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The effect of diet-induced serum hypercholesterolemia on the surfactant system and the development of lung injury



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ABSTRACT

Acute respiratory distress syndrome (ARDS) is a pulmonary disorder associated with alterations to the pulmonary surfactant system. Recent studies showed that supra-physiological levels of cholesterol in surfactant contribute to impaired function. Since cholesterol is incorporated into surfactant within the alveolar type II cells which derives its cholesterol from serum, it was hypothesized that serum hypercholesterolemia would predispose the host to the development of lung injury due to alterations of cholesterol content in the surfactant system.

Wistar rats were randomized to a standard lab diet or a high cholesterol diet for 17–20 days. Animals were then exposed to one of three models of lung injury: i) acid aspiration ii) ventilation induced lung injury, and iii) surfactant depletion. Following physiological monitoring, lungs were lavaged to obtain and analyze the surfactant system.

The physiological results showed there was no effect of the high cholesterol diet on the severity of lung injury in any of the three models of injury. There was also no effect of the diet on surfactant cholesterol composition. Rats fed a high cholesterol diet had a significant impairment in surface tension reducing capabilities of isolated surfactant compared to those fed a standard diet exposed to the surfactant depletion injury. In addition, only rats that were exposed to ventilation induced lung injury had elevated levels of surfactant associated cholesterol compared to non-injured rats.

It is concluded that serum hypercholesterolemia does not predispose rats to altered surfactant cholesterol composition or to lung injury. Elevated cholesterol within surfactant may be a marker for ventilation induced lung damage.

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1. Introduction

The Acute Respiratory Distress Syndrome (ARDS) is a pulmonary disorder caused by one or multiple insults to the lung and is characterized by severe hypoxemia ($\text{PaO}_2:\text{FiO}_2 < 200$ mmHg) with radiographic bilateral lung infiltrates [1]. ARDS carries a mortality rate of approximately 40% and in lieu of effective pharmacological therapies, the mainstay of treatment remains supportive through the use of low tidal volume mechanical ventilation to maintain adequate arterial oxygenation [2,3]. The pathophysiology of ARDS is complex and involves multiple contributing factors including lung edema formation, maladaptive inflammation, and alterations to the pulmonary surfactant system [4,5].

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Even prior to the development of ARDS, it has been suggested that certain individuals may be at an increased risk for the susceptibility to disease, and the development of poorer outcomes. An example of such susceptibility is chronic alcohol intake, which may be related to an increased vulnerability to some of these pathogenic mechanisms described above [6].

Among the various pathophysiological processes occurring in ARDS, alterations and dysfunction of the pulmonary surfactant system has been consistently observed across all causes. Dysfunctional surfactant not only contributes directly to altered lung physiology but also impacts local inflammatory responses [5,7,8]. Pulmonary surfactant is a lipoprotein complex synthesized and secreted by alveolar type II cells into the alveolar space where it reduces the work of breathing by stabilizing the lung through reducing the surface tension, especially during low lung volumes [9,10]. Obtained from bronchoalveolar lavage material, two extracellular surfactant subtypes can be isolated; the surface active large aggregates (LA) and the inactive small aggregates (SA) [11].

The functional LA are composed of phospholipids (80–90%), neutral lipids (3–10%) – the majority of which is cholesterol, and surfactant associated proteins (5–10%). The precise balance of surfactant's constituent components is paramount to the material's biophysical properties and changes to the composition can impair surfactant's ability to function [10,12].

In the setting of ARDS, numerous studies have demonstrated profound alterations to the surfactant phospholipid species composition, surfactant proteins levels, and decreased levels of the LA surfactant subtype [5,7,8]. These changes, together with biophysical inhibition by serum proteins that have leaked into the lung, have traditionally thought to be the primary contributors to surfactant dysfunction [12–14]. Interestingly, recent *in vitro* biophysical studies and experiments with animal models of lung injury have demonstrated that elevated cholesterol within surfactant represents a distinct mechanism of surfactant dysfunction [15–17]. For example our group has previously shown that in a rat model of ventilator-induced lung injury (VILI) there was increased surfactant associated cholesterol in LA, contributing directly to surfactant's biophysical dysfunction. Furthermore, a reduction of cholesterol to baseline physiological levels could restore isolated surfactants innate biophysical function [17]. The mechanisms by which cholesterol content within surfactant increases in the setting of ARDS are unknown. It has been shown that under normal physiological conditions the majority of cholesterol found within surfactant is derived by uptake from the circulation [18,19], with subsequent intracellular regulation of this cholesterol to allow for the incorporation into surfactant [20,21]. It was therefore hypothesized that serum hypercholesterolemia would predispose the host to the development of severe lung injury due to changes to the cholesterol content within pulmonary surfactant. In order to test this hypothesis rats fed a standard and high cholesterol diet were subjected to three independent experimental models of lung injury.

2. Materials and methods

2.1. Animal procedures

Eighty-six 6-week old male Wistar rats (Charles River, St. Constant, Quebec, Canada) were used for these experiments. All procedures were approved by the Animal Care Committee at Western University and are in agreement with the guidelines of the Canadian Council of Animal Care. Animals were acclimatized for three days and had *ad libitum* access to water and a standard laboratory diet. Following acclimatization, animals were randomized to either a standard or a high-cholesterol diet (0.5% cholic acid and 1.25% cholesterol by mass; Harlan Teklad, Madison, WI, USA) and allowed food *ad libitum*. Rats were fed their respective

diet for 17–20 days. Animals were subsequently exposed to one of three different models of experimental lung injury: 1) acid aspiration, 2) high-tidal volume mechanical ventilation, or 3) whole-lung lavage. In order to obtain non-injured controls, 5–6 animals from each diet group were euthanized by intraperitoneal sodium pentobarbital overdose (110 mg/kg) for determination of baseline serum cholesterol levels and lung lavage analysis as described below. High density lipoprotein (HDL) and triglycerides levels in serum were determined via enzymatic colorimetric assays by London Laboratory Services Group (London On).

At the outset of each experimental model of lung injury, surgical procedures were utilized for anesthesia and instrumentation as previously reported by Maruscak et. al [22]. Briefly, animals were anesthetized via intraperitoneal injection of 75 mg/mL ketamine and 5 mg/mL xylazine in sterile saline. After appropriate sedation, animals were given a subcutaneous injection of 0.1 mg/kg buprenorphine and a 0.2 mL subcutaneous injection of a 0.5% local anesthetic sensorcaine at the ventral neck area. After exposure of the ventral neck area, the left and right jugular veins and the right carotid artery were exposed and catheterized with PE-50 tubing. The left jugular catheter was used to deliver anesthetic/analgesic (0.5–2.5 mg/100 g/h propofol) and the right jugular catheter was used to continuously deliver fluid (sterile saline with 100 IU heparin/L at 0.5–1.0 mg/100 g/h). The carotid artery catheter was used to measure blood pressure, heart rate, collect blood for blood gas measurements (ABL 500 Radiometer, Copenhagen, DK), and continuously deliver fluids (sterile saline with 100 IU heparin/L at 0.5–1.0 mg/100 g/h). All fluids and anesthetics were delivered via infusion pumps (Harvard Instruments, St. Laurent, QC, Canada). After exposure of the trachea, a 14-gauge endotracheal tube was inserted and firmly secured.

Following these surgical preparations, animals were given a 0.1 mL IV bolus of 2 mg/mL neuromuscular inhibitor pancuronium bromide and then immediately connected to a volume-cycled rodent ventilator (Harvard Instruments, St. Laurent, Quebec, Canada) via endotracheal tube. The ventilator was set to initially deliver: 8 mL/kg tidal volume (Vt), respiratory rate (RR) of 54–58 breaths per minute (bpm), 5 cmH₂O positive end-expiratory pressure (PEEP), and a fraction of inspired oxygen (FiO₂) of 1.0. Animals were ventilated for fifteen minutes prior to initial baseline blood-gas measurements. To ensure animals did not have pre-existing lung injury, the baseline (BL) inclusion criteria following this procedure was PaO₂/FiO₂ ≥ 400 mmHg.

2.2. Experimental models of lung injury

2.2.1. Acid aspiration

A model of acid-aspiration injury was utilized as described previously [23]. Following the initial surgical procedure, rats meeting the BL inclusion criteria were randomized to receive an intra-tracheal

Table 1

Baseline characteristics of non-injured rats fed a standard or high cholesterol diet for 17–20 days.

Baseline characteristics	Standard diet (n=6)	High cholesterol diet (n=5)
Weight (g)	368.58 ± 4.97	362.63 ± 4.98
Serum analysis		
Cholesterol (mmol/L)	1.50 ± 0.14	7.97 ± 1.57
High density lipoprotein (mmol/L)	1.12 ± 0.08	1.94 ± 0.20*
Triglycerides (mmol/L)	0.735 ± 0.13	2.05 ± 0.20*
Lavage analysis		
Total surfactant (mg/kg BW)	2.92 ± 0.30	2.28 ± 0.32
Large aggregates (LA) (mg/kg BW)	1.79 ± 0.20	1.26 ± 0.14
Small aggregates (SA) (mg/kg BW)	1.13 ± 0.20	1.02 ± 0.18
% LA (% of total surfactant)	61.42 ± 4.93%	56.48 ± 2.67%
Cholesterol (% of LA)	7.31 ± 0.40%	9.34 ± 1.51%
Protein content (mg/kg weight)	12.17 ± 1.62	10.83 ± 1.39

* P < 0.05 Standard versus high cholesterol diet.

instillation of either 1 M HCl (1 mL/kg) or control air bolus (1 mL/kg). Following instillation, rats were continued to be ventilated ($V_t=8$ mL/kg, RR=54–58 bpm, PEEP=5 cmH₂O and $FiO_2=1.0$) for 120 min with airway pressure, hemodynamics, and blood gas measurements monitored and recorded every 15–30 min.

2.2.2. High-tidal volume mechanical ventilation

A high-tidal volume mechanical ventilation model of VILI was used as previously described [15]. After reaching BL inclusion criteria, standard and high cholesterol diet animals were switched from baseline ventilator settings to a high-stretch ventilation strategy ($V_t=30$ mL/kg, RR=16–18 bpm, PEEP=0 cmH₂O, $FiO_2=1.0$) and mechanically ventilated for 180 min [17,22]. Airway pressure, hemodynamics, and blood gas measurements were monitored every 15–30 min during ventilation.

2.2.3. Whole lung lavage model of lung injury

A third experimental model of surfactant depletion was utilized as previously described [24]. Animals meeting the BL inclusion criteria were disconnected from the ventilator and subjected to a whole lung lavage using 10 mL saline via syringe attached to the endotracheal tube. The peak inspiratory pressure (PIP) was measured prior to and subsequently after lavage, and volume of saline administered and recovered were recorded. After withdrawal of the lavage material, the rat was re-attached to the ventilator and given a single breath hold and a blood sample was taken. The lavage protocol was repeated a minimum of four times (to a maximum of 6) until the inclusion criteria of $PaO_2 \leq 250$ mmHg and $PIP \leq 16$ cmH₂O were achieved. Rats were then ventilated for an additional 120 min ($V_t=8$ mL/kg, RR=54–58 bpm, PEEP=5 cmH₂O and $FiO_2=1.0$) with monitoring as described above.

2.3. Surfactant isolation, processing, and analysis

At the completion of ventilation, a blood sample was obtained to verify serum cholesterol levels and animals were subsequently euthanized with an IV overdose of sodium pentobarbital (110 mg/kg). Animals were exsanguinated and a midline sternectomy was performed. Lungs were lavaged previously described [17,22]. The lavage samples were then centrifuged at 150xg for 10 min and supernatant containing the total surfactant was aliquoted and stored at -20°C . An aliquot of the freshly isolated surfactant was centrifuged at 40,000xg for 15 min to separate the supernatant containing the SA sub-fraction and the pellet containing LA. The pellet was re-suspended in saline and both sub-fractions were stored at -20°C . A modified Duck-Chong phosphorous assay was performed on lipid extracts from the LA and SA surfactant samples to determine the phospholipid content [25,26]. Free cholesterol in LA samples was determined by a Free Cholesterol-E kit according to the manufacturer's instructions (Wako Chemicals, Richmond, VA, USA).

Total protein in lavage samples were measured via micro BCA protein assay according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). IL-6 levels in lavage were measured using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, Calif., USA), according to manufacturer's instructions.

2.4. Biophysical analysis

For analysis of isolated surfactant's surface tension reducing ability, LA samples were re-suspended at a phospholipid concentration of 2 mg/mL in buffer containing 2.5 mM HEPES, 1.5 mM CaCl₂, and 140 mM NaCl, pH=7.4. Samples were incubated at 37°C for at least 1 h prior to assessing the surface tension reducing properties using a constrained sessile drop surfactometer (CSD) as previously reported [27]. Briefly, the system involved a pedestal,

3 mm in diameter with a 1 mm pinhole, placed within a chamber incubated at 37°C . Approximately 9–10 μL of surfactant sample was deposited onto the top of the pre-wet pedestal and allowed to equilibrate for 3 min to allow surfactant adsorption. Following equilibration, dynamic cycling was performed through repeated

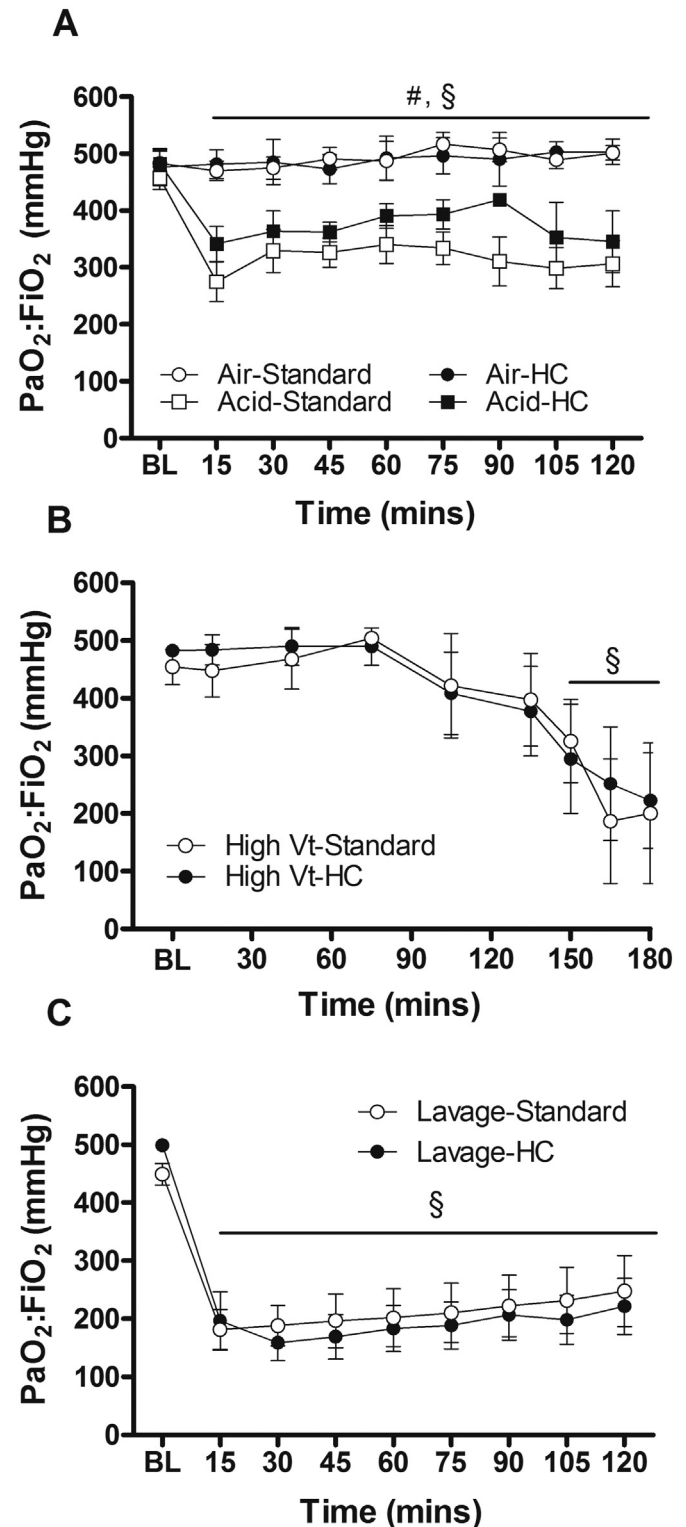


Fig. 1. Arterial partial pressure of oxygen (PaO_2) to fractional inspired oxygenation (FiO_2) ratio during the ventilation of rats fed a standard or high cholesterol diet exposed to acid/air aspiration (A) high-tidal volume mechanical ventilation (B) and whole lung lavage (C). #= $P < 0.05$ acid versus air, §= $P < 0.05$ versus time baseline within group. HC=high cholesterol, V_t – Tidal Volume.

Table 2
Physiology of rats that underwent one of three models of experimental lung injury.

Physiology parameter	Peak inspiratory pressure (cmH ₂ O)		Heart rate (beats per min)		Blood pressure (mmHg)		PaCO ₂ in blood (mmHg)	
	Standard	HC	Standard	HC	Standard	HC	Standard	HC
Diet								
Air control								
Baseline–air	11.4 ± 0.4	11.0 ± 0.2	269 ± 13	268 ± 7	74.0 ± 4.5	67.3 ± 3.8	35.9 ± 2.6	30.1 ± 2.7
120 min–air	14.2 ± 0.5 [§]	14.1 ± 0.8 [§]	282 ± 13	304 ± 7	88.7 ± 13	76.5 ± 16.9	42.2 ± 1.8	37.5 ± 3.4
Acid aspiration								
Baseline–acid	11.2 ± 0.4	11.7 ± 0.5	283 ± 8	265 ± 15	62.7 ± 4.0	67.3 ± 2.9	37.3 ± 1.7	38.3 ± 1.4
120 min–acid	17.4 ± 0.5 ^{#,§}	18.6 ± 1.6 ^{#,§}	283 ± 11	274 ± 15	63.9 ± 4.6	65.8 ± 10.5	42.8 ± 1.7	44.9 ± 2.5
High tidal volume mechanical ventilation								
Baseline–VILI	11.3 ± 0.4	10.6 ± 0.5	242 ± 11	283 ± 12	82.5 ± 18.0	79.3 ± 7.0	34.8 ± 2.2	38.0 ± 3.0
180 min–VILI	31.8 ± 2.9 [§]	31.4 ± 2.2 [§]	279 ± 9	308 ± 21	39.3 ± 7.0	27.8 ± 7.8 [§]	43.2 ± 14.9	22.9 ± 7.3
Lavage								
Baseline –lavage	11.0 ± 0.3	11.0 ± 0.4	260 ± 8.6	256 ± 5.3	73.1 ± 6.8	86.9 ± 4.9	34.7 ± 1.2	36.7 ± 2.2
120 min –lavage	19.3 ± 1.2 [§]	20.4 ± 1.6 [§]	285 ± 10.2	262 ± 8.2	58.0 ± 9.0 [§]	64.0 ± 7.2 [§]	53.7 ± 3.0 [§]	58.9 ± 2.8 [§]

PaCO₂ – partial pressure carbon dioxide in arterial blood, HC – high cholesterol.

[§] P < 0.05 versus time baseline within treatment group.

[#] P < 0.05 acid versus air instillation.

compression and expansions of the surfactant drop using a computer controlled stepping motor (model LTA-HS actuator, Newport Corporation, Irvine, CA) to control fluid drop volume. Samples underwent 20 compression/expansion cycles at a rate of 30 cycles per minute during which images of the drop were taken at a rate of 10 frames per second. Samples were consistently compressed to 72–78% of original area at each cycle. Axisymmetric Drop Shape Analysis was used to determine sample area and surface tensions of each picture taken during the dynamic compression-expansion cycles.

2.5. Statistics

Data are expressed as means ± standard error (SE). Differences between diets in experimental groups measured over time was done by a two-way repeated measures analysis of variance (ANCOVA) with Bonferroni post-hoc test. An unpaired two-way student's *T*-test was used to determine significance for individual time point comparisons between the two diets in the non-ventilated rats, the high tidal volume mechanical ventilation rats, and in the surfactant depletion rats. For comparisons between air instilled and acid instilled rats from either diet, a two-way ANOVA followed by Bonferroni post-hoc test was used. All statistics were performed using statistical analysis program GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Probability (*p*) values of less than 0.05 were considered statistically significant.

3. Results

3.1. Baseline characteristics

Baseline characteristics including weights, serum cholesterol profile, and lavage surfactant characteristics of animals fed standard and high-cholesterol diet groups are shown in Table 1. A significant increase in total serum cholesterol, triglycerides, and HDL levels were observed in animals consuming a high-cholesterol diet. At baseline, lavage and surfactant composition including cholesterol concentration within surfactant was not significantly different between the two diets groups.

3.2. Lung physiology: assessment of lung injury

The oxygenation responses of animals within the different experimental models of lung injury are depicted in Fig. 1. In the first model of injury, rats instilled with acid showed a rapid decrease in

oxygenation that was significantly different from baseline values throughout the course of ventilation (Fig. 1(A)). Furthermore, the PaO₂:FiO₂ values were significantly lower in acid instilled rats compared rats receiving air throughout the duration of mechanical ventilation. No significant differences in oxygenation in either acid or air instilled animals were observed between standard or high-cholesterol diet groups. Oxygenation response during 180 min of high tidal volume mechanical ventilation is shown in Fig. 1(B). Significant decreases in blood oxygenation values compared to baseline were observed from 150 min onwards in both diet groups; however, there was no significant difference between animals fed a standard or high-cholesterol diet. Finally, in the model of whole lung lavage, a significant decrease in oxygenation compared to baseline was observed following the lavage procedure with no significant differences observed between standard and high-cholesterol diet groups (Fig. 1(C)).

Physiological measurements of injury from baseline and the end of the ventilation period are shown in Table 2. Although several significant physiologic effects were observed in each of the experimental injury groups comparing baseline values to those at the end of ventilation, no significant differences were observed between standard and high-cholesterol diet fed animals in any of the experimental models.

3.3. Surfactant analysis

Fig. 2 shows the amount of surfactant recovered from rat lungs by lavage. There was no significant difference in the amount of functional LA (Fig. 2(A)) or inactive SA (Fig. 2(B)), recovered from rats that received acid aspiration versus the air control. Moreover, there was no difference observed between standard and high-cholesterol diet animals with respect to each surfactant subtype recovered in any of the three models of experimental lung injury. Further comparisons of the LA as a percentage of total surfactant (LA/LA+SA) recovered yielded no statistically significant differences between rats that were exposed to either diet (Fig. 2(C)).

Quantification of free cholesterol in the surfactant LA fraction is shown in Fig. 3. Within each model of experimental lung injury there was no difference in surfactant associated cholesterol between rats fed either diet. It was observed that only rats that were subjected to high-tidal volume mechanical ventilation had elevated levels of cholesterol in surfactant compared to baseline non injured animals.

Table 3 shows values of total protein and IL-6 concentrations measured in lavage fluid isolated from each of the experimental models of lung injury with comparisons made between the two

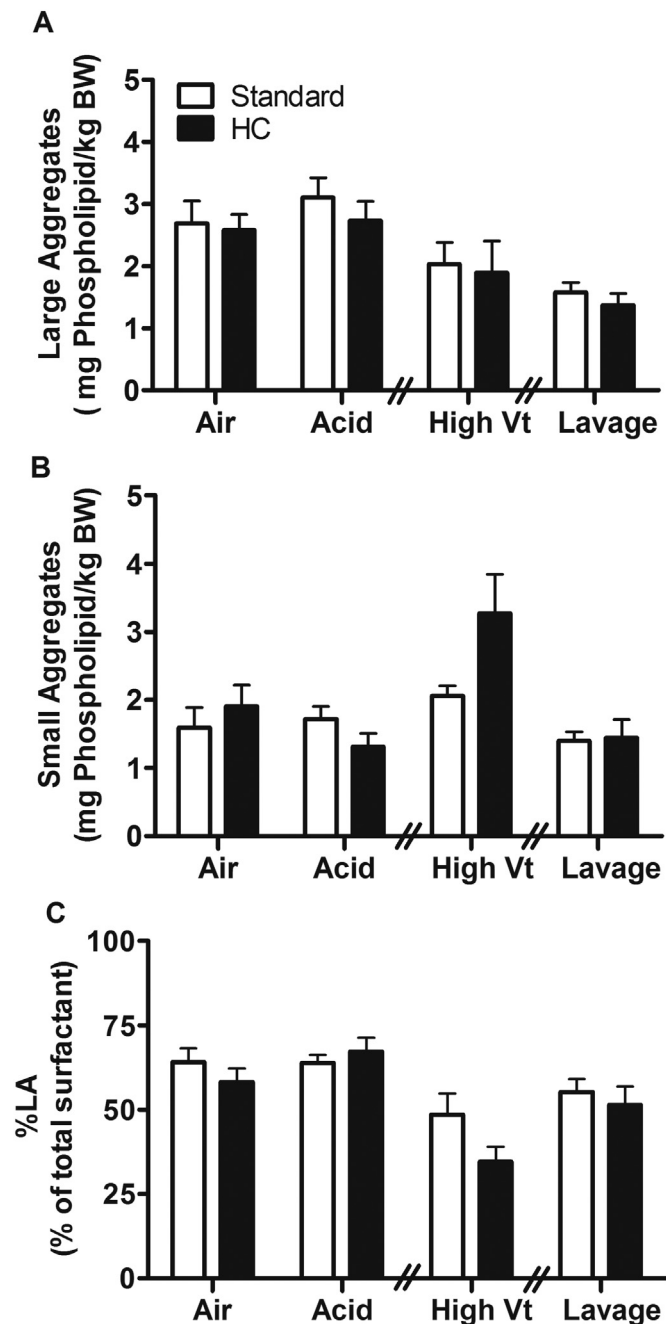


Fig. 2. Surfactant recovered via lavage from rats fed a standard or high cholesterol diet and exposed to one of three models of experimental lung injury. Surfactant recovered as large aggregates (A), small aggregates (B), and percent large aggregates of total surfactant (C). No statistical significance, $P > 0.05$. HC-high cholesterol, Vt-Tidal Volume, LA-large aggregates, BW-body weight.

diets. Total protein values were used as a marker of alveolar-capillary permeability, while lavage IL-6 levels were utilized as a marker of alveolar inflammation. In general, within each model of lung injury, no differences were observed between standard and high cholesterol diet animals with respect to lavage protein or IL-6 concentrations.

3.4. Surfactant biophysical function

Minimum surface tension achieved during compression of the sample was used as the primary indicator of biophysical function. Minimum surface tensions (mN/m) achieved at cycles 1, 5, 10, 15,

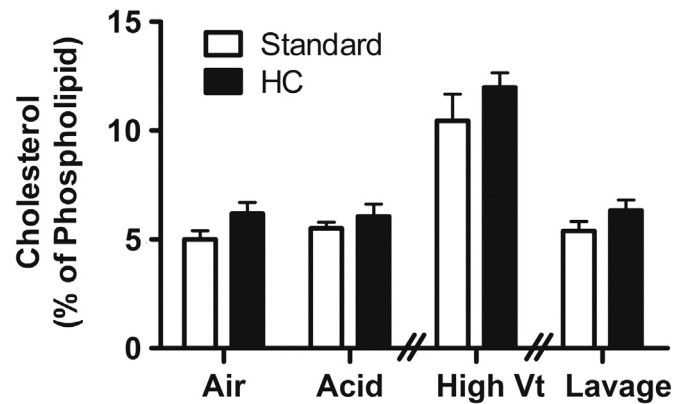


Fig. 3. Cholesterol content as a percentage of lavage LA phospholipids isolated from rats fed a standard or a high cholesterol diet exposed to one of three models of experimental lung injury. No statistical significance, $P > 0.05$. HC-High cholesterol, Vt-Tidal Volume.

20 are shown in Fig. 4. Firstly, rats that were fed a high cholesterol diet trended to have higher minimum surface tensions achieved compared to standard diet counterparts for respective acid or air treatments, although this did not reach statistical significance for any cycle (Fig. 4(A)). Secondly, there was no difference in surface tension reducing capabilities observed for rats exposed to high-tidal volume mechanical ventilation fed either diet at any cycle (Fig. 4(B)). Lastly, rats that were exposed to a whole lung lavage had significantly impaired surfactant surface tension reductions when fed a high cholesterol diet compared to rats fed a standard diet at later compression cycles (Fig. 4(C)).

4. Discussion/conclusion

Prior studies have implicated an increase in surfactant-associated cholesterol content as a key contributor to surfactant dysfunction in the context of lung injury [17,28]; however, the impact of circulating serum cholesterol levels on surfactant composition and potential downstream implications for lung injury were unknown. It was therefore hypothesized that serum hypercholesterolemia would predispose a host to a relatively more severe lung injury as a result of changes in the pulmonary surfactant system. Our primary findings demonstrate that despite achieving a four-fold increase in serum cholesterol through a high-cholesterol diet, no statistically significant differences were observed between hyper- and normocholesterolemic rats with respect to conventional markers of lung injury including changes in PIP, PaO₂, lavage protein, and lavage IL-6 concentration across three different models of ARDS. Furthermore, surfactant cholesterol levels and surfactant pool sizes were not significantly altered in rats fed a standard or high cholesterol diets. Additionally there was no functional difference in minimum surface tension achievement observed in surfactant isolated from rats fed both diets in two of the three experimental models of injury. Thus, it is concluded that diet-induced serum hypercholesterolemia does not lead to a direct increase in cholesterol content within extracellular pulmonary surfactant, and does not predispose, nor contribute to the severity of lung injury.

The first observation made in the current study was that there was no statistical difference between standard and high-cholesterol diet in our baseline control rats with respect to cholesterol content within surfactant. This finding was somewhat surprising in light of an earlier study conducted by McCrae and colleagues which examined the surfactant system from adolescent female mice fed a high-cholesterol diet [29]. These mice had a significant

Table 3

Protein and IL-6 concentration in lavage from rats exposed to three models of experimental lung injury.

	Total protein (mg/kg BW)		IL-6 (pg/mL)	
	Standard	HC	Standard	HC
Diet				
Air control	14.6 ± 1.6	16.0 ± 3.3	457.4 ± 48.6	733.2 ± 121.0
Acid aspiration	71.4 ± 4.5 [#]	102.9 ± 17.1 [#]	1605.5 ± 253.1 [#]	1470.6 ± 282.3
High Vt	150.5 ± 41.5	196.6 ± 67.4	3149.3 ± 787.4	3579.2 ± 1157
Lavage	34.5 ± 7.9	45.1 ± 8.3	2600.7 ± 283.9	2175.5 ± 244.7

HC – High Cholesterol, Vt – Tidal Volume.

[#] P < 0.001 acid versus air aspiration.

increase in both their serum cholesterol levels and the amount of cholesterol in their surfactant without marked changes to their lamellar body content or lipid droplets in the lipofibroblast [29]. Furthermore, assessment of surfactant biophysical function using a capillary surfactometer demonstrated decreased airway patency and reduced surface film activity in hypercholesterolemic mice. Potential explanations for the observed differences between our results and those of McCrae include species specific differences (rats versus mice), sex differences (male versus female), and differences in the duration of exposure of animals to a high-cholesterol diet (14–20 days versus 16 weeks). Furthermore these experimental differences have to take the complexity of cholesterol metabolism within the type II cell into consideration. In this regard, future studies examining the consequences of acute and chronic hypercholesterolemia across multiple species of both sexes, with a more detailed focus on the intracellular cholesterol metabolism, are warranted.

In order to thoroughly investigate the effects of a high cholesterol diet we utilized three distinct experimental models of lung injury [30]. Acid aspiration, reflecting gastric aspiration as it occurs in patients, provided a clinically relevant model to assess the effect of heightened serum cholesterol on lung injury. Pathophysiologically this injury develops due to a chemical burn and the associated inflammation [31], and does not typically resolve spontaneously over the two hour ventilation period. We investigated VILI using high tidal volume mechanical ventilation since our previous studies had demonstrated significantly elevated cholesterol levels within the surfactant after exposure to this type of injury [17]. This injury develops over a 3 h time period due to repeated over stretching and collapse of the lung alveoli associated with the ventilation strategy. Finally, in the whole lung lavage model of surfactant depletion, the absence of extracellular surfactant is responsible for the development of lung injury [20] and the immediate need for de novo synthesis and secretion of surfactant following surfactant depletion would have provided the most likely opportunity to observe diet induced changes in surfactant-associated cholesterol content. Despite the differences in initiating insults leading to injury, the various severity of lung injury, and the physiological changes over time, all three models provide consistent evidence in support of the conclusion that hypercholesterolemia does not impact the cholesterol content of surfactant or the severity of lung injury.

In spite of the absence of differences observed between standard and high-cholesterol fed animals with respect to lung injury outcomes, it is notable that differences were observed among the three different models of lung injury. Specifically, only the VILI model led to increase in surfactant-associated cholesterol content in the lavage as compared to the values observed in non-injured animals. Given the consistency of this observation across this and other reported studies [17,22,28], it is suggested that this cholesterol effect is related to the damaging result of injurious mechanical ventilation specifically rather than simply a generic effect of lung injury and associated inflammation. Mechanistically, VILI appears to occur due to repeated collapse and overstretching of

the alveoli and in vitro studies have demonstrated that overstretching of various lung cells, including the surfactant producing alveolar type II cells, can lead to detrimental intracellular effects [32–34]. The main focus of these studies has been on inflammation but it is possible that stretch induces effects on cholesterol and surfactant metabolism that occur within the type II cell. From a clinical perspective, the finding that elevated surfactant cholesterol is, potentially, a ventilation specific phenomenon, makes it tempting to speculate that surfactant-associated cholesterol levels may provide a valid biomarker for the damaging effects of mechanical ventilation.

Our data, demonstrating a lack of differences between our standard and high-cholesterol fed animals has one further implication related to the mechanism by which cholesterol increases within surfactant; namely it suggest that the cholesterol does not simply leak into the alveolar space from the serum. Thus surfactant cholesterol metabolism is altered during VILI specifically and, since our data excludes leak and serum cholesterol levels as having a major influence on this finding, future studies are required to investigate which mechanisms are involved. It is possible that surfactant cholesterol is increased in VILI due to reduced clearance by alveolar macrophages from the airspace. However, considering that during normal clearance only 20–30% of cholesterol is removed in 24 h [35], such a mechanism seems unlikely. A more likely scenario is an alteration in intracellular metabolism of cholesterol and surfactant within the alveolar type II cell. This intracellular metabolism of cholesterol involves a variety of differentially regulated proteins associated with import and transport of cholesterol into the lamellar body [20,21] which may be altered due to VILI. A specific focus for future studies may be proteins like as NPC1, NPC2, and ABCA1 since these proteins are responsible for cholesterol transport in the alveolar type II cell. Moreover, knockout animals of these proteins produce elevated cholesterol within intracellular surfactant and extracellular surfactant [36,37].

Of the various outcomes measured within our study, statistical significance between the two diets was only observed in measurements of surface tension reducing properties of LA-surfactant isolated from the lavage model with a similar trend being observed in the acid aspiration group. Importantly, these differences in biophysical function were evidently not of a magnitude that induced differences in physiologic outcomes. It is speculated that the high cholesterol diet may have had effects on the composition of other surfactant components, such as relative amounts of phospholipid species, which may have impacted surfactant function following injury. Previous studies, demonstrating significant changes to the pulmonary surfactant system following altered diet [38,39], are consistent with such a mechanism.

Although we were successful in the development of hypercholesterolemic rats due to a diet high in cholesterol, there is only limited information available on the lungs response to such condition. It is possible that the lungs respond to such a condition through a compensatory mechanism involving lipid storage in pulmonary lipofibroblasts [29] with potential impact on surfactant only in a more chronic condition. A limitation of our study

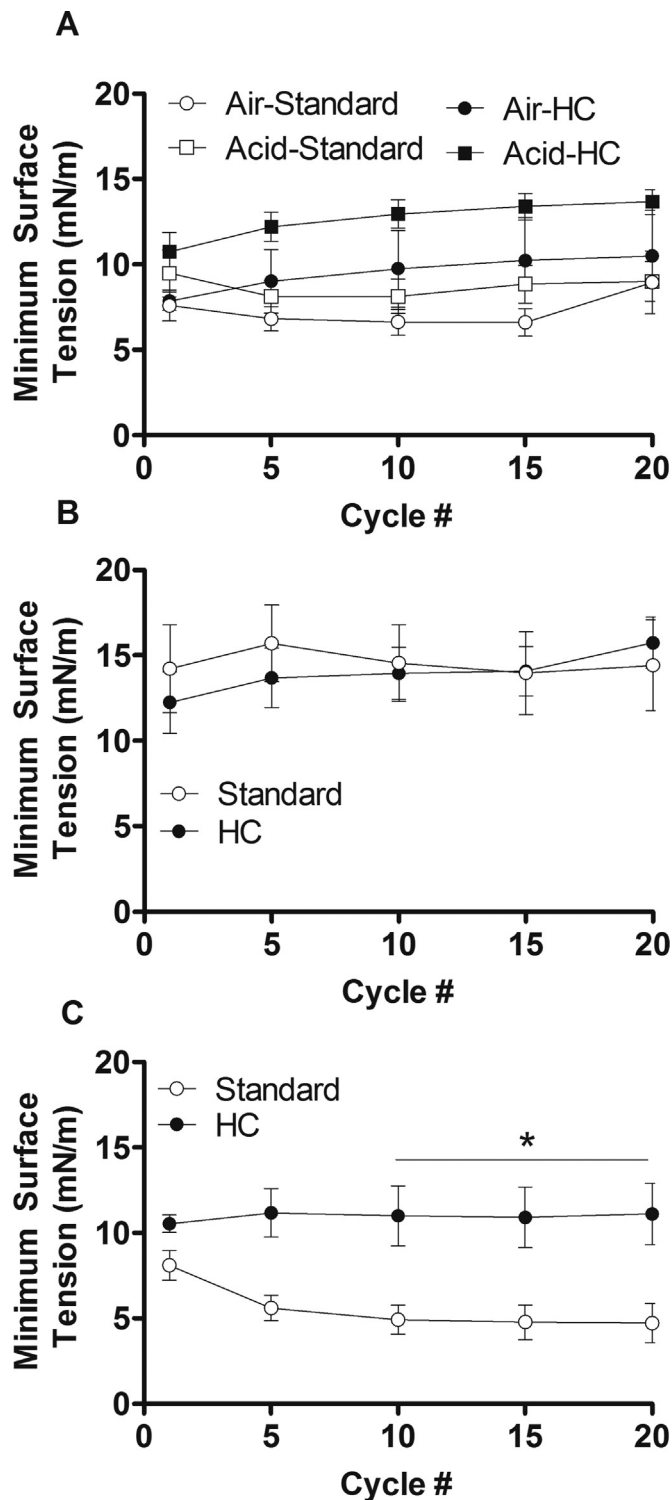


Fig. 4. Minimum surface tension (mN/m) achieved during compression cycles 1, 5, 10, 15, and 20 of surfactant isolated from rats by lavage and concentrated 2 mg/mL phospholipid. Rats were fed a standard or high cholesterol diet and were then exposed to acid/air aspiration (A), high-tidal volume mechanical ventilation (B), or whole lung lavage (C). * = $P < 0.05$ high cholesterol versus standard diet. HC – high cholesterol, Vt – Tidal volume.

therefore, was that the onset of their hypercholesterolemia was acute and does not represent the human development of the condition due to chronic ingestion of high-fat and high-cholesterol foods. It should be noted however, that this short term model was selected to avoid possible confounding variables such as obesity or

comorbid cardiovascular disease in longer term dietary models. A second limitation inherent to the majority of experimental models of lung injury is the acute, short term nature of the experimental protocols in contrast to the prolonged course of lung injury and mechanical ventilation which may occur over a time from of days to weeks [4,40]. Therefore, the impact of hypercholesterolemia during such a prolonged period of ventilation requires further investigation.

In summary, diet-induced serum hypercholesterolemia did not predispose rats to the development of more severe lung injury, nor did it affect their surfactant composition. Increases in surfactant cholesterol levels were observed only with the use of high-tidal volume mechanical ventilation leading to the suggestion that repeated collapse and over-distension of alveoli may be responsible for increased cholesterol within the surfactant. These findings provide further insight into the damaging effect of mechanical ventilation and the potential use of surfactant-associated cholesterol levels as a marker of ventilator-induced lung injury. Overall, our data argues against hypercholesterolemia as a predisposing factor affecting surfactant associated cholesterol levels during injury and the development of lung injury.

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Appendix A. Transparency document

Transparency Document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.06.009>.

References

- [1] V.M. Ranieri, G.D. Rubenfeld, B.T. Thompson, N.D. Ferguson, E. Caldwell, E. Fan, L. Camporota, A.S. Slutsky, Acute respiratory distress syndrome: the Berlin definition, *J. Am. Med. Assoc.* 307 (2012) 2526–2533.
- [2] K.J. Bosma, R. Taneja, J.F. Lewis, Pharmacotherapy for prevention and treatment of acute respiratory distress syndrome: current and experimental approaches, *Drugs* 70 (2010) 1255–1282.
- [3] J. Villar, J. Blanco, R.M. Kacmarek, Current incidence and outcome of the acute respiratory distress syndrome, *Curr. Opin. Crit. Care* 22 (2016) 1–6.
- [4] M.A. Matthay, L.B. Ware, G.A. Zimmerman, The acute respiratory distress syndrome, *J. Clin. Invest.* 122 (2012) 2731–2740.
- [5] A. Gunther, C. Ruppert, R. Schmidt, P. Markart, F. Grimminger, D. Walmrath, W. Seeger, a Günther, Surfactant alteration and replacement in acute respiratory distress syndrome, *Respir. Res.* 2 (2001) 353–364.
- [6] F. Holguin, I. Moss, L.A.S. Brown, D.M. Guidot, Chronic ethanol ingestion impairs alveolar type ii cell glutathione homeostasis and function and predisposes to endotoxin – mediated acute edematous lung injury in rats, *J. Clin. Invest.* 101 (1998) 761–768.
- [7] R.A. Veldhuizen, L.A. McCaig, T. Akino, J.F. Lewis, Pulmonary surfactant sub-fractions in patients with the acute respiratory distress syndrome, *Am. J. Respir. Crit. Care Med.* 152 (1995) 1867–1871.
- [8] U. Pison, W. Seeger, R. Buchhorn, T. Joka, M. Brand, U. Obertacke, H. Neuhof, Surfactant abnormalities in patients with respiratory failure after multiple trauma, *Am. Rev. Respir. Dis.* 140 (1989) 1033–1039.
- [9] J. Goerke, Pulmonary surfactant: functions and molecular composition, *Biochim. Biophys. Acta* 1408 (1998) 79–89.
- [10] E. Parra, J. Pérez-Gil, Composition, structure and mechanical properties define performance of pulmonary surfactant membranes and films, *Chem. Phys. Lipids* 185 (2015) 153–175.
- [11] J.F. Lewis, M. Ikegami, A.H. Jobe, Altered surfactant function and metabolism in rabbits with acute lung injury, *J. Appl. Physiol.* 69 (1990) 2303–2310.
- [12] Y.Y. Zuo, R.A.W. Veldhuizen, a W. Neumann, N.O. Petersen, F. Possmayer, Current perspectives in pulmonary surfactant – Inhibition, enhancement and

- evaluation, *Biochim. Biophys. Acta* 2008 (1778) 1947–1977.
- [13] I. Frerking, A. Gunther, W. Seeger, U. Pison, Pulmonary surfactant: functions, abnormalities and therapeutic options, *Intensive Care Med.* 27 (2001) 1699–1717.
- [14] B.A. Holm, G. Enhorning, R.H. Notter, A biophysical mechanism by which plasma proteins inhibit lung surfactant activity, *Chem. Phys. Lipids* 49 (1988) 49–55.
- [15] E. Keating, L. Rahman, J. Francis, A. Petersen, F. Possmayer, R. Veldhuizen, N. O. Petersen, Effect of cholesterol on the biophysical and physiological properties of a clinical pulmonary surfactant, *Biophys. J.* 93 (2007) 1391–1401.
- [16] L. Gunasekara, S. Schurch, W.M. Schoel, K. Nag, Z. Leonenko, M. Haufs, M. Amrein, S. Schürch, S. Schu, Pulmonary surfactant function is abolished by an elevated proportion of cholesterol, *Biochim. Biophys. Acta* 2005 (1737) 27–35.
- [17] D. Vockeroth, L. Gunasekara, M. Amrein, F. Possmayer, F. Lewis, R.A. W. Veldhuizen, J.F. Lewis, Role of cholesterol in the biophysical dysfunction of surfactant in ventilator-induced lung injury, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 298 (2010) L117–L125.
- [18] M.A. Hass, W.J. Longmore, Regulation of lung surfactant cholesterol metabolism by serum lipoproteins, *Lipids* 15 (1980) 401–406.
- [19] M.A. Hass, W.J. Longmore, Surfactant cholesterol metabolism of the isolated perfused rat lung, *Biochim. Biophys. Acta* 573 (1979) 166–174.
- [20] S. Orgeig, C.B. Daniels, The roles of cholesterol in pulmonary surfactant: insights from comparative and evolutionary studies, *Comp. Biochem. Physiol. – Mol. Integr. Physiol.* 129 (2001) 75–89.
- [21] S. Orgeig, H.A. Barr, T.E. Nicholas, Effect of hyperpnea on the cholesterol to disaturated phospholipid ratio in alveolar surfactant of rats, *Exp. Lung Res.* 21 (1995) 157–174.
- [22] A.A. Maruscak, D.W. Vockeroth, B. Girardi, T. Sheikh, F. Possmayer, J.F. Lewis, R. A.W. Veldhuizen, Alterations to surfactant precede physiological deterioration during high tidal volume ventilation, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 294 (2008) L974–L983.
- [23] A.J. Aspros, C.G. Coto, J.F. Lewis, R.A.W. Veldhuizen, High-frequency oscillation and surfactant treatment in an acid aspiration model, *Can. J. Physiol. Pharmacol.* 88 (2010) 14–20.
- [24] T.C. Bailey, K. a Da Silva, J.F. Lewis, K. Rodriguez-Capote, F. Possmayer, R.A. W. Veldhuizen, Physiological and inflammatory response to instillation of an oxidized surfactant in a rat model of surfactant deficiency, *J. Appl. Physiol.* 96 (2004) 1674–1680.
- [25] C.G. Duck-Chong, A rapid sensitive method for determining phospholipid phosphorus involving digestion with magnesium nitrate, *Lipids* 14 (1979) 492–497.
- [26] E.G. Blich, W.J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.* 37 (1959) 911–917.
- [27] L. Ge, X. Liu, R. Chen, Y. Xu, Y.Y. Zuo, R.N. Cooney, G. Wang, Differential susceptibility of transgenic mice expressing human surfactant protein B genetic variants to *Pseudomonas aeruginosa* induced pneumonia, *Biochem. Biophys. Res. Commun.* 469 (2016) 171–175.
- [28] J.Q. Hiansen, E. Keating, A. Aspros, L.-J. Yao, K.J. Bosma, C.M. Yamashita, J. F. Lewis, R.A.W. Veldhuizen, Cholesterol-mediated surfactant dysfunction is mitigated by surfactant protein A, *Biochim. Biophys. Acta* 2015 (1848) 813–820.
- [29] K.C. McCrae, B. Weltman, S. Alyward, R.A. Shaw, M.G. Sowa, H.W. Unruh, T. G. Rand, Ja Thliveris, J.E. Scott, The effect of elevated dietary cholesterol on pulmonary surfactant function in adolescent mice, *Pediatr. Pulmonol.* 43 (2008) 426–434.
- [30] G. Matute-Bello, C.W. Frevert, T.R. Martin, Animal models of acute lung injury, *Am. J. Physiol. Cell. Mol. Physiol.* 295 (2008) L379–L399.
- [31] L.K. Reiss, U. Uhlig, S. Uhlig, Models and mechanisms of acute lung injury caused by direct insults, *Eur. J. Cell Biol.* 91 (2012) 590–601.
- [32] J. Pugin, I. Dunn, P. Jolliet, D. Tassaux, J.L. Magnenat, L.P. Nicod, J.C. Chevrolat, Activation of human macrophages by mechanical ventilation in vitro, *Am. J. Physiol.* 275 (1998) L1040–L1050.
- [33] C.C. dos Santos, B. Han, C.F. Andrade, X. Bai, S. Uhlig, R. Hubmayr, M. Tsang, M. Lodyga, S. Keshavjee, A.S. Slutsky, M. Liu, DNA microarray analysis of gene expression in alveolar epithelial cells in response to TNFalpha, LPS, and cyclic stretch, *Physiol. Genom.* 19 (2004) 331–342.
- [34] N.E. Vlahakis, M. a Schroeder, A.H. Limper, R.D. Hubmayr, Stretch induces cytokine release by alveolar epithelial cells in vitro, *Am. J. Physiol.* 277 (1999) L167–L173.
- [35] A. Pettenazzo, A. Jobe, M. Ikegami, R. Abra, E. Hogue, P. Mihalko, Clearance of phosphatidylcholine and cholesterol from liposomes, liposomes loaded with metaprotefenol, and rabbit surfactant from adult rabbit lungs, *Am. Rev. Respir. Dis.* 139 (1989) 752–758.
- [36] B.R. Roszell, J.-Q. Tao, K.J. Yu, S. Huang, S.R. Bates, Characterization of the Niemann-Pick C pathway in alveolar type II cells and lamellar bodies of the lung, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 302 (2012) L919–L932.
- [37] S.R. Bates, J.-Q. Tao, H.L. Collins, O.L. Francone, G.H. Rothblat, Pulmonary abnormalities due to ABCA1 deficiency in mice, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 289 (2005) L980–L989.
- [38] R.R. Wolfe, W.Z. Martini, O. Irtun, H.K. Hawkins, R.E. Barrow, Dietary fat composition alters pulmonary function in pigs, *Nutrition* 18 (2002) 647–653.
- [39] M.J. Murray, G. Kanazi, K. Moukabary, H.D. Tazelaar, S.J. DeMichele, Effects of eicosapentaenoic and gamma-linolenic acids (dietary lipids) on pulmonary surfactant composition and function during porcine endotoxemia, *Chest* 117 (2000) 1720–1727.
- [40] K.G. Hickling, Ventilatory management of ARDS: can it affect the outcome? *Intensive Care Med.* 16 (1990) 219–226.