lung injury (Lohser and Slinger, 2015). Accordingly, a previous clinical study found a correlation between the duration of OLV and the extent of oxidative lung injury (Garcia-de-la-Asuncion et al., 2016). Besides, there is evidence that the attenuation of inflammatory responses following thoracic surgery may decrease the prevalence of postoperative pulmonary complications and improve outcome (De Conno et al., 2009).

Protective ventilatory settings have been estabished to prevent the lungs from mechanical damage provoked by shear stress and increased airway pressures. But still, chemical issues might also be worthwhile to look at to attenuate the release of pro-inflammatory cytokines and support anti-inflammatory pathways. In this context, RIP is one of the most promising features (Ravingerova et al., 2016).

However, the mechanisms of RIP are still not fully elucidated, but likely act *via* multiple pathways. Short episodes of ischemia applied to one limb activate kinases and transcription factors (Hussein et al., 2016) which are released into the circulation, enabling remote organs to resist ischemia at a later point in time (Botker and Schmidt, 2015). The expression of anti-oxidative enzymes and the suppression of oxygen radicals (ROS) seem to play a role as well as the transcription and expression of anti-inflammatory markers, which activate intracellular pathways after binding to their receptor (Kleinbongard et al., 2017), leading to the opening of potassium-dependent ATP-channels and preventing permeability-pores from opening, thus protecting the mitochondria and lowering the electrical potential of the membrane (Herajarvi et al., 2017). Eventually, the cells will activate their self-repair-mechanisms and improve their survival, preventing ischemic injury and thus inflammation and apoptosis (Sivaraman et al., 2015).

The wet lung / dry lung weight ratio is reduced after remote

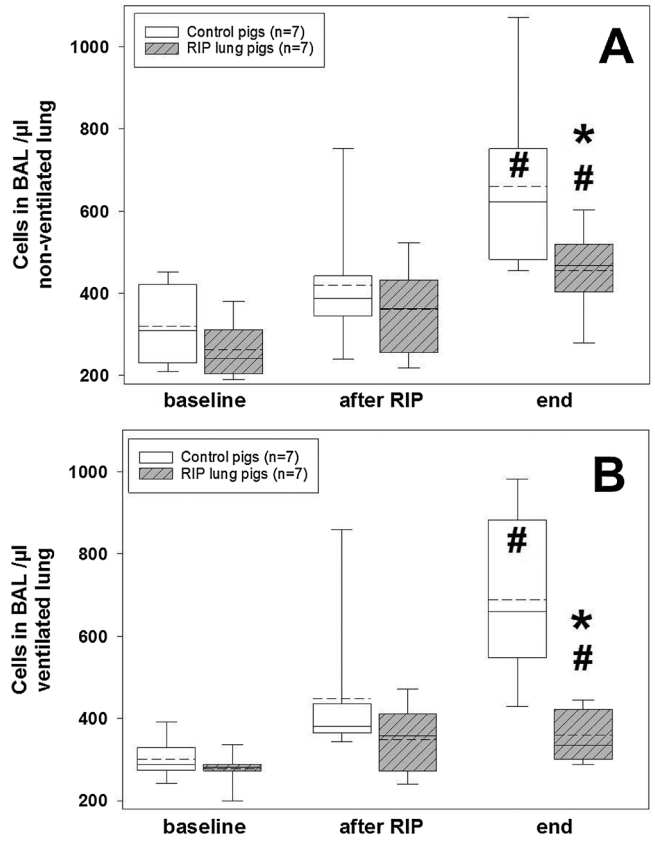

Fig. 4. A, B Total numbers of cells in the BAL fluid of the non-ventilated lung (upper, A) and ventilated lung (lower, B) at baseline, after RIP, and at the end of the experiment are separately displayed for controls and RIP pigs. * indicates the differences in comparison of controls and RIP pigs ($p < 0.05$); # gives the difference as compared to baseline ($p < 0.05$). (Abbreviations: BAL bronchoalveolar lavage, RIP remote ischemic preconditioning).

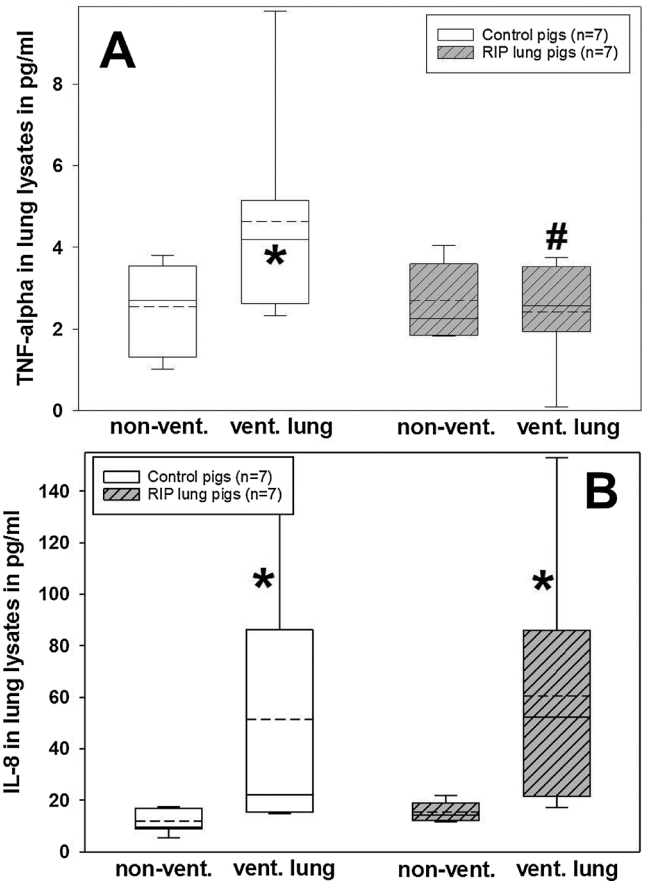

Fig. 5. A, B Concentrations of TNF-α (upper, A) and interleukin-8 (lower, B) in lung lysates of the non-ventilated and ventilated lungs. * indicates the differences between the non-ventilated and ventilated lung, # marks the different TNF-α concentrations in the ventilated lung of controls and RIP pigs ($p < 0.05$). (Abbreviations: TNF tumor necrosis factor, IL interleukin, RIP remote ischemic preconditioning).

ischemic preconditioning applied to the liver in a rat model, indicating that RIP has an effect on the development of edema (Luo et al., 2018). However, this is not exactly in line with our results, as we have found that alveolar edema is increased after RIP. This might hint that the site of RIP plays a role as well, as we performed RIP on the hind limb and not on an intestinal organ. And of course our model opens an interesting field for further studies that will analyze pulmonary edema formation but also may include mRNA-expression of different mediators.

Additional RIP-mechanisms might include cytokines and damage-associated molecular pattern (DAMP) molecules, which are intracellular danger signals such as high-mobility group box 1 (HMGB1) and a multigenic family of calcium modulated proteins involved in cellular regulatory activity (S100A8/A9) (Liu et al., 2018). These bind to toll-like receptors (TLR) or receptors for advanced glycation end-products (RAGE) and promote nuclear factor-κB (NF-κB) signaling and expression of cytokines functioning through their respective receptors (Abe et al., 2014). NF-κB-dependent proinflammatory cytokines upregulate the expression of DAMPs and RAGE, thus leading to a pathological cycle of inflammation (Nadatani et al., 2013). The downstream effects on macrophages were assessed in this study, using interleukins and TNF-α as surrogate markers for HMGB1 and S100, which are fairly easy to measure in porcine samples. In fact, the concentrations of proinflammatory TNF-α and of alveolar protein were lower in the ventilated lung in the RIP group as compared to the control group, which may suggest an anti-inflammatory effect of RIP. Thus, our findings agree with a clinical study on thoracic surgical patients (Bottiger et al., 2015). As IL-8 and TNF-α develop differently, it might be that the effects of RIP on the lungs are not consistent for all cytokines and/or that they rise and decline at different time points.

It is difficult to assess potential differences of pulmonary cytokines in a clinical setting. The differences of IL-8 in the ventilated side in the RIP group and unchanged IL-8 serum concentrations may indicate that RIP has a direct effect on the pulmonary immune system. In healthy volunteers, RIP attenuated normal hypoxic increase in pulmonary artery systolic pressure, possibly by attenuation of hypoxic pulmonary vasoconstriction (HPV) (Foster et al., 2011). The production of reactive oxygen species is increased in hypoxic tissue in complex I and III of the mitochondrial chain, which may augment HPV (Ward, 2006). Thus, limb RIP ameliorates oxidative lung damage but may aggravate hyperperfusion of the ventilated lung during and after OLV (Garcia-de-la-Asuncion et al., 2017).

The leukocyte counts in BAL revealed that RIP markedly reduced alveolar sequestration of granulocytes. In contrast, the IL-8 release was high in both the control and the RIP group. However, the pathways regulating recruitment of circulating neutrophils and transmigration into the alveolar compartment are different from the pathways regulating IL8 release. (Reutershan and Ley, 2004). The inflammatory response to lung resection and OLV including neutrophil sequestration may reach a maximum in the postoperative course (Fink-Neuboeck et al., 2016) but the present study was not designed to look at a prolonged time period after OLV.

Granulocytes are the main source of TNF-α and were decreased in BAL fluid after RIP. Therefore, RIP may indirectly affect pulmonary immune function by attenuating neutrophil sequestration. Is is important to notice that the anesthetics might influence the effect of RIP.

Table 3
I. Proinflammatory serum cytokine concentrations displayed as medians and interquartile ranges (IQR, P_{25}-P_{75}), separately for the control group (n = 7) and RIP pigs (n = 7). # p < 0.05 in comparison to baseline. # indicates differences (p < 0.05) in comparison with baseline.

Group / Variable	Baseline		After RIP		End	
	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)	Controls (n = 7)	RIP (n = 7)
TNF-α [pg/ml]	70.4 (51.5 - 83.1)	66.0 (52.6 - 75.4)	65 (58.9 - 82.3)	68.0 (61.6 – 76.8)	52.0 (47.0 – 58.0)	73.0 (49.3 – 76.5)
IL-8 [pg/ml]	85.6 (30.9 – 184.7)	74.0 (64.6 – 480.0)	82.6 (40.3 – 146.1)	123.7 (46.7 – 463.8) #	116.8 (109.3 – 127) #	148.9 (41.8 – 288.6) #
IL-1 [pg/ml]	1.8 (1.2 – 2.9)	1.1 (0.7 – 1.3)	2.1 (1.2 – 2.8)	2.4 (1.3 – 2.9)	3.3 (1.8 – 3.5) #	3.6 (3.1 – 3.8) #

IL: Interleukin; TNF: tumor necrosis factor.

Thus, highly lipid-soluble drugs like propofol, as we used, may influence *e.g.* neutrophil activation and inhibit respiratory burst in the lung. Volatile anesthetics seem to be more immune depressive. In fact, we have previously demonstrated in a number of clinical and experimental studies that the volatile anesthetics desflurane and sevoflurane suppress the pro-inflammatory response in the ventilated lung after OLV more than propofol does (Schilling et al., 2011, 2013). In contrast, propofol does not exert this alleviating effect on alveolar cytokines. Consequently, a recent clinical study provided clear evidence for the efficacy of limb RIPC even under propofol–remifentanil anesthesia (Li et al., 2014).

Mechanical ventilation including two hours of OLV results in significantly increased DAD scores, which confirms earlier histologic studies using a corresponding experimental protocol (Kozian et al., 2010) (Fig. 10 in Ref. Bergmann et al., 2018). In addition, clinical studies revealed that lung resection surgery is associated with increased markers of oxidative stress and inflammation (de la Gala et al., 2015; Misthos et al., 2005, 2006). Previous data obtained by bronchoscopic microsampling from each lung showed that IL-1β, IL-6, and IL-8 are significantly increased both in the ventilated and in the non-ventilated lung at the end of lung resection surgery (Sugasawa et al., 2011). Moreover, the production and release of superoxide anions and cytokines may be induced by hypoxic pulmonary vasoconstriction in the collapsed lung, and OLV has been shown to increase concentrations of 8-isoprostane, NO_2^- and NO_3^- and H_2O_2 in exhaled breath condensate (Garcia-de-la-Asuncion et al., 2016). However, in the present study, RIP was not associated with different DAD scores, indicating that early alveolar damage is not prevented or attenuated by RIP. Histologic analysis revealed slightly higher degrees of microhemorrhage and alveolar edema in the ventilated lung suggesting that RIP may even increase pulmonary capillary perfusion.

In the present study, oxygenation was increased in the RIP group after OLV agreeing with previous studies (Garcia-de-la-Asuncion et al., 2017). This effect can be related to the modulation of regional lung perfusion, resulting in improved ventilation-perfusion matching. Persistent hyperperfusion of the ventilated lung after OLV has been demonstrated by single photon emission tomography (Kozian et al., 2008). Therefore, RIP may affect pulmonary vasoconstriction in the previously collapsed lung and thereby improve oxygenation.

There are limitations in the current study, including the restrictions of the animal model *per se*, the sample size of the study group, the fixed ventilation setup for mechanical ventilation and the short post-interventional observation period. Thus, prolonged effects of RIP could not be assessed.

The intention of this experiment was to induce a significant alveolar injury by OLV, which was stable and reproducible. Despite hemodynamic parameters were similar in both groups, increased MAP in the RIP group can be related to high individual scatter. Thus, the applied ventilation settings and time schedule may cover a multitude of clinical procedures. This study was closely related to previous experiments on OLV-induced pulmonary changes in pigs (Kozian et al., 2008, 2010). By using the established protocol, it becomes possible to validate and compare the present data to previous results. Moreover, it was not the principal aim of the present study to demonstrate effects of protective ventilation but to analyze potential effects of RIP in an animal model of OLV. Whether RIP further augments a lung protective approach in thoracic surgical patients can be addressed in future studies.

5. Conclusions

In conclusion, the pulmonary effects of RIP in an experimental model of mechanical ventilation may include decreased proinflammatory response and alveolar leukocyte sequestration into both lungs. It cannot be said wether RIP in itself is beneficial to the lung or not, as the overall DAD score remains unchanged and two aspects of it – microhemmorrhage and alveolar edema – are even increased after RIP. This suggests that pulmonary capillary perfusion is affected. RIP thus may have several impacts on the lungs and should be investigated in further studies.

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