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Published in:
Transplantology

DOI:
[10.3390/transplantology4040018](https://doi.org/10.3390/transplantology4040018)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2023

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Moes, C. A., Gan, C. T., Venema, L. H., Hoffmann, R. F., Melgert, B. N., Kerstjens, H. A. M., Olinga, P., & Ruigrok, M. J. R. (2023). Modelling Acid-Induced Lung Damage in Precision-Cut Lung Slices: An Ex Vivo Animal Model. *Transplantology*, 4(4), 185-196. <https://doi.org/10.3390/transplantology4040018>

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Article

Modelling Acid-Induced Lung Damage in Precision-Cut Lung Slices: An Ex Vivo Animal Model

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Abstract: *Background:* Donor lungs are often discarded, with gastric aspiration accounting for ~9% of lungs unsuitable for transplantation. To increase the donor pool, it is important to understand the pathophysiology of aspiration-induced lung damage (AILD) and to assess its treatment. *Methods:* Precision-cut lung slices (PCLS) were prepared from murine lungs and exposed to acid—pH 1.5 to 5.5—for 15 min. We also investigated whether acid-exposed slices (pH 3.5) could affect unexposed slices. In addition, we investigated whether dexamethasone (0.5 or 1 µM) could mitigate and treat the damage in each group. In each experiment ($n = 3$), we analyzed cell viability (ATP/protein content) and markers of inflammation (IL-1 β , IL-6, TNF- α , TRAIL). *Results:* PCLS subjected to pH 1.5–3.5 had a significantly reduced amount of ATP, albeit no increase in inflammation markers. There was no interaction of secretions from acid-exposed slices on unexposed slices. Dexamethasone had no beneficial effects in either group. *Conclusion:* Direct exposure to acid in the PCLS leads to a decrease in cell viability. Acid-exposed slices had no effect on the cell viability of unexposed slices. Treatment with dexamethasone offered no mitigation. More studies have to be performed to elucidate the pathophysiology of AILD and the possible treatment of aspiration-induced injury.

Keywords: lung transplantation; lung injury; animal model; precision-cut lung slices; aspiration



Citation: Moes, C.A.; Gan, C.T.; Venema, L.H.; Hoffmann, R.F.; Melgert, B.N.; Kerstjens, H.A.M.; Olinga, P.; Ruigrok, M.J.R. Modelling Acid-Induced Lung Damage in Precision-Cut Lung Slices: An Ex Vivo Animal Model. *Transplantology* **2023**, *4*, 185–196. <https://doi.org/10.3390/transplantology4040018>

Academic Editor: Norihisa Shigemura

Received: 31 May 2023

Revised: 7 August 2023

Accepted: 16 October 2023

Published: 25 October 2023



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1. Introduction

Lung transplantation is a life-saving surgical procedure for patients with end-stage lung disease [1]. Currently, there is a shortage of donor lungs, leading to increased waiting times, resulting in approximately 20% of patients on the waiting list dying [2–4]. The utilization rate of donor lungs (10–15%) is lower than that of the kidneys and the liver (combined 80–85%) [2,5–8]. Multiple studies have shown that the primary reason for discarding donor lungs was due to quality issues (45–50%) [9], with gastric aspiration contributing to 8–10% of discarded cases [10,11]. Gastric aspiration is defined as the inhalation of oropharyngeal or gastric content into the larynx and lower respiratory tract [12].

The aspiration of gastric contents commonly occurs in organ donors who have suffered from a neurological insult, and can also occur secondary to resuscitation. As gastric contents enter the tracheobronchial tree, the epithelium and alveoli become exposed to strongly acidic material [13–15], as stomach acid ranges from pH 1.5 to 3.5 [16]. In turn, this might result in lung injuries like chemical pneumonitis or acute respiratory distress syndrome

(ARDS) [17,18]. An important complication of gastric aspiration is the development of secondary bacterial pneumonia [12,19,20]. Severe primary graft dysfunction (PGD) is more likely to occur if these lungs are transplanted. This is due to the added damage to the lungs caused by ischemia-reperfusion injury [15]. Therefore, aspiration-damaged lungs are usually regarded as unsuitable for transplantation.

Despite the significance of aspiration-induced lung damage (AILD), the pathophysiological mechanisms are unclear and not fully understood. Currently, the literature on the topic is limited [14,21–23]. Previous studies have suggested that corticosteroids could be beneficial in the treatment of AILD [24,25]. New insights might lead to the development of new therapeutic interventions in the future. The ability to repair such lungs, in the context of transplantation medicine, could eventually lead to an increase in the donor pool, thereby reducing premature death among patients on waiting lists.

To get insights into the pathophysiology, precision-cut lung slices (PCLS) could be used. PCLS are viable explants of animal or human tissue which can be cultured *ex vivo*. Anatomical structures and cell interactions of the lung are maintained. This technique has already proven its success in the fields of physiology, pharmacology, and toxicology [26–28]. In addition, mice have been accepted as a relatively accurate representation in the context of acute lung injury [26,29].

The aim of this study was to model aspiration-induced lung damage by exploring the effect of acid on PCLS. We hypothesized that the lower the pH, the more damage is done in terms of cell viability (denoted by the ATP/protein content) and the secretion of inflammatory cytokines (IL-1 β , IL-6, TRAIL and TNF- α). Furthermore, we also aimed to explore whether such damage would transfer to slices that were not exposed to acid, and what role corticosteroids might play in the prevention and treatment of acid-induced lung damage.

2. Materials and Methods

2.1. Animals

This is an *ex vivo* animal study. Lung tissue was obtained from male C57BL/6 mice (8–11 weeks old; 22–30 g). The animals were housed under controlled conditions, including a 12 h day/night cycle and unlimited access to water and food (Central Animal Facility, University Medical Centre Groningen, Groningen, The Netherlands). Mice were anaesthetized with isoflurane/O₂ (Nicolas Piramal, London, UK) and sacrificed by exsanguination via the inferior vena cava followed by perforation of the diaphragm. Lungs were then inflated *in situ* with 1 mL of liquefied and pre-warmed (37 °C) inflation medium containing 0.9% NaCl (Merck Millipore, Darmstadt, Germany), Ultrapure Milli-Q water and 1.5% low-gelling-temperature agarose (Sigma-Aldrich, Zwijndrecht, The Netherlands). Subsequently, the lungs were excised and immediately transferred to ice-cold University of Wisconsin preservation solution (UW) (DuPont Critical Care, Waukegan, IL, USA) where they were stored on ice until further use [30]. The animal experiments were approved by the Central Authority for Scientific Procedures on Animals (permit number: 20171290) and conducted conforming to criteria set out in national and international legislation.

2.2. Precision-Cut Lung Slices

After the lungs were obtained, tissue cores were prepared with a 5 mm biopsy punch and stored in ice-cold UW solution. Slices (wet weight of 4–5 mg; thickness of 250–350 μ m; diameter of 5 mm) were prepared using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA). Before slicing, the slicer was filled with ice-cold Krebs–Henseleit buffer which was supplemented with 10 mM HEPES (MP Biomedicals, Irvine, CA, USA), 25 mM D-Glucose (Merck Millipore) and 25 mM NaHCO₃ (Merck Millipore), and saturated with carbogen gas (95% O₂ and 5% CO₂), which was then adjusted to a pH of 7.4 [30]. After slicing, slices were stored in ice-cold UW.

2.3. Experimental Set-Up

We performed four consecutive experiments. In each experiment, three mice were used. Per mouse, on average, 30–40 lung slices were cut, so on average there were 4 slices for each experimental condition per mouse. Slices were selected based on macroscopic morphology as well as weight (4–5 mg). A schematic diagram of the experimental set-up is visualized in Figure 1.

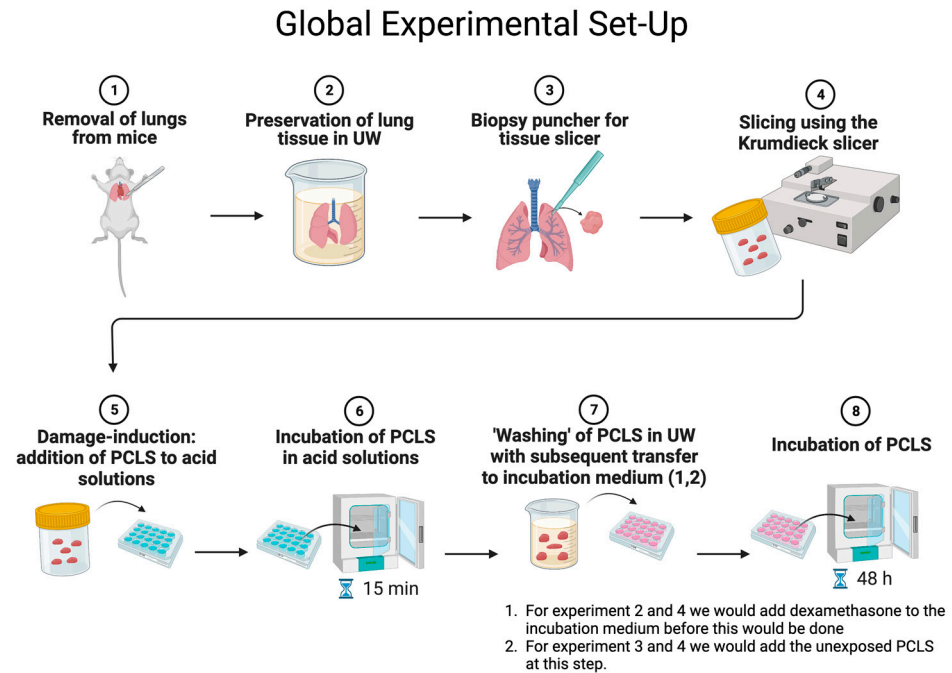


Figure 1. A schematic diagram of the experimental set-up.

In the first experiment, acid injury was induced by incubating the slices in saline solutions adjusted with HCl to a range of different pHs, from 1.5 to 5.5. Slices were transferred to a 12-well plate with pre-warmed (37 °C) adjusted saline solution containing 0.9% NaCl (Merk Millipore) and Ultrapure Mili-Q water, which was titrated with 5 M HCl to the different experimental pHs (4 slices/2 mL saline/well). The slices were then incubated for 15 min at 37 °C, 20% O₂ and 5% CO₂ whilst being gently shaken at 90 cycles/min. Then, the slices were briefly washed with University of Wisconsin (UW) solution (DuPont Critical Care) before being transferred to culture plates with fresh and pre-warmed (37 °C) culture medium (1 mL/well) consisting of Advanced DMEM/F-12 (Fisher Scientific, Amsterdam, The Netherlands) which was supplemented with 2 mM GlutaMAX, 10 mM HEPES, 100 U/mL penicillin-streptomycin, and 50 µg/mL of gentamicin. The culture plates with the slices were incubated for 48 h at 37 °C, 20% O₂ and 5% CO₂ whilst gently being shaken at 90 cycles/min. After incubation, samples were collected and stored.

In the second experiment, we wanted to determine whether dexamethasone could mitigate damage in acid-exposed slices (pH 3.5). Dexamethasone (dissolved in DMSO to either 0.5 µM or 1.0 µM) or DMSO (vehicle control) was added to the culture medium before incubation with the acid-exposed slices.

In the third experiment, we repeated the first experiment, and an additional unexposed control slice was incubated with an acid-exposed slice, i.e., the control slice was added after the acid-exposed slices had undergone damage induction, to determine whether excretions from acid-exposed slices (pH 3.5) could induce damage in control slices. The control slices were kept in ice-cold UW.

In the final experiment, we repeated the third experiment, but added dexamethasone (either 0.5 µM or 1.0 µM) or DMSO to the culture medium after acid incubation in order to

determine whether dexamethasone ameliorates damage from acid-exposed slices (pH 3.5) to control slices.

2.3.1. Evaluation of Cell Viability

The viability of PCLS was evaluated by assessing the ATP content. ATP is a measure of mitochondrial activity, which is needed for a cell to be viable [31]. Intracellular ATP was extracted from slices (2 per condition) using the ATP Bioluminescence Kit (Roche Diagnostics, Mannheim, Germany). Luminescence was measured using a luminometer (Packard LumiCount, Downers Grove, IL, USA). Calculated ATP values (pmol) were then normalized to the total amount of protein (μg) using the Pierce BCA Protein Assay Kit. This is a routine assay, as published by our group previously [27].

2.3.2. Cytokine Release

We assessed cytokines which play a crucial role in initiating and amplifying the immune response: interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), as well as TNF-related apoptosis-inducing ligand (TRAIL), which plays a role in inducing cell apoptosis. Medium samples were taken after 48 h incubation by collecting 1.5 mL culture media from 2 wells belonging to the ATP samples (750 μL /well). They were then analyzed using the Mouse IL-1 β DuoSet Enzyme-Linked Immunosorbent Assay (ELISA) Kit, Mouse IL-6 DuoSet ELISA Kit, Mouse TNF- α DuoSet ELISA kit and Mouse TRAIL DuoSet ELISA Kit (Bio-Techne, Abingdon, UK). The kits were used per the manufacturer's instructions. A BioTek Synergy HT Plate Reader (BioTek Instruments, Vermont, USA) was used to measure optical densities of each plate. Optical imperfections were corrected by subtracting readings at a wavelength of 540 nm from readings at a wavelength of 450 nm.

2.3.3. Stainings

Slices (2 per condition) were fixed in 4% formalin and stored in 70% ethanol for at least 24 h. The slices were then prepared for staining, which entailed enclosing the slices in cassettes, which could subsequently be stored in 70% ethanol. Tissues were processed and embedded in paraffin blocks. Consequently, the slices were embedded horizontally in paraffin. Sections (4 μm) were cut and mounted on glass slides. The glass slides were then left to dry in the drying stove at 37 $^{\circ}\text{C}$ for at least 24 h. Thereafter, the glass slides were ready to be stained. Haematoxylin and eosin (H&E) staining was performed to visualize the general morphology of each slice. Stained sections were scanned using a C9600 NanoZoomer (Hamamatsu Photonics, Hamamatsu, Japan).

2.4. Statistics

Statistical analyses were performed using IBM SPSS Statistics (version 27.0) and GraphPad Prism (version 9.0). Data were expressed as the mean \pm standard error of the mean (SEM). Continuous data were analyzed using one-way ANOVA followed by Dunnett's post hoc test or a two-way ANOVA followed by Bonferroni's post hoc test. Differences between groups were statistically significant when $p < 0.05$.

3. Results

3.1. Modelling an Aspiration Event by Incubating PCLS with Acidic Saline Solutions

3.1.1. General Cell Viability

Slices exposed to pH 1.5–3.5 had a significantly reduced ATP content ($p < 0.0001$, $p < 0.0001$ and $p = 0.0076$, respectively) and a significantly lower ATP/protein content ($p < 0.0001$, $p < 0.0001$ and $p = 0.0051$, respectively) compared to the control (Figure 2A–C). With pH 4.5 and 5.5, we found no significant reduction in ATP ($p = 0.1102$ and $p = 0.9989$, respectively) and ATP/protein content compared to the control ($p = 0.0724$ and $p = 0.3589$, respectively). The total protein content remained similar between all conditions.

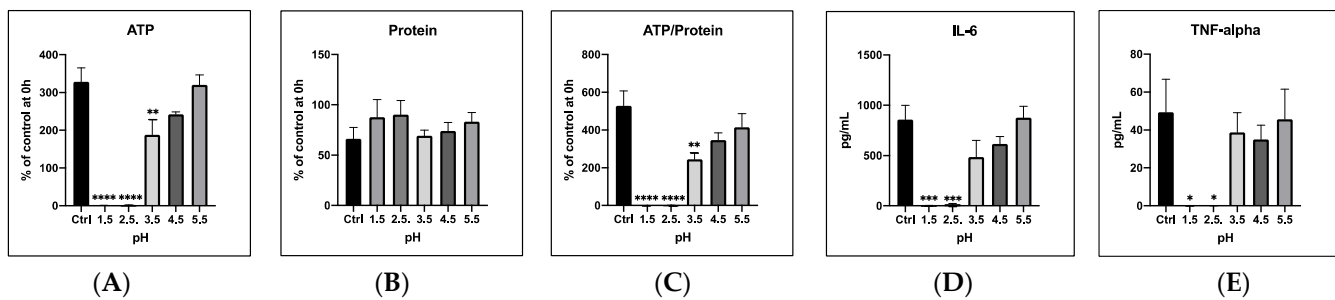


Figure 2. Effect of HCl-adjusted saline solutions of various pHs on the viability and cytokine release of slices. Slices were collected after 48 h incubation ($n = 3$). The ATP (panel A), protein (panel B) and ATP/protein (panel C) contents were measured to determine the viability of the PCLS. An ELISA was performed to assess IL-6 (panel D) and TNF- α (panel E). Values represent individual experiments performed in triplicate and are accompanied by the mean (bar) \pm standard error of the mean (error bars). * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$ and **** $p < 0.0001$.

3.1.2. Cytokine Release

The exposure of slices to saline solutions of various pHs resulted in a significantly reduced secretion of IL-6 for slices damaged by pH 1.5 and 2.5 (Figure 2D, $p = 0.0004$ for both). A similar trend is true for TNF- α (Figure 2E), where there was also a significant lack of cytokine secretion ($p = 0.0304$ for both). For IL-1 β and TRAIL, the secretion was below the biological detection limit.

3.1.3. Tissue Damage

To evaluate the extent of tissue damage in the airways and lung parenchyma, we performed H&E staining (Figure 3). Slices damaged by pH 1.5–3.5 showed a larger extent of damage than the slices damaged by pH 4.5 and 5.5. This coincided with the results of the ATP and ATP/protein content. With respect to the airways (upper panel), there was karyolysis and the nuclei faded in slices exposed to pH 1.5–3.5. For the slices exposed to pH 4.5 and 5.5, the opposite was true. Moreover, the lung parenchyma (lower panel) also showed substantial differences in tissue damage for slices treated with a lower pH compared to those treated with a high pH. For slices treated with pH 1.5–3.5, there was karyolysis (nuclei dissolution) and karyorrhexis (nuclei fragmentation), whilst the slices exposed to pH 4.5 and 5.5 showed apoptotic bodies and pyknosis (nuclei shrinkage).

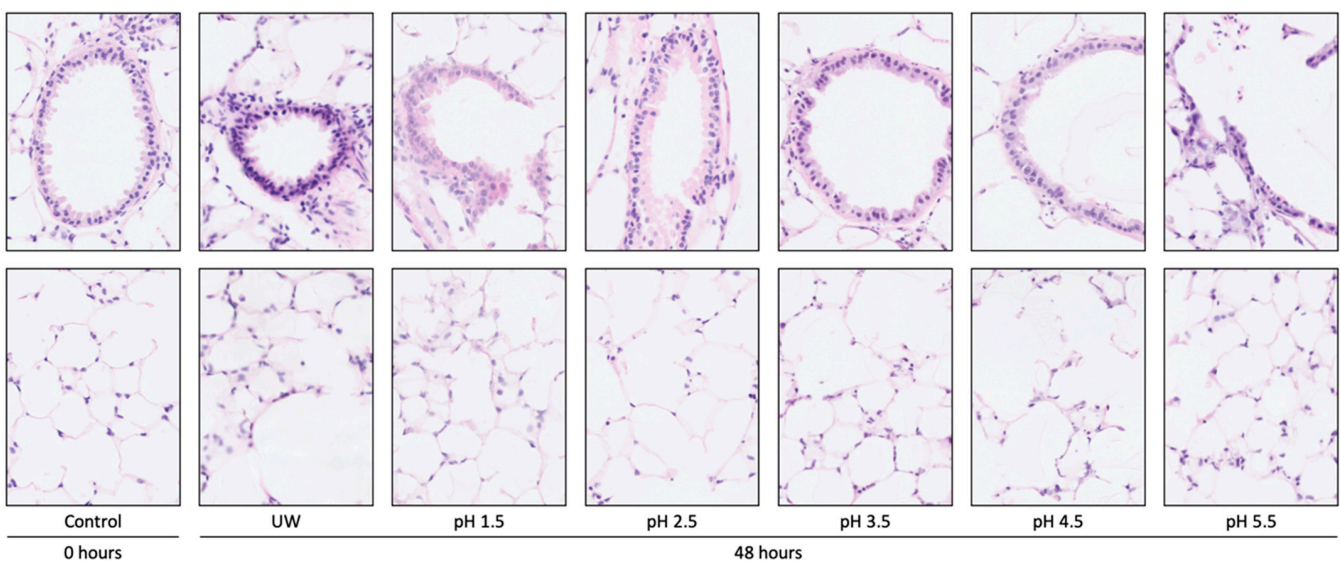


Figure 3. HE staining was carried out to assess tissue damage in the airways (upper panel) and the lung parenchyma (lower panel) (scale: 150 μ m).

3.2. Treatment of Acid-Exposed (pH 3.5) PCLS with Dexamethasone and DMSO

3.2.1. General Cell Viability

DMSO itself had no effect on ATP or ATP/protein content, as there was no significant difference compared to the control—see Figure 4A–C. Dexamethasone did not elicit a significant increase (or decrease) in ATP or ATP/protein, though at the higher dexamethasone concentration there was a negative trend.

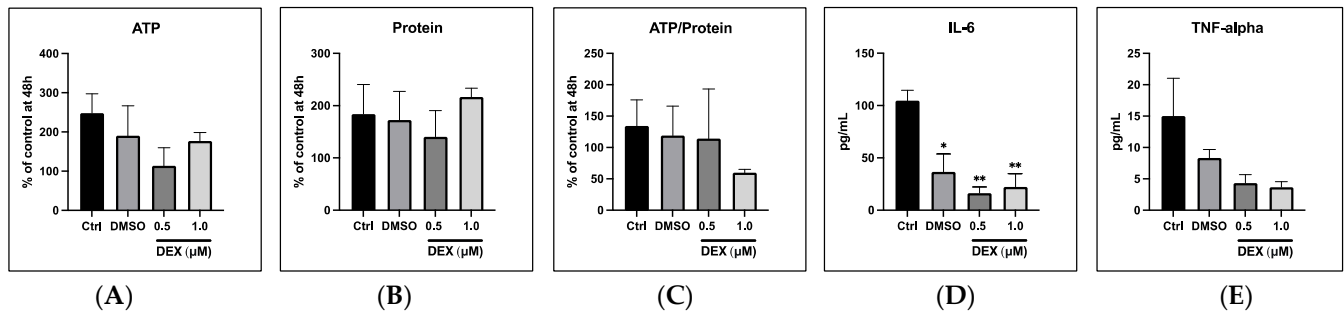


Figure 4. Effect of acidic saline solution (pH = 3.5) with subsequent incubation with various concentrations of dexamethasone/DMSO on the viability and cytokine release of slices. Slices were collected after 48 h incubation ($n = 3$). The ATP (panel A), protein (panel B) and ATP/protein (panel C) contents were measured to determine the viability of the PCLS. An ELISA was performed to assess IL-6 (panel D) and TNF- α (panel E). Values represent individual experiments performed in triplicate and are accompanied by the mean (bar) \pm standard error of the mean (error bars). * $p < 0.0332$, ** $p < 0.0021$.

3.2.2. Cytokine Release

The secretion of IL-6 was significantly reduced in all groups compared to the control (DMSO: $p = 0.0106$, 0.5 DEX: 0.0023; 1.0 DEX: $p = 0.0035$), but the differences between these three exposures were not significant, which was also compatible with an effect of DMSO—see Figure 4D. A similar pattern was visible for TNF- α , although the reduction was not significant for any group—see Figure 4E). As with experiment 1, the proteins of IL-1 β and TRAIL were below the biological detection limit.

3.3. The Effects of Acid-Exposed PCLS to Unexposed Control PCLS

3.3.1. General Cell Viability

Slices that were acid-exposed showed the same trends as in experiment 1, whilst the slices exposed to the acid-exposed slices, but unexposed to the acid itself, had no change in ATP or ATP/protein in each group—see Figure 5A–C.

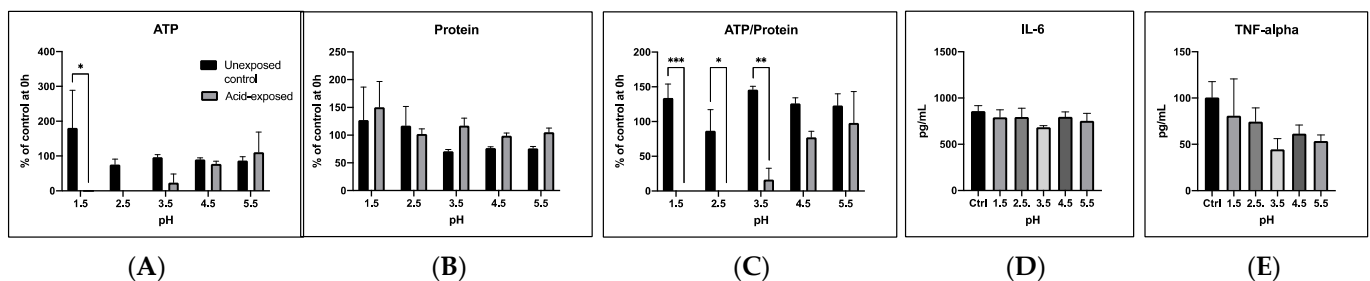


Figure 5. Effect of incubation of acid-exposed slices with control slices on the viability and cytokines of slices. Slices were collected after 48 h incubation ($n = 3$). The ATP (panel A), protein (panel B) and ATP/protein (panel C) contents were measured to determine the viability of the PCLS. An ELISA was performed to assess IL-6 (panel D) and TNF- α (panel E). Values represent individual experiments performed in triplicate and are accompanied by the mean (bar) \pm standard error of the mean (error bars). * $p < 0.0332$, ** $p < 0.0021$, and *** $p < 0.0002$.

In addition, there was a significant difference in ATP content for pH 1.5 ($p = 0.0236$) between the acid-exposed slices and the unexposed control slices. The ATP/protein content showed significant differences between the unexposed slices compared to the slices exposed to pH 1.5, 2.5 and 3.5 ($p = 0.0007$, $p = 0.0332$ and $p = 0.0010$, respectively). The protein content was similar for all the conditions and no significant differences were found.

3.3.2. Cytokine Release

The release of IL-6 was similar for each condition, whilst for TNF- α the amount of secretion decreased as the pH increased. However, for both cytokines, these results were not statistically significant—see Figure 5D,E. Again, for IL-1 β and TRAIL, the secretion was below the detection limit of the ELISA.

3.4. Dexamethasone to Prevent Damage from Acid-Exposed PCLS to Unexposed Control PCLS

3.4.1. General Cell Viability

No significant interaction was found between the acid-exposed and the unexposed slices in ATP or ATP/protein after incubation with 0.5 and 1.0 μM dexamethasone. These results are represented by Figure 6A–C.

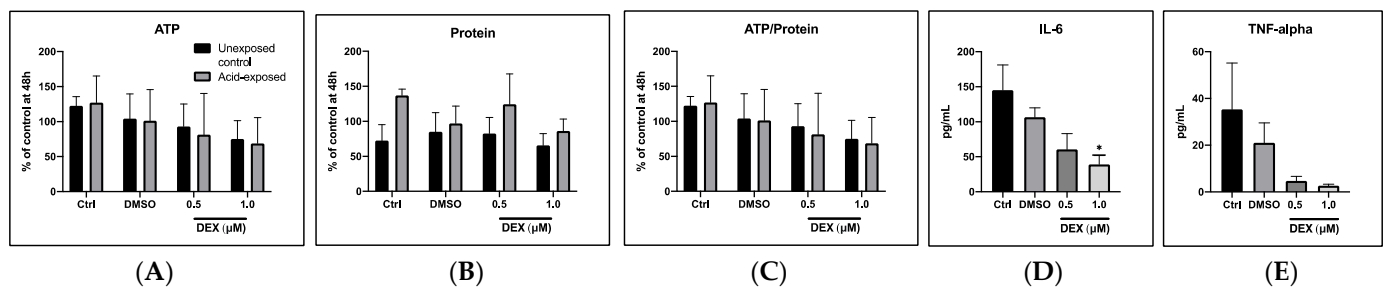


Figure 6. Effect of incubation of acid-exposed slices with control slices and dexamethasone/DMSO on the viability and cytokine release of slices. Slices were collected after 48 h incubation ($n = 3$). The ATP (panel A), protein (panel B) and ATP/protein (panel C) contents were measured to determine the viability of the PCLS. An ELISA was performed to assess IL-6 (panel D) and TNF- α (panel E). Values represent individual experiments performed in triplicate and are accompanied by the mean (bar) \pm standard error of the mean (error bars). * $p < 0.0332$.

3.4.2. Cytokine Release

We found a similar downward trend as with experiment 2. With the use of DMSO and dexamethasone, especially with a higher concentration, the secretion of IL-6 was reduced. This reduction was only significant for the groups exposed to 1.0 μM dexamethasone ($p = 0.0296$)—see Figure 6D. For TNF- α , there was also a reduction in the amount secreted, with less being secreted when there was a higher concentration of dexamethasone. However, this trend was not significant—see Figure 6E. Both IL-1 β and TRAIL secretion were below the detection limit of the ELISA.

4. Discussion

The main objective of this study was to model aspiration-induced lung damage by exploring the effect of acid on PCLS. We hypothesized that the lower the pH, the more damage done in terms of cell viability (denoted by the ATP/protein content) and the secretion of inflammatory cytokines (IL-1 β , IL-6, TRAIL and TNF- α). Furthermore, we also aimed to explore whether secretions after damage could be transferred to tissue unexposed to acid, and what role corticosteroids might play in the prevention and treatment of acid-induced lung damage.

Our main findings were that the incubation of PCLS with saline solutions adjusted to pH 1.5 and 2.5 caused more cell death compared to PCLS exposed to pH 3.5–5.5. In addition, the treatment of acid-exposed slices (pH 3.5) with dexamethasone had no effect

on cell viability. Furthermore, in our study setting, the incubation of unexposed control slices in co-culture with acid-exposed slices (pH 1.5–5.5) did not decrease cell viability in the unexposed control slices. Dexamethasone did not lead to a significant increase in the control slices' cell viability.

4.1. Incubation of PCLS with Acidic Saline Solutions Causes Cell Death

The effects of 15 min of acid exposure (pH 1.5–5.5) on PCLS caused cell death; the lower the pH the less viable the cells were. We found that the ATP and ATP/protein content in slices exposed to pH 1.5 and 2.5 was 0, indicating no mitochondrial activity. This led to the inability of cells to sustain homeostasis [31]. PCLS exposed to pH 3.5–5.5 showed no significant differences in ATP and ATP/protein compared to the control group. Upon histological analysis, there were signs of necrosis (karyolysis and karyorrhexis). Interestingly, the slices that were exposed to less acidic saline solutions (4.5 and 5.5) showed signs of apoptosis rather than necrosis. Similar observations have been made in an *in vitro* study by Chen et al., where cells treated with HCl (pH 4.0) for 15 min showed decreased cell viability with increased IL-8 and LDH release, and cell apoptosis [32]. In other studies, cell necrosis has also been demonstrated after the intratracheal administration of HCl (pH 1.0–1.2) in pigs and mice [33–35]. Teabeaut et al. found that the pH of the aspirate plays an important role in the development of aspiration pneumonitis [17]. The presence of food material in the aspirate results in even more severe lung injury [19].

Nonetheless, it is still unknown when exactly the slices die; upon direct exposure to acidic saline solutions, or during the 48 h incubation. We suggest that the cells die within 15 min of incubation, as it was observed that the slices become discolored. Moreover, there were no signs of acute inflammation found in the slices exposed to very acidic saline (pH 1.5–2.5) solutions, suggesting that cytokine-producing cells such as macrophages ceased to function, most likely due to cell death. In a rat model by Knight et al., it was demonstrated that there was indeed a downregulated macrophage response upon HCl instillation [18]. In addition, other studies have suggested that there was a delayed inflammatory response characterized by neutrophil activation and the release of pro-inflammatory mediators—which could also lead to further injury [19,21,22]. PCLS, as an *ex vivo* model, has the advantage of offering controlled experimental conditions. However, they may not fully capture the complex immune responses seen *in vivo* due to their lack of neutrophils, which presents an opportunity for the further refinement of the model.

4.2. Treatment of Acid-Exposed PCLS with Dexamethasone Has No Effect on Cell Viability

After damaging the slices with pH 3.5, we investigated whether dexamethasone could treat the damage as it is used in the clinic to treat patients with aspiration pneumonitis [23]. We hypothesized that dexamethasone would result in a higher ATP/protein content through its anti-inflammatory effects, causing less cell damage and thus less cell death.

Dexamethasone had no marked effect on improving or maintaining cell viability in PCLS damaged by pH 3.5. This is substantiated by the fact that there was no significant difference between the control group, where slices were only exposed to pH 3.5. Moreover, there was a significant decrease in IL-6 release and for TNF- α there was a lower amount detected in the medium. These results could suggest that dexamethasone might play a role in decreasing the pro-inflammatory response. However, when compared with the DMSO vehicle control we saw no significant differences. This could suggest that the observed dexamethasone effects were in fact due to DMSO, or were of similar magnitude as DMSO without synergism.

Since Mendelson's report on aspiration pneumonitis in 1946, the beneficial effects of corticosteroids have been both studied and observed in case reports and animal studies in rabbits. [24,25,36,37]. Bannister and Sattilaro found that rabbits treated with corticosteroids showed less acid-induced lung damage than rabbits without corticosteroid treatment [38]. In the literature, we found that 1.0 μ M of dexamethasone significantly reduced inflammatory cytokine production in the PCLS of rats with bleomycin-induced lung injury [39]. In

a more recent study performed by Zhao et al., stroke patients diagnosed with aspiration-related ARDS had reduced hospital mortality after low-dose and short-term treatment with corticosteroids [40]. Although these studies did not directly investigate acid damage, they involved similar inflammatory processes. Therefore, we speculate that acid damage causes an inflammatory process in the lungs and dexamethasone might still have a beneficial effect on the outcomes after aspiration by downregulating the inflammatory response. The latter might be dose–response related, or related to the timing of the measurement, neither of which we investigated in this study.

Moreover, it would be of benefit to study other corticosteroids further. The main reason we chose dexamethasone was due to the fact that it is widely available and used in current clinical practice in our center. Another corticosteroid of interest is budesonide. Budesonide is known as the cause of a beneficial reduction in inflammation, as well as apoptosis, leading to greater survival outcomes [41,42].

4.3. Incubation of Unexposed Control PCLS with Acid-Exposed PCLS Has No Effect on Control PCLS Viability

We showed that co-incubation with acid-exposed PCLS did not affect acid-unexposed control PCLS. There was a significant difference in ATP and ATP/protein content between control and acid-exposed slices. The interaction of acid-exposed slices with unexposed slices did not confer damage. The amount of ATP and the ATP/protein content was similar in each control slice regardless of which acid-exposed slice they were incubated with. Thus, the acid-exposed slices did not appear to affect the control slice. It must be noted that since the slices were not in direct contact with each other, the transfer of damage was solely dependent on the diffusion of chemokines, cytokines, and DAMPS from the acid-exposed slice into the control slice.

In a study by Kim et al., murine lung slices were spatially injured with HCl (pH 1.1) for 1 min leading to an isolated region of acid damage [43]. The acid-exposed region contained a high proportion of dead cells with a small number of live cells. In the same slice, the uninjured region contained a similar proportion of live cells as the control. This corroborates the idea that acid damage does not spread easily. The reverse reasoning could also be put forward: the unexposed control slices did not mitigate cell damage in acid-exposed slices.

The lack of interaction between acid-exposed slices and unexposed slices rendered the last experiment with dexamethasone incubation uninformative.

4.4. Advantages and Disadvantages of Murine PCLS

The main advantage of using PCLS is that they reflect the microenvironment of the respiratory tract by maintaining the 3D architecture of the lung. This provides a platform for studying organ-specific cellular mechanisms. Another advantage is that multiple experimental conditions can be tested using a single animal, thereby reducing the need for animal testing whilst keeping biological differences to a minimum [44,45].

However, there are some limitations. Firstly, we lack the full immune response, such as the influx of inflammatory cells and especially neutrophils from the blood. Secondly, we cannot evaluate how lung functioning is affected as, in the setting of lung transplantation, this is defined as the arterial oxygen tension (PaO₂) divided by the fraction of inspired oxygen (FiO₂). In our experimental setting, this cannot be measured.

4.5. Limitations of the Study

To start off, in vivo only a section of the lung would be damaged whilst, with our experiments, the whole slice is damaged. This is due to gravitational forces. Furthermore, in most aspiration events the exposure time of the tissue to aspirate might be significantly longer than 15 min, as such events can also go unnoticed, allowing the damage to continue.

To continue, this study had a relatively small sample size. Increasing the number of biological replicates would allow us to interfere with minute differences within the

population. In addition, the results were primarily based on analyses of cell viability and a select number of cytokines. In addition, we do not know when exactly the slices died, as we only took samples after 48 h. By taking samples at multiple time intervals, we could have established more precisely when cell death occurred.

Furthermore, when we examined whether the damage could be transferred to the control tissue, the control slices did not physically interact with the acid-exposed slices. Damage transfer solely depended on the secretion of DAMPs, cytokines and chemokines into the incubation medium, and on these molecules reaching the control slices. In addition, cytokines can have a rather short half-life. This needs to be considered during future experiments.

Moreover, a dose–response experiment needed to be performed to see which concentration of dexamethasone would be optimal for the slices.

However, with these pilot experiments, we were able to illustrate the value of murine PCLS as a model for lung and transplantation research. This model will be further optimized in future studies, keeping in mind the aforementioned factors.

5. Conclusions

Our study has shown that PCLS are indeed a suitable ex vivo model for lung research. Our findings provide considerable insights: a brief exposure to acidic saline solutions (pH 1.5 and 2.5) causes more cell damage than exposure to less acidic solutions (pH 4.5 and 5.5). In addition, damage does not appear to be transferable to unexposed control PCLS. Lastly, dexamethasone does not appear to play a role in the prevention or treatment of acid damage in unexposed PCLS. For slices damaged by pH 3.5 (corresponding to the pH of stomach acid), there is no significant difference with the unexposed control and this might suggest that there is still a potential for this damaged lung tissue to be rehabilitated. Insights from our research are beneficial to both lung transplant patients and researchers. If we could rehabilitate acid-exposed donor lungs, this should increase the donor pool and consequently reduce waitlist mortality. Also, outside of the context of lung transplantation, a better understanding of aspiration will be beneficial to other patient categories with increased aspiration risk.

Author Contributions: Conceptualization, C.A.M., M.J.R.R., L.H.V. and C.T.G.; methodology, M.J.R.R. and P.O.; resources, P.O.; data curation, C.A.M., M.J.R.R. and B.N.M.; writing—original draft preparation, C.A.M.; writing—review and editing, M.J.R.R., C.T.G., R.F.H., B.N.M., P.O., H.A.M.K. and L.H.V.; visualization, C.A.M.; supervision, M.J.R.R. and C.T.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Ethical approval for the animal experiments was given by the Central Authority for Scientific Procedures on Animals (permit number: AVD1050020171290, approved on 23 September 2022). Experiments were conducted conforming to criteria set out in national and international legislation.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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