"Nitric Oxide Releasing Hydrogel Nanoparticles Decreases Epithelial Cell Injuries Associated With Airway Reopening"

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Background

Acute respiratory distress syndrome (ARDS) is an acute inflammatory lung condition. It is characterized by disruption of gas exchange inside the alveoli, accumulation of protein edema, and an increase in lung stiffness. One major cause of ARDS is a lung infection, such as SARS-COV-2 infection. Lungs of ARDS patients need to be mechanically ventilated for airway reopening. Consequently, ventilation might damage delicate lung tissue leading to excess edema, known as ventilator-induced lung injury (VILI). Mortality of COVID-19 patients under VILI seems to be higher than non-COVID patients, necessitating effective preventative therapies. VILI occurs when small air bubbles form in the alveoli, injuring epithelial cells (EPC) due to shear stress. Nitric oxide (NO) inhalation was suggested as a therapy for ARDS, however, it was shown that it is not effective because of the extremely short half-life of NO. In this study, NO-releasing nanoparticles were produced and tested in an in vitro model, representing airways in the deep lung. Cellular injuries were quantified via fluorescent live/dead assay. Atomic force microscopy (AFM) was used to assess cell morphology. qRT-PCR was performed to assess the expression of inflammatory markers, specifically IL6 and CCL2. ELISA was performed to assess IL6 and confirm qRT-PCR results at the protein level. Finally, ROS levels were assessed in all groups. Here, we show that NO delivery via nanoparticles enhanced EPC survival and recovery, AFM measurements revealed that NO exposure affect cell morphology, while qRT-PCR demonstrated a significant downregulation in IL6 and CCL2 expression when treating the cells to NO both before and after shear exposure. ELISA results for IL6 confirmed qRT-PCR data. ROS experiment results support our findings from previous experiments. These findings demonstrate that NO-releasing nanoparticles can be used as an effective delivery approach of NO to deep lung to prevent/reduce ARDS associated inflammation and cell injuries. This information is particularly useful to treat severe ARDS due to COVID-19 infection. These nanoparticles will be useful when clinically administrated to COVID-19 patients to reduce the symptoms originating from lung distress.

Objectives

- **Establishing an in-vitro model for exposing lung cells to sheer stress.**
- **Testing the effect of sheer stress on lung cells.**
- **Testing NO releasing particles on reducing cell injury that is induced by sheer stress as a mechanism of airway reopening via analyzing cell stiffness ,inflammatory markers and ROS.**

Methedology

We have developed an in-vitro model of airway reopening to expose lung EPCs to injurious stresses associated with mechanical ventilation using parallel-plate flow chamber.

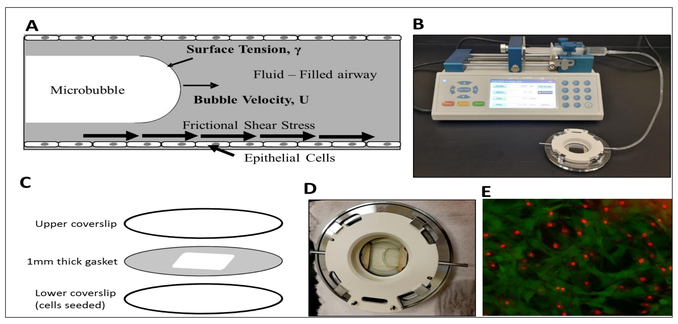

Figure 1: Experimental setup. A. We expose epithelial cells to frictional shear stress relevant to airway reopening in our setup. B. A single bubble is propagated over cell monolayer by filling the chamber and then retracting fluid over the cells. C. Parallel plate flow chamber is exposed of an upper cover slip, cell seeded lower coverslip, and membrane sandwiched between two coverslips. D. A propogating bubble is seen. E. Fluorescent live/dead stain method used to assess viability. Here, green are calcein stained live cells, and red are ethidium-stained dead cells.

Cell culture:
L2 (ATCC® CCL149™) lung epithelial cells were grown on circular coverslips until confluence

Study group categorization:
- Control 1: No chemicals or stress (-ve control)
- Control 2: Cells subjected to stress by the flow chamber only (Bubble control)
- Control 3: cells subjected to nitric oxide (NO) nanoparticles only (NO control)
- Experimental group 1: Cells subjected to stress by the flow chamber and then treated with NO nanoparticles.
- Experimental group 2: Cells treated with NO nanoparticles and then subjected to stress.

NO Solution preparation:
(NO-8 particles were used).
A concentration of 5mg/ml of NO beads was prepared and 2 ml of solution was added to cell cultures and incubated for one hour for maximum NO release.

- Actin polymerization/depolymerization assessment
- AFM for stiffness measurement
- RT-PCR for inflammatory markers
- Rat IL-6 immunoassay (ELISA) quantification
- ROS measurement

Results & discussion

Cell viability data

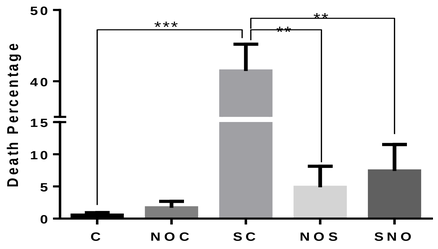

Figure 2: Cells survival rate. Lung epithelial cells were treated with media as control (C), nitric oxide (NOC), exposed to shear (SC), nitric oxide then shear (NOS) or shear then nitric oxide (SNO).There was a significant decrease in the death percentage with the treatment with nitric oxide pre and post sheer exposure.

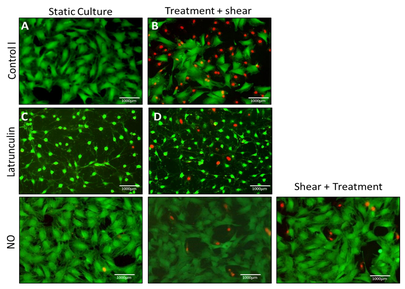

Figure 3: Live/dead staining results for the nanoparticles tested on L2 (ATCC® CCL-149™) lung epithelial cells.

Cell morphology & stiffness

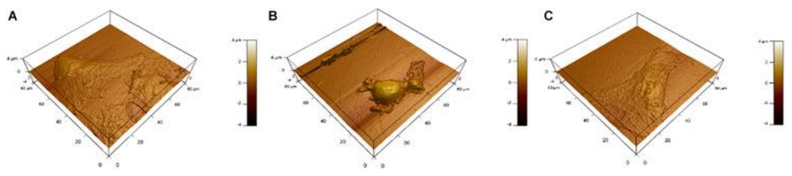

Figure 4: Topography via AFM. (A) Control, (B) latrunculin, and (C) NO treated.

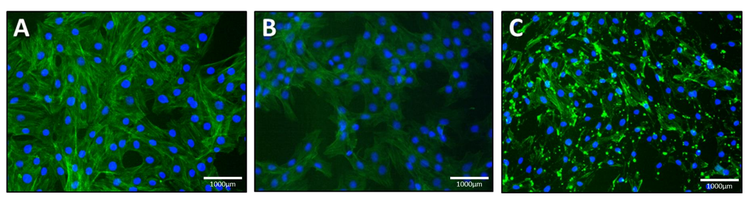

Figure 5: Lung epithelial cells stained with cytoskeleton staining. **A**: control, **B**: NO treated cells and **C:** latrunculin treated cells. Green represents the actin stained cells whereas the blue is DAPI labeled nuclei.

PCR & Gene expression

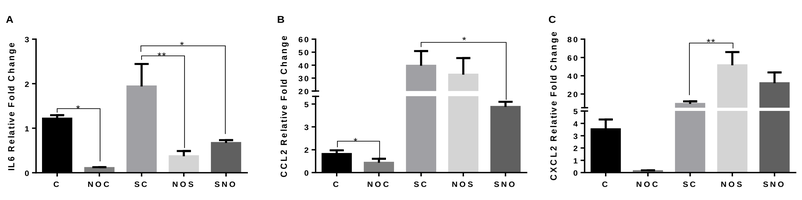

Figure 6:Relative gene expression of inflammatory markers. Lung epithelial cells gene expression of A. Interleukin 6 (IL6), B. chemokine (C-C motif) ligand 2 (CCL2), and C. Chemokine (C-X-C motif) ligand 2 (CXCL2). Cells were cultured until 80% confluence. There was a significant decrease in IL6 and CCL2 when cells were treated NO pre and post sheer and a significant increase in CXCL2 between SC and NOS

Results & discussion

IL-6 ELISA results

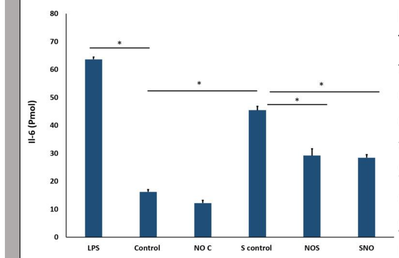

Figure 7: ELISA result for IL-6. The signal is reported as the total detected signal in (Pmol) divided by the cell count for each condition. NOC Analysis was by one-way-ANOVA with Sidak *post hoc* test for multiple comparisons between the group. Data is presented as mean ± SEM. *N* = 4 for all groups. *Significant.

ROS Measurement

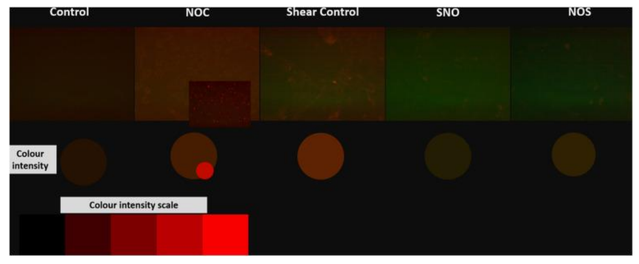

Figure 8: Microscopic images for different groups stained with orange cell ROX stain. The color intensity was compared to the red color intensity saturation scale.

Conclusion

Delivering NO particles with the aid of biotechnology might be an effective treatment for ARDS, considering the positive effects of increasing softness and reducing inflammatory markers. NO must be delivered at low concentrations with a steady rate, otherwise, a toxic effect might be induced.

References

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