Neutrophils in respiratory syncytial virus disease
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Citation for published version (APA):
Cortjens, B. (2017). Neutrophils in respiratory syncytial virus disease: Untangling the NET

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Chapter 7

Local dornase alfa Treatment reduces NETs-induced Airway Obstruction during severe RSV Infection


* Both authors contributed equally

Accepted with minor revisions in Thorax
Abstract

Respiratory syncytial virus (RSV) infection is characterized by airway obstruction with mucus plugs, containing extensive DNA networks in the form of neutrophil extracellular traps (NETs). We investigated the effect of dornase alfa (DNase) on histopathological NETs-induced airway obstruction and viral load in an age-relevant calf model of severe bovine RSV disease. As compared to the control animals, dornase alfa treatment resulted in a strong reduction of NETs-induced airway obstruction. Viral load in the lower respiratory tract was not different between the two groups. We conclude that NETs form a relevant target for treatment of airway obstruction in severe RSV disease.
Introduction

Respiratory syncytial virus (RSV) is among the most important respiratory pathogens in children worldwide.\(^1\) Small airway obstruction by dense plugs, composed of mucus and cellular debris from neutrophils and sloughed bronchial epithelial cells, is a key histopathological feature in children with severe RSV-lower respiratory tract disease (LRTD).\(^2\) Recently, we have implicated neutrophil extracellular traps (NETs) in the development of airway obstruction in severe RSV-LRTD.\(^3\)

NETs are networks of extracellular DNA covered with antimicrobial peptides and histones, which contribute to the capture and inactivation of bacteria, fungi and viruses, including RSV.\(^4\) However, there appears a delicate balance between aid and damage to the host, as accumulating evidence now suggests that NETs can cause lung injury and may contribute to airway obstruction by trapping mucus into large, viscous plugs.\(^4\) As such, pharmacological targeting of NETs, for example by degradation of the DNA backbone by DNases (dornase alfa), may be of therapeutic benefit.

In this study, we hypothesized that local dornase alfa treatment reduces NETs-induced airway obstruction in the airways during experimental severe RSV-LRTD. To test this hypothesis we used the well-established cognate host-virus model of bovine RSV (bRSV) infection in calves, which is characterized by neutrophilic inflammation with NETs-formation and histopathological evidence of (small) airway obstruction, similar to severe RSV-LRTD in children.\(^5\)\(^7\)

Methods

Twelve 4-week old, colostrum-deprived dairy calves were infected on day 0 with 3.6 log\(_{10}\) TCID\(_{50}\) of bRSV (Odijk strain, seventh in vivo passage) in a 2 mL volume. Next, the calves (6 per group) received either twice daily 5mL dornase alfa (treatment group, Pulmozyme\(^\oplus\) 1 mg/mL, Hoffmann-La Roche, Basel, Switzerland) or 0.9% NaCl (control group), starting on day 5 after viral inoculation. Further details can be found in the supplemental methods section.

Results

Overall, there was a significant reduction of NETs in the dornase alfa treated animals, as compared to the control group (Fig. 1A-D, \(P = 0.02\)). On average, the percentage of obstructed airways in the dornase alfa group was 31% lower as compared to normal saline group (41.8 ± 6.4% versus 60.7 ± 3.3% respectively, fig. 1E, \(P = 0.03\)), with a highest improvement of 51% in the left cranial lung area (36.3 ± 8.4% versus 74.5 ± 8.4%, fig. 1E, \(P = 0.03\)). There was no difference in the viral loads between the calves treated with dornase alfa versus normal saline.
Local dornase alfa degrades NETs during any of the treatment days (Fig. 2). Further results on lung inflammation and clinical responses can be found in the supplemental results section.

![Figure 1: Neutrophil extracellular traps (NETs) degradation by dornase alfa treatment.](image)

Immunohistochemistry of lung tissue sections, stained for citrullinated histone H3 (CitH3) to detect NETs formation. (A) Severe airway obstruction by NETs-rich plugs (black arrowheads and black square) in a normal saline (control) treated calf (representative image, magnification 100×), with (B) magnification (450×) of the square. (C) Open airways (asterisks) with absence of NETs-rich obstructing plugs in a dornase alfa treated calf (representative image, magnification 100×). (D) Percentages of intra-luminal airway CitH3-positive pixels for the five different lung locations (see supplemental fig. 1) in control calves (N = 6, black bars) and dornase alfa treated calves (N = 6, white bars), average (Avg) of the five locations: P = 0.04. (E) Percentages of partially and completely obstructed airways counted in whole lung tissue sections for the five different locations (see supplemental fig. 1) in control calves (N = 6, black bars) as compared to dornase alfa treated calves (N = 6, white bars), average (Avg) of the five locations: P = 0.03, location 4: P = 0.03.

![Figure 2: Lung viral load](image)

Viral load in broncho-alveolar lavage (BAL) from control (black circles) and dornase alfa treated (white circles) calves during bovine respiratory syncytial virus (bRSV) lower respiratory tract disease, as detected by RT-PCR. Data are expressed as mean ± SD of the ΔCt (total number PCR cycles minus threshold cycle). N = 6 calves per group, P = 0.1 between groups.
Discussion

Over the last years, NETs are increasingly being implicated in the pathophysiology of multiple respiratory diseases, including cystic fibrosis, COPD, acute lung injury and pulmonary infections. Specifically, the formation of NETs in the respiratory tract may contribute to airway obstruction, by deposition of large web-like networks with high DNA content, thereby increasing mucus viscosity. Previously, we have shown the formation of NETs in bronchoalveolar lavage fluid (BALF) from infants with severe RSV-LRTD, as well as in mucus plugs obstructing the airways of bRSV-infected calves. In the present study, we confirmed the hypothesis that NETs are actively involved in airway obstruction during severe bRSV-LRTD. Local dornase alfa treatment strongly reduced the amount of NETs in the airways (Fig. 1A-D), which was associated with a reduction in airway occlusion on histopathological evaluation (Fig. 1E). Interestingly, the lysis of NETs led to increased DNA content in BALF, suggesting fragmentation and ‘freeing’ the DNA from disintegrated mucus plugs (supplemental results), similar to reports in cystic fibrosis patients.

On the other hand, disruption of the protective functions of NETs might also be disadvantageous. NETs are able to capture RSV particles in vitro, and as such, a theoretical concern may be renewed release and enhanced dissemination of immobilized virions upon lysis of NETs. However, there was no increased viral dissemination (Fig. 2), nor evidence of enhanced direct viral-induced lung pathology or inflammation (supplemental results section) in our in vivo bRSV calf model. These data suggest a relatively limited role of NETs in anti-viral defence in the respiratory tract, but future studies must continue to evaluate these potential risks.

In our ‘proof of principle’ study focussed on targeting NETs (see supplemental limitations section), we observed improvement of clinical indices, most prominently a relevant reduction in the extent of hypercapnia, upon dornase alfa treatment (see supplemental results section). Although these findings need to be interpreted with care because our study was not specifically powered to detect robust clinical effects, this may form the basis of further testing of a potential clinical benefit of dornase alfa and other future interventions in NETs biology (e.g. PAD4 inhibitors). The clinical use of nebulized dornase alfa treatment during mild to moderate RSV disease in infants has previously been investigated. While one prospective randomized trial showed a strong reduction in chest X-ray abnormalities, including atelectasis, no clinical benefit, and even a statistically non-significant prolonged hospitalization, of the routine use of nebulized dornase alfa was demonstrated in two subsequent randomized placebo-controlled trials and meta-analysis. However, in a case series in mechanically ventilated children with severe RSV-LRTD, a population that suffers much more from the typical airway mucus obstruction with atelectasis, dornase alfa nebulization was of benefit with both clinical
Local dornase alfa degrades NETs and radiological improvement, which is a finding in line with observations in daily clinical practice. Future studies powered for clinical effect must determine if targeting of NETs is of actual clinical benefit during severe RSV-LRTD.

In conclusion, local dornase alfa treatment strongly reduces the amount of NETs in the respiratory tract, leading to less airway obstruction in calves with bRSV-LRTD. These results suggest that NETs contribute to the pathogenesis of airway obstruction during severe RSV-LRTD. Targeting NETs formation could prove a promising new treatment to alleviate airway obstruction in severe RSV-LRTD.

Acknowledgements

We would like to thank N. Stockhofe-Zurwieden and S. Vreman, Wageningen Bioveterinary Research, Lelystad, The Netherlands, for expert lung pathology analysis.

Statement of contribution

BC, JW, RJ, AA and RB designed the study. BC, RJ, JB and AA conceived and carried out the animals experiments. BC, RJ and JB carried out the BAL measurements and immunohistochemistry and analysed the data. BC, RJ, AA, JW and RB interpreted the results. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Funding statement

This work was supported by the Emma Children’s Hospital Support Foundation [CC200001], and the Amsterdam Economic Board [EZ1311 ALOHA RSV].
References

Supplementary methods

Animals

This study was conducted under legislation of the Dutch Central Authority for Scientific procedures on Animals and after approval of the Body of Animal Welfare of Wageningen University and Research. Twelve 4-week old, colostrum-deprived dairy calves were transported to and raised in an isolated environment after birth until the start of the study. All calves were tested free of antibodies against bRSV (Priocheck ELISA bRSV, Thermo Scientific, Rockford, IL, USA). In addition, after the study we tested a selection of broncho-alveolar lavage samples by PCR to exclude co-infection with bovine respiratory pathogenic bacteria, including: Pasteurella multocida, Histophilus somni, Mannheimia haemolytica, Arcanobacterium pyogenes and Mycoplasma species.

Virus

Intranasal inoculation was performed on day zero using an air-jet nebulizer as described before.1 The viral inoculum contained 3.6 log10 TCID50 of bRSV (Odijk strain, seventh in vivo passage) in a 2 mL volume. The Odijk isolate (subtype A) was obtained during a field outbreak in Odijk, The Netherlands2,3 and induces in our experience severe disease in calves.

Experimental design

Calves were randomized into the dornase alfa (treatment) or normal saline (control) group, with six calves per group. Starting on day 5 after viral inoculation at the onset of LRTD symptoms, thereby mimicking a therapeutic approach, the experimental group received twice daily dornase alfa (Pulmozyme® 1 mg/mL, Hoffmann-La Roche, Basel, Switzerland) and the control group received twice daily 0.9% NaCl. The treatments were performed in the morning (intratracheal) and afternoon (intranasal). During the morning session, the calves received either 5 mL dornase alfa in 15 mL 0.9%NaCl (experimental group) or 20 mL 0.9%NaCl (control group) by direct intratracheal instillation. The instillation was performed after a broncho-alveolar lavage (BAL, see below). For deposition into both lungs, the intratracheal BAL catheter was repositioned just above the carina. The required length of the catheter was determined beforehand by a bronchoscopy on study day -13. In the afternoon, 5 mL dornase alfa (experimental group) or 5 mL 0.9% NaCl (control group) was aerosolized using a portable nebulizer (AT-Neb, Atlantean Corp., Chubay City, Taiwan) and mask.

Monitoring and Sampling

The animals were followed for a maximum of 9 days after viral inoculation or until reaching the pre-defined humane endpoint (see below). Daily clinical observations were performed by a veterinarian blinded for the treatment groups, according to the scoring system outlined in...
Local dornase alfa degrades NETs

supplemental table 1. Animals were sacrificed if they reached the predetermined endpoint (single severity score of 4 for general illness or for LRTD, or four subsequent scores of 3 for general illness and LRTD).

Because of rapid health deterioration of more than half of the animals from day 7 after viral inoculation (see supplemental results), daily sampling of blood and BAL was completed for all calves of both treatment groups from day -2 up to day 7. Arterial blood pCO₂, pO₂ and haemoglobin saturation values were measured on a blood gas analyser (GEM3000, Instrumentation Laboratories, Bedford, USA) after puncture of the auricular artery. BAL was performed according to the method described by Fogarty et al. with 50 mL D-PBS (Gibco, Grand Island, NY, USA). BAL total cell counts were performed on the Coulter Counter (Beckman Coulter, Brea, USA). Cells were centrifuged to a slide in the Shandon cytospin 3 (Thermo Scientific), stained with a modified Wrights staining and a total of 400 cells were evaluated by microscope for leukocyte differential counts. DNA concentration in BAL was measured by UV-spectrophotometer (NanoDrop 2000, Thermo Scientific).

**Supplemental table 1: Clinical scoring system**

<table>
<thead>
<tr>
<th>Score</th>
<th>General Illness</th>
<th>Upper Respiratory Tract Disease</th>
<th>Lower Respiratory Tract Disease</th>
</tr>
</thead>
</table>
| 0 (absent) | Bright, alert  
Normal appetite  
Normal behaviour | No nasal discharge  
No coughing  
No ocular discharge | Normal respiration  
RR < 50 (breathings per minute) |
| 1 (mild) | Reduced responsiveness  
Decreased appetite  
Otherwise normal behaviour | Nasal or ocular discharge  
Intermittent watery – mucus  
Occasional spontaneous dry cough – induced cough (unproductive) present | Increased respiration  
RR 51-70 (breathings per minute) |
| 2 (moderate) | Dull  
Decreased appetite  
Retreats | Increased nasal or ocular discharge  
Persistent mucoid – mucopurulent discharge  
Frequent spontaneous productive cough – induced productive cough present | Abnormal respiration  
obvious abdominal breathing  
RR 71-100 (breathings per minute) |
| 3 (severe) | Lethargic  
Anorexia  
Stays down | Severe nasal or ocular discharge  
Persistent purulent – haemorrhagic discharge  
Frequent spontaneous productive cough – induced productive cough present, prolonged when induced | Dyspnoeic  
severe abdominal breathing  
e.g. stretched neck and/or accessory breathing sounds  
RR >100 (breathings per minute) |
| 4 (severe) | Soporific (non-responsive)  
Anorexia  
Unable to stand without assistance | | Asphyxia  
e.g. mouth breathing / frothing |
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**Lung tissue sample collection**

Lung tissue was stored in 10% neutral buffered formalin. Blinded histological evaluation (of the lung samples) was performed independently by two veterinary pathologist (and discordant results were re-evaluated with multiheaded microscope to reach consensus), using a pathology score from 0 (minimal) to 4 (very severe) in the following categories: bronchitis/-iolitis, peribronchitis, interstitial pneumonia and alveolitis, adapted from described before.\(^5\)

**Histopathology and immunohistochemistry**

Upon reaching the humane end point or on day 9 after virus inoculation, calves were euthanized with an overdose of pentobarbital followed by exsanguination. Lung tissue was collected from five designated sites, as indicated in supplemental fig. 1 and described before.\(^5\) To detect NETs and airway obstruction in the respiratory tract, lung sections were stained with rabbit anti-human citrullinated anti-Histone H3 (CitH3, Abcam, Cambridge, UK), anti-PAD4 (Abcam) or mouse anti-cytokeratin Pan ab1 (Thermo Scientific) as described before.\(^6\) Lung sections were dewaxed and rehydrated, followed by antigen retrieval. The sections were subsequently incubated with the primary antibody and detected with HRP conjugated anti rabbit-Ig polymer (Immunologic, Duiven, The Netherlands). HRP activity was visualized with Novared (Vector Laboratories, Peterborough, UK). All sections were mounted with glycerol and scanned using the Philips Ultra Fast Scanner 1.6RA (Philips, Eindhoven, The Netherlands). Negative controls (including both lung tissue sections from non-infected calves and from bRSV infected calves in which the specific primary antibody had been omitted) were always included. Immunostaining of whole lung tissue sections was analysed using a colour deconvolution plugin (ImageJ) followed by quantitative thresholding. CitH3-positive pixels within the airways were counted and divided by total amount of tissue pixels to calculate the percentage of positive NET staining in each specimen. To quantitate the degree of histopathological airway obstruction, the percentage of open, closed or partially closed (small) airways was evaluated by visual counting of the whole lung tissue section using the counter plug-in in ImageJ.

**Viral load measurement**

All BAL samples were tested by reverse transcription (RT)-PCR to determine the viral load as described before.\(^6\) From 200µl BAL sample, total nucleic acid was isolated using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Hoffmann-La Roche). The conserved bRSV N-gene was detected with a primer/taqman probe mix by realtime RT-PCR using the QuantiFast Multiplex Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. All assays included reverse transcription of RNA into cDNA and were run on the Applied Biosystems 7500 under optimized cycling conditions (40 cycles).
Local dornase alfa degrades NETs

Statistics

Statistical analysis was performed using Graphpad Prism 6 (Graphpad Software Inc, La Jolla, CA, USA). Results are presented as mean with SD, or as mean with individual values. Results between groups were compared using the Mann–Whitney U–test or unpaired student t-test where appropriate, based on normality. Results between groups in time with multiple measurements were compared using mixed 2-way ANOVA. P < 0.05 was considered statistically significant.

References


Supplemental results

Severe bRSV-LRTD is associated with PAD4 activation and NETs formation in the airways

First, we confirmed our previous findings of widespread presence of NETs in mucus plugs residing in the lumen of (small) airways in severe bRSV-LRTD in calves (supplemental fig. 1A). To further establish our animal model of NETosis, we additionally evaluated local PAD4 expression, demonstrating both intra- and extracellular PAD4 immunostaining within the NETs-positive mucus plugs (supplemental fig. 1B). This is consistent with the current understanding of the localisation of PAD4 during NETosis in inflammatory processes.2

Supplemental fig. 1: Intra-luminal airway neutrophil extracellular traps formation.

Representative images of immunohistochemistry stainings for citrullinated histone H3 (CitH3, A) and protein arginine deiminase type 4 (PAD4, B) in lung tissue sections of a calf with severe bovine respiratory syncytial virus (bRSV)-induced lower respiratory tract disease, magnification 200×.
Local dornase alfa degrades NETs

Local dornase alfa treatment reduces the amount of NETs in the respiratory tract

Next, we investigated whether local treatment with dornase alfa degrades the NETs in the airways during the course of severe bRSV-LRTD. To assess the spatial distribution of NETs throughout the lungs, we collected left and right cranial and caudal, and central area lung tissue samples (supplemental fig. 2). Overall, there was a significant reduction of NETs in the dornase alfa treated animals, as compared to the control group (Fig. 1A-D, Avg: \( P = 0.02 \)). In the lung areas which showed most NETs in control animals (right/left cranial and central tissue samples), NETs were almost completely destroyed by dornase alfa treatment. Importantly, the large difference in NETs content between the two treatment groups was not related to the timing of autopsy at peak disease on either day 7 or 8 (supplemental fig. 3A).

Supplemental fig. 2: Lung tissue sampling locations

Dorsal overview of the five pre-determined lung tissue locations sampled for histology, including cranial right (1), cranial left (4), caudal right (2), caudal left (3) and central areas (5).

Supplemental fig. 3: Neutrophil extracellular trap formation at different time-points.

(A) Percentages of positive citrullinated histone H3 (CitH3) airway pixels (average of the 5 locations) in control animals (black circles, \( N = 6 \)) and dornase alfa treated animals (white circles, \( N = 6 \)) on the day of sacrifice. Importantly, at peak disease (day 7-8), when reaching the humane endpoint, dornase alfa treated animals showed very limited positive staining, as compared to marked positive staining in control animals. (B) Percentage of partial or complete airway obstruction in control animals (black circles, \( N = 6 \)) and dornase alfa treated animals (white circles, \( N = 6 \)) on the day of sacrifice.
Local dornase alfa degrades NETs

**NETs lysis by dornase alfa treatment reduces histopathological airway obstruction**

NETs are web-like ‘sticky’ structures that may hold together mucus plugs within the airways. To determine if degradation of NETs indeed leads to less airway obstruction, we counted the number of open or partially/completely obstructed airways within all lung tissue sections. On average, the percentage of obstructed airways in the dornase alfa group was 31% lower as compared to normal saline group (41.8 ± 6.4% versus 60.7 ± 3.3% respectively, fig. 1E, avg: P = 0.03), with a highest improvement of 51% in the left cranial area (36.3 ± 8.4% versus 74.5 ± 8.4%, fig. 1E, P = 0.03). In line with the hypothesis that NETs are immobilized within airway mucus plugs, the DNA content in BAL at peak disease was increased by dornase alfa treatment, (supplemental fig. 4, P = 0.03), indicative of active DNA-rich mucusplug lysis.

![Graph](image)

Supplemental fig. 4: Broncho-alveolar lavage DNA content.

DNA concentration (µg/mL) in broncho-alveolar lavage (BAL) in control calves (N = 6, black circles) and dornase alfa treated calves (N = 6, white circles). Data are expressed as mean + individual values, * P = 0.03.

**Local dornase alfa treatment does not affect bRSV-induced lung inflammation**

To detect any influence of dornase alfa treatment on the lung inflammatory response to bRSV, we analysed BAL cells and the degree of cellular infiltration in airway and alveolar pathology samples. There were no significant differences in the total BAL cell counts or the number of neutrophils and macrophages between both groups (supplemental fig. 5A-C). Lymphocytes were near absent in both groups (data not shown). Although lung pathology scores revealed slightly less peri-bronchiolar and interstitial cellular infiltrates in the dornase alfa treated group, as compared to the control group (supplemental fig. 6A-B, supplemental table 2), the animals in both groups had evidence of widespread intra-alveolar changes (supplemental fig. 6C-D), indicating a strong lung inflammatory response to bRSV infection as described before.
Local dornase alfa degrades NETs

Supplemental fig. 5: The lung inflammatory response.

Total white blood cells (A), neutrophils (B) and macrophages (C) in broncho-alveolar lavage (BAL) during the course of severe bovine respiratory syncytial virus (bRSV)-induced lower respiratory tract disease in control animals (N = 6, black circles) and dornase alfa treated animals (N = 6, white circles). Differences not significant, data are shown as mean + individual data points.

Supplemental fig. 6: Lung pathology

Representative images of haematxylin and eosin stained lung tissue sections (location 1, see supplemental fig. 2) during severe bovine respiratory syncytial virus (bRSV)-induced lower respiratory tract disease. Bronchiolitis in dornase alfa treated (A) and control animals (B), magnification 40×. Alveoli showing diffuse alveolar injury consisting of cellular alveolitis, alveolar wall thickening, capillary congestion and haemorrhage with intra-luminal deposition of protein-rich oedema, present in dornase alfa treated (C) and control animals (D), magnification 100×.
Local dornase alfa degrades NETs

Supplemental table 2. Histopathology scoring of lung sections.

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Nb. Individual values are the average of the five lung locations sampled, scored by two blinded pathologists. * P = 0.01

Local dornase alfa treatment partially improves clinical airway obstruction

BRSV infection induced severe LRTD in all calves, with an onset of symptoms at day 5 after inoculation (supplemental fig. 7A). The animals treated with dornase alfa had a trend towards lower LRTD scores (P = 0.07). Interestingly, 4 out of the 6 animals in the control group had to be sacrificed prematurely due to reaching their humane end-point based on acute deterioration (to a maximal clinical disease score of 4): 3 calves on day 7 and 1 calf on day 8, as opposed to 2 out of 6 calves in the dornase alfa group based on persistent severe disease (with four subsequent clinical scores of 3, both on day 8). However, this difference between the groups did not reach statistical significance in survival analysis (supplemental fig. 7B). The remaining animals (2 in the control groups versus 4 in the dornase alfa group) were slowly recovering from their LRTD on day 9 at the study ending. The average weight gain per day was 150 gram/day higher in the dornase alfa treated group as compared to the control group (supplemental fig. 7C, 508 ± 114 gram versus 363 ± 146 gram respectively, P = 0.08).

In addition to our histopathological observation that dornase alfa treatment causes substantial decrease in NETs-induced airway obstruction, the extent of hypercapnia was significantly reduced in dornase alfa treated calves at peak disease (day 7), as compared to saline treated animals of which three calves suffered from acute ventilatory failure (51.5 ± 2.8 mmHg versus 61.7 ± 4.7 mmHg, supplemental fig. 7D, P = 0.04). Blood oxygenation
parameters were however not different between groups (supplemental fig. 7E-F), which could be explained from the widespread alveolar histopathological changes regardless of treatment.

Supplemental fig. 7: Clinical disease severity and ventilation and oxygenation parameters

(A) Mean (± SD) clinical scores of lower respiratory tract disease (LRTD) severity (supplemental table 1) in dornase alfa (white circles) and control (black circles) treated calves infected with bovine respiratory syncytial virus (bRSV). (N = 6 per group, \( P = 0.07 \)). (B) Kaplan Meier curve of survival of control calves (black circles) and dornase alfa treated calves (white circles) during the study (\( P = 0.16 \)). (C) Average (± SD) weight gain in kg per day during the study (study day -9 until the day of sacrifice) in dornase alfa treated animals (white circles) and control calves (black circles, \( P = 0.08 \)). (D) Arterial blood gas analysis for the levels of pCO\(_2\), pO\(_2\) (E) and Haemoglobin-saturation (F) during severe bovine respiratory syncytial virus (RSV)-induced lower respiratory tract disease in control calves (black circles) and dornase alfa treated calves (white circles). N = 6 calves per group. Data are represented as mean + individual data points. * \( P = 0.04 \).

References

Supplemental limitation section

This is a preliminary single-experiment animal study, and as such, it has several limitations. First, we need to interpret its findings with caution in the context of a clinical human setting. The primary focus of this study was on the pathophysiological concept of NETs, and therefore it was not powered to detect a difference in clinical disease between treatment groups. In addition, none of the current existing animal models, including our used bovine model, mimics all the features of human RSV disease.1 Finally, dornase alfa may also affect ‘normal’ extracellular DNA from necrotic cells in addition to NETs. Taken together, more studies are needed to address the role of NETs and potential clinical benefit to target them (e.g. by dornase alfa).

A second limitation of our study was the unexpected need to sacrifice this number of animals prematurely as a result of rapid LRTD deterioration at day 7-8, as observed mostly in the control group. This led to a complete dataset of blood and BAL samples up to day 7, and to different time points of histological analysis. However, as mentioned and shown in the supplemental data, the observed differences between the dornase alfa and control treated animals were not attributable to this.

In the present study we used the clinical available (and cystic fibrosis FDA-registered) human recombinant dornase alfa (Pulmozyme®) as treatment. The dose was within therapeutic range for humans, delivered directly intratracheally2 and by nebulisation. Due to ethical reasons we could not deliver both administrations intratracheally. Thus we chose to administer the second dose by nebulization to ascertain maximal treatment effect. Future research must determine if single dose or double dose via nebulization only, will result in equal NET-lysis.

References