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Tauroursodeoxycholic acid inhibits apoptosis associated with ventilator-induced lung injury in rabbits

AF Broccard[1*], AB Adams[2], E Korbach[2], RE Castro[3], CJ Steer[4], CM P Rodrigues[3]

Abstract

Introduction

Apoptosis has been implicated in the process of lung injury. A drug that inhibits apoptosis could be very helpful to understand the role of programmed cell death in ventilator-induced lung injury (VILI). We examined whether tauroursodeoxycholic acid (TUDCA), an anti-apoptotic bile acid prevents ventilator-induced apoptosis in rabbit model of VILI.

Materials and methods

Following median thoracotomy (open chest model), rabbits (n=15) were randomly assigned. Controls were ventilated for 4 h with Pressure Control Ventilation (PCV) using a low peak pressure of 10 cm H2O (group 1). Experimental animals received PCV using a high peak pressure of 25 cm H2O (groups 2 and 3). Group 3 also received TUDCA via a 100mg/kg bolus followed by 50 mg/kg/h maintenance dose. At the end of the protocol, the animals were euthanized, the left lung was removed for wet weight to dry weight (WW/DW) determinations and kidney/right lung samples were stored for TUNEL assay of apoptosis. Primary endpoints included apoptosis. How TUDCA affected WW/DW, PaO2/FIO2 ratios and the haemodynamics were secondary end-points.

Results

High levels of apoptosis were seen in the lungs of group 2 but not in the kidneys of any group. The addition of TUDCA in group 3 reduced apoptosis in the lung to a level similar to controls. There were significant differences in WW/DW and PaO2/FIO2 between the control and the 2 experimental groups but not between groups 2 and 3. TUDCA appears to reduce the rise in pulmonary vascular resistance associated with VILI and help maintain cardiac output.

Conclusion

Ventilation with high pressure induced cellular apoptosis in lung but not kidneys. TUDCA significantly reduced ventilator-induced apoptosis in the lungs. This study is the first to demonstrate that the reduced cardiac output, increased pulmonary vascular resistance and apoptosis in the lungs associated with VILI is reduced by TUDCA.

*Corresponding author
Email: brocc001@umn.edu

[1] Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, University of Minnesota, Minneapolis, USA
[2] Regions Hospital, St. Paul, USA
[3] University of Lisbon, Lisbon, Portugal
[4] Departments of Medicine and Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, USA

Introduction

Experimental data have clearly demonstrated that ventilation with an excessive tidal volume causes lung injury similar to the acute respiratory distress syndrome (ARDS)[1]. This form of lung injury termed ventilator-induced lung injury (VILI) has been well characterized in animal studies[1]. The clinical relevance of VILI gained wide acceptance following the ARDSnet trial in which avoidance of large tidal volume ventilation was associated with reduced mortality in patients with ARDS[2]. Despite intensive investigation, many questions remain about how mechanical ventilation produces lung injury at cellular and molecular level[3]. In addition, the adaptive or maladaptive role of many phenomenon associated with VILI is not well characterized, one of which is apoptosis.

Apoptosis is an energy-requiring cellular process of regulated cell death[4]. Apoptosis in the lungs can be induced by various forms of injury[5,6] including excessive mechanical stress/ strain[7]. In the latter setting, apoptosis may not be limited to the lungs and can occur in distant organs such as the kidneys, at least when excessive mechanical stress is combined with another form of lung injury[8].

A key question remains unanswered: is apoptosis in the lungs an adaptive and protective response to injury that prevents necrosis[9], or a pathway that plays a central role in the genesis of VILI that should be blocked? Although in experimental models of VILI, reduced apoptosis has generally been associated with less injury[10,11,12,13], the opposite association between apoptosis and injury has also been reported[14].

Further investigations in clinically relevant models and ultimately in human subjects are thus needed. Given that apoptosis is a possible therapeutic target for a wide variety of lung diseases[15], it would thus be very helpful to confirm or refute the experimental data regarding the role of apoptosis in lung injury in general and VILI in particular, to have a drug with an excellent safety profile for future human trials to reduce apoptosis in the lung.

Tauroursodeoxycholic acid (TUDCA) is an endogenous bile acid, known to be a potent anti-apoptotic agent that blocks the classic death (apoptotic) pathways and enhances survival pathways involving PI3K, ERK, and MAPK in the liver and in the brain in animal models of neurodegenerative diseases[16,17].

TUDCA has little known effects on the lungs apart from potential beneficial effects on pulmonary hypertension as recently reported, where it acts a chemical chaperone able to suppress endoplasmic reticulum stress signalling[18]; however, TUDCA has not be tested regarding its potential effects on lung apoptosis and VILI. In contrast

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to TUDCA, other drugs that could potentially be used to assess in future clinical trials the role of apoptosis and its blockage in the setting of VILI such as nitric oxide[13], N-acetylcysteine (NAC)[11] or captopril[19] have additional biological effects on the lungs that make teasing out the role of apoptosis and its modulation on VILI more difficult. Finally, TUDCA is used clinically for the treatment of primary biliary cirrhosis, has an excellent safety profile[20], with few significant side effects[21].

We hypothesized that TUDCA, as an anti-apoptotic agent, would block the development of apoptosis associated with VILI. If so, TUDCA could be helpful to understand the role of apoptosis in the pathogenesis of experimental VILI and to confirm or refute a role for blocking ventilator-induced apoptosis in future clinical trials, should the experimental data support a protective effects of blocking this pathway. We conducted our study in a rabbit model of VILI and assessed whether TUDCA was able to block ventilator-induced apoptosis in the lungs and in the kidneys. We have also looked at the impact of TUDCA on lung oedema, gas exchange and the haemodynamics, as knowledge of TUDCA effects on those variables if any, could help in the design of future studies.

Materials and methods

Ethics statement

All techniques and procedures were approved by the Animal Care and Use Committee of Regions Hospital, St. Paul, MN. Animals received humane care according to the criteria outlined in the ''Guide for the Care and Use of Laboratory Animals'' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23; revised 1985).

Preparation

This study was approved by the Animal Care and Use Committee of Regions Hospital, St. Paul, Minnesota. White male New Zealand rabbits (3.0-3.4 kg) were premedicated with an intramuscular injection of ketamine (35 mg/kg), xylazine (5 mg/kg) and acepromazine solution (0.75 mg/kg), and deep anaesthesia was maintained with a continuous drip of pentobarbital (3-6 mg/kg/h).

Subsequently, a customized rigid endotracheal tube was inserted and secured via a tracheostomy.

Vessels were cannulated for central venous pressure monitoring/fluid administration and continuous blood pressure monitoring/blood draws. An ABG analysis catheter (Paratrend, Diametrics, Roseville, MN) was inserted via femoral artery to continuously monitor PaCO2, PaO2, pH and temperature. Rabbits were ventilated (Babylog 8000, Drager) in the supine position using a neonatal circuit. At baseline in all groups and throughout the entire protocol in the control group only, the ventilatory settings were: Pressure Control Ventilation (PCV), peak inspiratory pressure 10cm H2O and PEEP 2cm H2O, frequency =30/min, inspiratory time 1 second (I/E ratio 1:1), and FIO2 0.60.

An open chest model was created by equating pleural pressure with ambient pressure to standardize transpulmonary pressure between animals within groups. The abdomen was incised opened and the diaphragm was penetrated from the abdominal side to create bilateral pneumothoraces - the lung collapse helped to prevent damage to the lungs during the median sternotomy that followed.

The chest wall and diaphragm were retracted to equate extrapulmonary pressure with atmospheric pressure and the chest and abdomen were kept open for the duration of the study. The pericardial space was opened, the ascending aorta was isolated and a doppler flow probe (Transonic) was placed around the aorta for continuous cardiac output measurement.

Experimental Protocol

The animals were randomized to receive either TUDCA or normal saline (placebo) prior to initiating high pressure ventilation. Controls (group 1) were ventilated for 4 h with PCV at 10/2 cmH2O, f = 30/min, I:E = 1:1 and an FIO2 of 0.60, while experimental animals (groups 2, 3) received high pressure ventilation (Ppeak = 25cm H2O or peak Ptranspulmonary = 25cm H2O) and PEEP = 3cm H2O. Group 2 received an infusion of phosphate buffered saline (PBS) as a placebo, and group 3 received TUDCA in PBS at 100 mg/kg bolus and at 50 mg/kg/h maintenance. Intracircuit CO2 was insufflated in groups 2, 3 to maintain normocapnia. In all groups, the FIO2 was maintained at 0.60 unless PaO2 fell below 40 torr, where FIO2 was then titrated up to maintain PaO2 greater than 40 torr. Preliminary studies found the following predetermined strategy was required for fluid and haemodynamic management.

Lactated Ringers was infused at 200 cc/h throughout protocol. Boluses of 25-50 ml were infused to maintain a systolic blood pressure (SBP) > 40 mmHg; and if SBP could not be maintained with fluids, a norepinephrine infusion was begun at 2 mcg/kg/min and titrated to achieve SBP > 40 mmHg. Airway pressure, flow tracings, tidal volume, ABGs, arterial and CVP pressures as well as ascending aortic flow (CO) were recorded every 30 minutes. At the completion of the protocol period, pulmonary artery (PA) and left ventricular end diastolic pressures (LVEDP) were determined via pressure sensing needle-probe.

Outcome variables for apoptosis

Following the 4 h protocol, animals were euthanized and specimens from the kidney and right lower lung that appeared injured were taken for terminal transferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL) assay of apoptosis[22].

Apoptotic cells were quantified using the TUNEL assay. Tissue sections 10 μm thick were fixed with 1% formaldehyde and processed. An Apoptag in situ apoptosis detection kit (Serologicals Corp., Norcross, GA) was used for TUNEL staining. In brief, samples were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After adding the equilibration buffer, sections were treated with terminal

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deoxynucleotidyltransferase (TdT) and digoxigenin- dNTPs for 60 min at 37ºC. Specimens were then incubated with anti-digoxigenin-peroxidase for 30 min at 37ºC, colorized with 3,3'-diaminobenzidine (DAB) substrate, and counterstained with 0.5% methyl green. Finally, slides were rinsed, dehydrated, and mounted. A negative control was prepared by omitting the TdT enzyme to control for non-specific incorporation of nucleotides or binding of enzyme-conjugate. The specimens were examined by an investigator blinded to the specimen-source using a bright-field microscope (Zeiss Axioskop; Carl Zeiss GmbH, Jena, Germany), and the data expressed as a percentage of the total area of tissue from at least three random fields that show TUNEL-positive cells.

Secondary outcome variables

The left lung was removed for wet weight to dry weight (WW/DW) assessment (post extraction lung weight/lung weight after 5 days at 130^0 and dessication. PaO2/FIO2 as an indirect marker of oedema formation was also recorded throughout the study period. Cardiac output and arterial blood pressures were measured throughout the protocol and pulmonary artery (PA) and left ventricular end diastolic pressures (LVEDP) were determined at the end of it.

Statistics

A Kruskal-Wallis test was used to examine the association between treatment group and the variables of interest. This test is a nonparametric alternative to one-way ANOVA, and can be considered an analogue of the Wilcoxon test for more than two groups. Primary outcomes tested were lung apoptosis and kidney apoptosis.

The null hypothesis under examination was each group contributed about as many high or low values as the other two; in other words, if the values are ranked from high to low, about as many high and low ranks come from each group. The secondary outcome variables wet

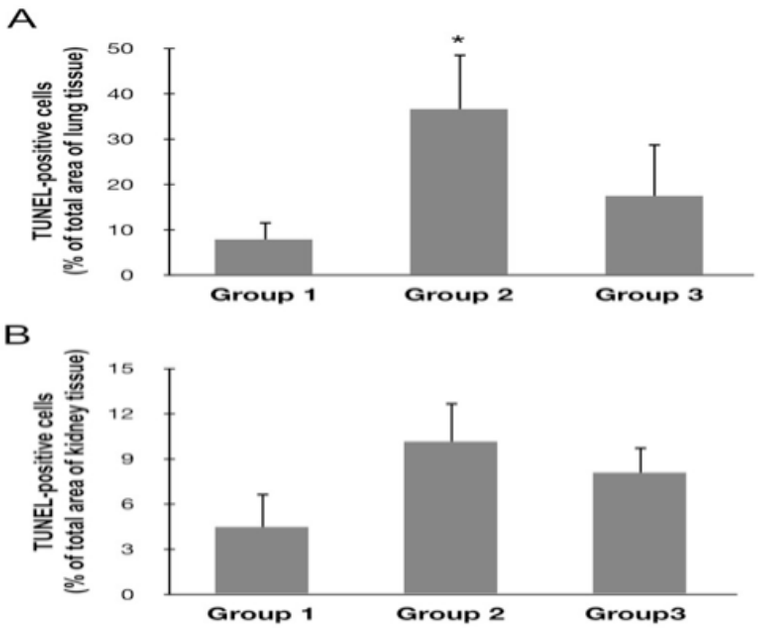

Figure 1: Levels of apoptosis in lung (A) and kidney (B) tissue following lung injury compared to controls (group 1). Apoptosis was significantly increased in lung tissue following VILI in group 2 ($p < 0.05$, T-test) while the increase was not significant in group 3. Overall, group differences in lung apoptosis were not apparent by non-parametric test ($p = .102$, Kruskall-Wallis). Apoptosis was insignificantly increased in kidney tissue following VILI ($p = .196$, Kruskal-Wallis). Results are expressed as mean +/- SEM.

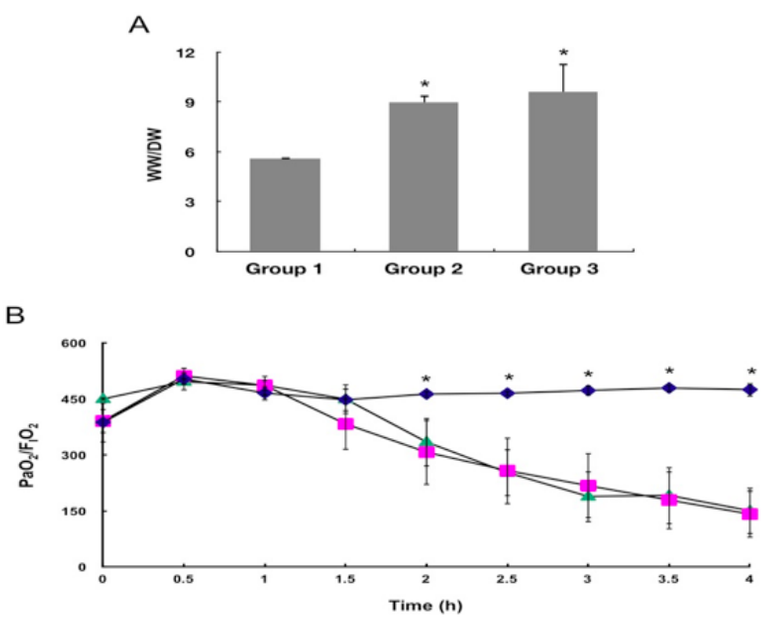

Figure 2: (A) WW/DW for control and experimental (high pressure) groups. WW/DW was significantly increased by 4 h of high pressure ventilation (groups 2, 3) ($p < 0.05$, T-test). (B) Time course of PaO2/FIO2 over the 4 h study period. The control group (group 1, diamond) receiving low pressure ventilation had no change in PaO2/FIO2 throughout the study period. Both experimental groups (group 2, square; group 3, triangle) receiving high pressure ventilation of 25/3 cmH2O experienced significant reductions in PaO2/FIO2 after 2 hours of ventilation ($p < 0.05$, T-test). There was no significant difference between the TUDCA and non-TUDCA groups in PaO2/FIO2 throughout the study.

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Table 1: Cardiopulmonary Variables.

Variable	Group 1 Control	Group 2 High Pressure	Group 3 High Pressure + TUDCA
After Randomization			
Pplat (cmH_2O)	10	25	25
PMean (cmH_2O)	5.6 +/- 0.3	10.7 +/- 0.6	10.7 +/- 0.5
pH	7.45 +/- 05	7.43 +/- .04	7.41 +/- .03
CO_2 (mmHg)	37.5 +/- 6.9	38.9 +/- 3.8	39.1 +/- 3.3
PaO_2/F_IO_2	494 +/- 43	498 +/- 51	505 +/- 54
V_T/kg (ml/Kg)	10.4 +/- 2.1	22.8 +/- 3.0*	20.9 +/- 0.9*
Compliance (ml/cmH_2O)	3.9 +/- 0.7	3.3 +/- 0.7	3.6 +/- 0.6
CO (ml/min)	259 +/- 20	249 +/- 14	239 +/- 39
Post Protocol			
PaO_2/F_IO_2	476 +/- 33	151 +/- 111*	176 +/- 145*
V_T/kg (ml/Kg)	8.3 +/- 2.1	16.4 +/- 3.3*	17.5 +/- 1.2*
Compliance (ml/cmH_2O)	3.3 +/- 0.8	2.2 +/- 0.4*	2.4 +/- 0.1
CO (ml/min)	292 +/- 103	170 +/- 97#	258 +/- 53
LVEDP (mmHg)	2.8 +/- 1.9	1.0 +/- 2.0	1.7 +/- 1.6
PA mean (mmHg)	13 +/- 4.3	14.8 +/- 1.6	13.6 +/- 1.7
PVR ($dyne.s.cm^{-5}$)	2795 +/- 650	6495 +/- 752	3690 +/- 145

weight-to-dry weight ratio was analysed using the same method. The repeatedly measured PaO2/FIO2 ratio and the hemodynamic variables were analysed using mixed-effects linear regression and ANOVA for repeated measures.

Results

A total of 4 control, 6 high pressure plus placebo and 5 animals receiving high pressure plus TUDCA completed the protocol period – 2 animals died in preparation. The groups had no significant differences in gas exchange or haemodynamic variables between groups at baseline (Table 1). During the study period, CO tended to decrease in all group but more so in group 2 (Table 1 and Figure 3). Of interest, since the lower CO in group 2 was associated with similar LVEDP and mean pulmonary arterial pressure than those measured in group 1 and 2 (Table 1), the calculated pulmonary vascular resistance (PVR) was also the highest in group 2, which could have contributed to the greater drop in CO in this group.

Lung apoptosis was non-normally distributed between groups. The p-value for this data was 0.102, meaning that there was insufficient evidence to reject the null hypothesis of no difference in apoptosis at the 5% level. However, compared to group 1, lung apoptosis was increased in group 2 ($p < 0.05$), but not in group 3, as evaluated by simple t-tests (Figure 1).

Similar to lung apoptosis, kidney apoptosis exhibited a large degree of non-normality, and so the association between it and the treatment group was also examined with a Kruskal-Wallis test. The null hypothesis of no difference in ranks was not rejected in this case, as the p-value produced by the test was 0.196 (Figure 2).

Unlike apoptosis outcomes, WW/DW data did not appear to have significant skew or non-constant variances, so

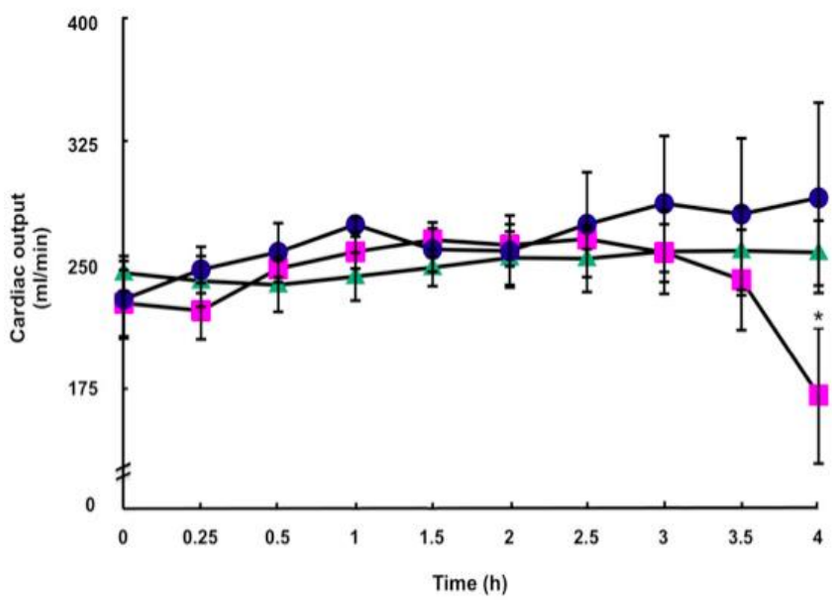

Figure 3: Time course of cardiac output (ml/min) over the 4 h study period. The group exposed to high airway pressure, which did not get TUDCA (group 2, square), experienced a significant drop in cardiac output compared to the other 2 groups (group 1, circle; group 3, triangle) ($p < 0.05$, repeated-measures Anova).

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assumptions made in one-way ANOVA may have been valid for this outcome. However, for the sake of simplicity and continuity, a Kruskal-Wallis test was also used to measure the association between WW/DW and treatment group. In this case, the resulting p-value was 0.012, which indicates that wet and dry weights are significantly different between groups, at the 95% level of confidence (Figure 3).

For PaO2/FIO2 ratio, two linear regression models were fit, because the analysis can potentially differ based upon the way PaO2/FIO2 changes with time (Table 2). The first linear model, model 1, assumes that PaO2/FIO2 decreases as a linear function of time, and tests if the regression coefficients of the best-fit lines of PaO2/FIO2 as a function of time differ between groups.

The second model, model 2, assumes that PaO2/FIO2 decreases quadratically with respect to time, and thus tests if the best fit parabolas of PaO2/FIO2 over time for each group are significantly different from one another.

Regardless of how it is assumed that PaO2/FIO2 changes with respect to time, there appears to be a significant association between the interaction of treatment group and time and PaO2/FIO2 ratio. Figure 4 shows that PaO2/FIO2 in groups 2 and 3 are not different from each other, while PaO2/FIO2 in group 1 remains stable throughout the study; which means that as intended the higher airway pressure caused lung injury and that TUDCA did appear to have altered gas exchange.

Table 2: Statistical Significance of Effects on PaO2/FIO2 from Regression Models.

Effect	Model 1 P-Value	Model 2 P-Value
Group (1 vs 2 vs 3)	0.773	0.999
Time	**<0.001**	0.640
$Time^2$	Not Tested	0.117
Group*Time	**<0.001**	Not Tested
Group*Time	Not Tested	**<0.001**

Discussion

In this short-term model of acute VILI, our main finding was that TUDCA, a drug used to treat primary biliary cirrhosis and known to block apoptosis in the brain and liver, also inhibits apoptosis in lungs exposed to mechanical stress. In contrast to what had been reported in a prior double hit model of lung injury (acid aspiration and injurious ventilation)[8], we did not observe any significant apoptosis in the kidney.

This could be due to the single hit model (mechanical stress) and the short protocol (4 versus 8 h in the prior study) used here. TUDCA was not associated with any significant difference in lung oedema and gas exchange in the high airway pressure group, which suggests that TUDCA had no significant direct impact on gas exchange and/or oedema formation. Interestingly and unexpectedly, TUDCA appears to have prevented the significant drop in cardiac output observed in the untreated high airway pressure group. Our study is the first to show that TUDCA is a drug suitable to study the role of apoptosis in clinically relevant models of VILI.

Further, the results suggest that this simple bile acid could be used for human studies if experimental studies can establish that blocking this pathway is helpful in preventing VILI under a precise timeline.

Our study controlled well for mechanical stress as the open chest model used here allows for precise matching of the trans-pulmonary pressure in the 2 treatment groups. In addition, we monitored closely cardiac output and haemodynamics as those variables have the potential to modulate VILI[23]. We are thus confident that observed apoptosis was driven by mechanical stress on the lung and was attenuated by TUDCA.

Our study has limitations, however. We used an acute short model of VILI and did not assess injury other that its consequence on oedema formation, as the former was not necessary to test our main hypothesis (TUDCA blocks ventilator-induced apoptosis). The model and study design precludes us from making inference on whether blocking apoptosis is helpful or perhaps even detrimental. Addressing this issue will require a different and more clinically relevant model (e.g., a lower degree of mechanical stress over a prolonged time period).

The realization that in addition to mechanical deformation, other factors including necrosis and apoptosis[3] were associated and/or involved in the development of VILI has opened the door to new therapeutic opportunities[15,24,25].

Whether apoptosis should be targeted and blocked remains uncertain in the setting of VILI. Indeed, although reducing apoptosis has been associated with a reduction in lung injury[10,11,12,13], different levels of VILI with similar degrees of apoptosis[26] and even an opposite association between apoptosis and injury have been reported[14]. This could be related to the fact that severe injury appears to be associated with necrosis while mild injury with frank apoptosis[8,27].

Given that apoptosis and necrosis may represent alternate pathways to cell death[9] and that apoptosis constitutes a "cleaner" cell death not associated with the inflammatory response seen in necrosis, one has to consider the possibility that blocking apoptosis may not necessarily always lead to cell survival. Through untold mechanisms, it may alternatively promote cell necrosis and inflammation and could thus enhance injury as suggested by the reported association between the degree of injury, apoptosis and necrosis[8,27].

One also needs to consider that if apoptosis of the cells which are

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instrumental in maintaining the alveolar-capillary barrier integrity (e.g. epithelial or endothelial cells) is undesirable[28], apoptosis of other cells may play an important adaptive role to reduce inflammation (e,g. roscovitine lung protective effect against VILI appears dependent on the induction of neutrophil apoptosis[29].

Later in the course of injury it could conceivably help with lung repair by preventing the epithelial to mesenchymal transition and the fibrotic remodelling that follows an acute mechanical insult to lungs[30]. In addition, the potential contribution of apoptosis to VILI is complicated by the fact that other relevant clinical factors such as sepsis[31], and oxidative stress[32] have the potential to modulate apoptosis and inflammation as well. Finally, different cell populations appear to exhibit varying trigger patterns of apoptosis[33].

The contribution of apoptosis to lung injury and the role of its blockage as a therapeutic target is thus far from established, and TUDCA could be very helpful to clarify some of those issues.
We were also interested to assess whether and how TUDCA could affect oedema formation, gas exchange and haemodynamics, as a detrimental effect on any of these variables could significantly limit the potential clinical use of TUDCA. In the current model, TUDCA appears to have blunted the drop in cardiac output and the increase in PVR associated with VILI without affecting oedema formation or gas exchange.

Pulmonary vascular resistances are known to rise in parallel with the degree of VILI[23,34], and the greater PVR observed in group 2 at the end of the study is the most likely explanation for the significant drop in CO observed in group 2 (increased right ventricle afterload).

There are a least three possible explanations for the observation that TUDCA helped preserve CO in the rabbits exposed to high tidal volumes: one could speculate that the degree of lung injury was reduced by blocking apoptosis as reported in some studies[10,11,12,13]; TUDCA altered the pulmonary vascular tone and the development of pulmonary hypertension; or had cardioprotective effects. These possibilities will require further investigation but are plausible contributory mechanisms for our observation given the recent reports showing that: (i) the increased right ventricular afterload including that associated with excessive lung inflation can result in cardiomyocyte apoptosis[35,36]; and (ii) TUDCA appears to also have a beneficial role in pulmonary hypertension[18]. Regardless of the mechanism, we found that UDCA has the potential to preserve CO and mitigate the development of high pulmonary vascular resistance as lung injury develops.

In VILI models, oedema present at the end of the study (WW/DW) is the net result of oedema formation on one end minus clearance at the other and does not appear to be affected much by a reduction in apoptosis in the lung probably because as reported by others[12], apoptosis impacts more fluid clearance than oedema formation possibly because alveolar type II cells which play an important role in oedema clearance[37], are prone to undergo apoptosis and/or necrosis when exposed to cyclic stretching[10,13], while capillary integrity and its opposite stress failure are a key driver of oedema formation[38]. The decreased cardiac output in group 2 might also have had a protective effect against effects of oedema formation[39]. Gas exchange abnormalities (PaO2/FIO2 ratio) are relatively insensitive to difference in degree of VILI and apoptosis present in rabbits[8] and paralleled the similar degree of oedema present in the 2 study groups. This suggests that TUDCA does not alter directly the degree of shunt and/or hypoxic vasoconstriction enough if at all, to significantly impact gas exchange.

Although other drugs currently used for other indications in humans have been used in experimental models to block apoptosis in VILI such N-acetylcysteine[11,40], captopril[10,41], losartan[42] or nitric oxide[13], all these drugs have, however, additional complex biological effects or like nitric oxide have the potential to enhance VILI[43].

In contrast, TUDCA is a molecule with potent anti-apoptotic, pro-survival properties and limited side effects which appears to have protective effects in other type of injuries encountered in critically ill patients including acute kidney injury[44], pancreatitis[45], toxin induced myonecrosis[46], acute brain injury[47] and others. Those effects together with the lack of known problematic side effects in the critically ill makes this drug well suited to study the contribution of apoptosis in models of critical illness and its modulation as a possible but yet uncertain therapeutic pathway that needs to be ultimately tested in humans.

Conclusion

TUDCA blocks ventilator-induced apoptosis in the lung and appears to offset at least partially the haemodynamic changes associated with VILI.

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