Local Effects of Two Intravenous Formulations of Pulmonary Vasodilators on Airway Epithelium

Bradley A. Kuch MHA, RRT-NPS, FAARC[1]; Rosalie Linssen MD[2]; Hiroki Yoshikawa MD[2]; Craig D. Smallwood PhD, RRT[3,4]; Michael D. Davis RRT, PhD, FAARC[3, 5]

[1] Department of Pediatric Critical Care Medicine and Respiratory Care Services, UPMC Children's Hospital of Pittsburgh, Pittsburg, PA

[2] Division of Pulmonary Medicine, Children's Hospital of Richmond at Virginia Commonwealth University, Richmond, VA

[3] Division of Critical Care Medicine, Department of Anesthesiology, Critical Care and Pain Medicine, Boston Children's Hospital, Boston, MA

[4] Harvard Medical School, Boston, MA

[5] Wells Center for Pediatric Research/Pulmonology, Allergy, and Sleep Medicine, Riley Hospital for Children at Indiana University, Indianapolis, IN

Author of correspondence:

Dr. Michael D. Davis RRT, PhD, FAARC

Assistant Professor

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Wells Center for Pediatric Research

Pulmonology, Allergy, and Sleep Medicine

Riley Hospital for Children at Indiana University

MDD1@IU.EDU

Author Contributions:

BK conducted a literature review and performed the majority of the writing of this manuscript. CS aided in the design and performance of experiments, data interpretation, and drafting of this manuscript. MD was the PI of the study and aided in experimental design, performance of experiments, and drafting of this manuscript. RL and HY performed experiments and aided in the editing of this manuscript.

Conflict of Interest/Funding Disclosure:

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Quick Look:

Current Knowledge: Intravenous formulations of epoprostenol are routinely delivered by nebulization to acutely ill patients and are effective as pulmonary vasodilators. Concerns exist regarding their safe delivery and local effects within the airways.

What This Paper Contributes to Our Knowledge: Exposure of intravenous formulations of epoprostenol that are commonly delivered by nebulizer to airway epithelium *in vitro* causes increased cell death and decreased ciliary beat frequency. These effects were due to the high pH of the intravenous formulation rather than the epoprostenol itself.

Keywords: Airway pH, inhaled epoprostenol, inhaled prostacyclin, pulmonary vasodilator

Airway Effects of Pulmonary Vasodilators

ABSTRACT:

Background: Intravenous formulations of epoprostenol are frequently delivered by nebulization to treat pulmonary hypertension in acutely ill patients. Although their efficacy as pulmonary vasodilators have been shown to be comparable to inhaled nitric oxide, the local effects of these compounds within the airways have not been determined. We hypothesized that the alkaline diluents of these compounds would lead to increased airway epithelial cell death and ciliary cessation. **Methods:** Human bronchial epithelial cells were exposed to epoprostenol in glycine and arginine diluents or control fluid. Ciliary beat frequency, lactate dehydrogenase, and total RNA levels were measured before and after exposure. Results were compared between exposure and control groups. **Results:** Ciliary beat frequency ceased immediately after exposure to epoprostenol with both diluents. Lactate dehydrogenase levels increased by 200% after exposure to epoprostenol and glycine diluent ($p < 0.05$). Total RNA levels were undetectable after exposure to epoprostenol and arginine, indicating complete cell death and lysis ($p < 0.05$). Ciliary beat frequency ceased after thirty seconds of exposure to epoprostenol and glycine ($p < 0.008$). There was no difference between cells exposed to epoprostenol and those exposed only to diluent. **Conclusion:** Exposure to intravenous formulations of epoprostenol in glycine and arginine that are commonly delivered by nebulizer causes increased cell death and ciliary cessation in bronchial epithelial cells. These findings suggest undesired local effects may occur when these compounds are delivered as inhaled aerosols to patients.

BACKGROUND:

Inhaled nitric oxide (iNO) and continuously nebulized inhaled epoprostenol (EPO) have been used to treat pulmonary hypertension in neonatal, pediatric, and adult populations[1, 2]. iNO and EPO have been demonstrated to have similar effectiveness at increasing oxygenation, decreasing pulmonary artery pressure, and decreasing right ventricular afterload in acute respiratory distress syndrome (ARDS)[3-7]. These effects have also been observed in cardiothoracic surgery patients[8-10]. In patients receiving heart or lung transplants, iNO and EPO are thought to decrease reperfusion injury while acting as a selective pulmonary vasodilator, improving hemodynamic indexes[11].

Airway Effects of Pulmonary Vasodilators

Currently, iNO is the most commonly used, FDA-approved inhaled pulmonary vasodilator and is approved for use in neonates (> 34 weeks gestation) with hypoxic respiratory failure and associated pulmonary hypertension[12]. Administration of iNO outside of this indication is off-label but common across all age groups. Increasing healthcare costs coupled with declining reimbursements rates have led to efforts to decrease direct-delivery costs of iNO. Continuously nebulized inhaled EPO may be a cost-effective alternative to iNO[2, 13]. It has been reported that the cost of iNO is approximately $220.46/hour compared to $2.01 - $10.05/hour for inhaled EPO for a 70 kg adult patient. These potential cost savings have led to interest in substituting inhaled EPO in patients who would otherwise receive iNO[10].

Although there is evidence that EPO offers similar clinical benefits to iNO, safety data are very limited. Significant safety concerns remain regarding the continuous nebulization of EPO, including ventilator malfunction, unrecognized nebulizer failure, and a lack of mandated back-up delivery systems[1, 2, 14]. Further, preparations of EPO with either glycine or sucrose/L-arginine buffers have exceedingly high pH ranging from 11 to 13 which may have negative effects on airway epithelia including increased inflammation, ciliary dysfunction, decreased transport of bronchodilators, and cell death[1, 15-20].

Due to these reported safety concerns, the alkaline properties of EPO and its unknown effect on airway epithelium, we sought to evaluate the effects of glycine-buffered epoprostenol (EPO-GLY) and sucrose/L-arginine-buffered epoprostenol (EPO-ARG) on inflammatory biomarkers in differentiated human airway cells. Our group has previously demonstrated that human airway epithelial cells can be grown in an air-liquid interface that allows them to fully differentiate into functioning segments of airway epithelium[21, 22]. This model allows for a controlled evaluation of

airway epithelial function and response without systemic artifact. We hypothesized that exposure to EPO-GLY and EPO-ARG would increase levels of lactate dehydrogenase (LDH), a validated marker of cell stress/death, and that the effects seen in the EPO groups will be caused by the alkaline GLY and ARG diluents as opposed to EPO[23, 24]. We further hypothesized that exposure to these compounds would decrease airway epithelial ciliary beat frequency.

METHODS:

Differentiated airway epithelial cell culture:

Primary normal human bronchial epithelial cells used for all experiments described were from a healthy, adult, non-smoker donor and were purchased from Lifeline Cell Technology (Lifeline Cell Technology, Connecticut, USA). Cell media used was BronchiaLife Basal Media (Lifeline Cell Technology, Connecticut, USA) during cell growth and previously-described, homemade differentiation media during cell differentiation[21, 22]. Cells were differentiated on 6 mm transwells (Corning, New York, USA) at 200,000 cells per well as previously described by our group and others[21, 22]. Differentiation as pseudostratified, ciliated, columnar epithelial cells was verified by direct visualization of ciliary movement by 20x microscope.

LDH measurement: LDH protein levels were evaluated in culture media using commercially available Pierce LDH cytotoxicity colorimetric assay kits (Thermo Fisher Scientific, Massachusetts, USA). Assays were run according to the manufacturer's specifications in undiluted media.

Total RNA measurement: Total RNA levels were evaluated in airway exposure fluids using a NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) as previously described by our group[21].

Ciliary beat frequency analysis: During 20x magnification by microscope, ciliary motion was directly visualized uniformly across all cell culture. We also recorded ciliary motion on video using an iPhone SE (Apple Inc., California, USA) mounted on a Gosky Universal Adapter Mount (GOSKY OPTICS, Zhejiang, China) after locking focus and exposure.

Each video was transformed to grayscale [0 = black, 1 = white]. Regions of interest (ROI) were identified for each video by recording the coordinates of clusters of cells that demonstrated motion and was used for subsequent ciliary beat frequency analysis. Preference for each ROI was given to cells where the cell wall was most clearly visible and not obscured by other cells. Next, individual regions were plotted (Figure 1) and the number of peaks was calculated over the measurement epoch. The frequency was determined by summing the number of peaks and dividing by the sampling time provided in the video (15 s for each video). In the cases where no cell motion could be detected by the software, this was noted in the study results.

Study Design: After epithelial segments were fully differentiated and ciliary motion was confirmed, transwells were divided equally into control and exposure groups. pH of all exposure fluids was measured immediately prior to exposure by a standard Mettler Toledo pH probe and were confirmed to be 7 (PBS), 12 (EPO-GLY and GLY), and 12.9 (EPO-ARG and ARG), as indicated on their package inserts. All groups other than the Dry Control group were exposed to 40uL of sterile phosphate-buffered saline (PBS) to allow for uniform ciliary beat visualization. 40uL was the minimum volume that uniformly-coated the airway segments. Video was recorded prior to exposure to establish a baseline. Following baseline establishment, 10uL of

PBS (Wet Control), GLY, ARG, EPO-GLY, or EPO-ARG was added to each group. 30,000 ng/mL concentration of EPO was used in both EPO groups because this concentration is commonly used *in vivo*. A Dry Control group was included in protein analysis to determine any cell stress caused solely by fluid exposure. Cells were visualized throughout the exposure by microscopy at 20x magnification and video was recorded. After two minutes of exposure, exposure fluids and cell culture media were removed and stored at -80^{o} Celsius until LDH and total RNA measurement occurred.

Statistical Analysis:

Sample sizes of four in each group were determined to be appropriate to achieve a level of significance of 0.05 and a power of 0.80 with an effect size of 0.9 (G*Power Software, Universitat Kiel, Germany). Statistical analysis was performed using SigmaStat (Systat Software, Inc., San Jose, CA). Student's unpaired, two-tailed t-test was used to evaluate LDH protein levels comparing control groups and stimulation groups. Wilcoxon rank-sum test was used for non-Gaussian distributed data as determined by the Shapiro-Wilk test. Wilcoxon matched-pairs signed rank test was used to assess differences in ciliary beat frequency between the control and treated groups. A p-value of less than 0.05 was considered statistically significant. A Bonferroni correction was used to account for multiplicity.

Results:

EPO-GLY, EPO-ARG, GLY, or ARG exposure increase airway epithelial cell death:

LDH levels in groups exposed to EPO-GLY and GLY were more than 200% higher than the levels in the Wet and Dry Control groups (Figure 2A, $p < 0.05$). Exposure to EPO-ARG and ARG did not

increase LDH levels compared to either control group, although ciliary cessation and cellular debris was noted within 30 seconds of exposure to these compounds. The exposure fluids of these groups were therefore analyzed for total RNA in order to determine if cell death occurred uniformly. Total RNA levels were undetectable in EPO-ARG and ARG exposure groups compared to control groups, indicating complete cell death and lysis (Figure 2B, $p < 0.05$).

EPO-GLY, EPO-ARG, GLY, or ARG exposure decrease airway ciliary beat frequency

After 30 seconds of exposure to EPO-GLY, EPO-ARG, GLY, and ARG, all ciliary motion ceased by direct visualization. When evaluated using digital video analysis, the GLY exposure resulted in a reduction in mean ciliary beat frequency from 4.7 ± 1.3 Hz to 0 ± 0 Hz in control (Figure 3, $P < 0.008$). Although ciliary motion was visually confirmed, the frequency analysis on the ARG exposure group could not be performed due to insufficient number of cilia detected by the software in these exposure videos.

Discussion:

In this *in vitro* study, we describe the effects of two preparations of EPO and their diluents on ciliary beat frequency and cell viability in healthy human bronchial epithelial cells. Apical exposure of EPO to pseudostratified, ciliated, columnar epithelium simulated conditions of airway exposure to these compounds from continuous aerosol delivery during invasive mechanical ventilation. Ciliary cell exposure to EPO-GLY, EPO-ARG, GLY, or ARG resulted in a statistically significant reduction in ciliary beat frequency and increased cell death compared to

control cultures. There was no difference in outcomes between EPO-GLY and GLY nor EPO-ARG and ARG, suggesting these results are caused by the ARG and GLY diluents rather than EPO itself, although this cannot be completely ruled out. To our knowledge, this is the first study to demonstrate the direct effect of these compounds on airway cells.

The efficacy of any aerosolized medication is highly variable due to the efficiency of different nebulizers, physicochemical properties of different medications and aerosol generation technologies (ultrasonic, vibrating mesh, and jet)[25, 26]. Drug delivery can be inconsistent. Nebulizers generate a range of particles sizes that have a direct effect on the effectiveness of the aerosolized medication[27, 28]. Particles > 5um generated by the nebulizer are more likely to be deposited in the ventilator circuit and large airways[29, 30]. Importantly, for nebulized medications that have a desired mechanism of action at the level of the alveolar-capillary membrane (such as pulmonary vasodilators), deposition in large airways offers limited or no clinical benefit and potential increased risk of side effects. Further, variability in nebulized particles size and the resultant changes in distribution of drug deposition lead to clinically important differences in physiologic response[1]. This is an inherent concern to delivery of any aerosolized medication unlike delivering medical gases, which are distributed based on ventilated regions of the lungs. However, further work in this area is warranted.

Previous studies have noted unwanted side effects such as a decrease in systolic blood pressure requiring clinical intervention in up to 30% of pediatric subjects receiving aerosolized EPO[31]. However, the hemodynamic profile of adult subjects receiving aerosolized EPO has been shown to be stable. This may be due to differences in volume distribution, surface area/body mass ratio, and differences in absorption kinetics of the neonatal and pediatric populations

compared to adults[8, 9, 11, 32]. An independent third-party report highlighted sources of technical error which include infusion pump misconnections, over or underdosing, a lack of alarms on nebulizers to identify stoppage of dose delivery, and ventilators component malfunction leading to auto-peep and pneumothorax[14].

The high pH diluents for EPO may have unwanted effects to airway epithelium[1, 15-20]. Large particles, ≥9.5um in size have been shown to have an increased deposition rate to the trachea[29]. This suggests that the proportion of aerosolized particles of large size would have an increased probability of collecting in the trachea and large conducting airways where ciliary function is essential. Poor nebulizer function and changes in aerosol output may increase heterogeneous deposition of medications with respiratory tract and increase the proportion of these droplets. Of note, a single 2.5mL nebulization of a glycine buffer formulated for inhalation and buffered to a pH of 9.8 has been shown to be sufficient to alter airway pH[33]. Our findings suggest that continuous exposure to these aerosolized diluents may result in airway cell death and decreased mucociliary clearance but that clinical studies are needed to assess this.

This is not the first report of therapeutic agents that have the potential to injure the airways when delivered as aerosols[34]. Unknown risks should be assumed when intravenous drug formulations are used for inhalational administration as the topical effects of these formulations on airway epithelium may not be known. We found a 200% increase in LDH levels in cells exposed to EPO-GLY and GLY and complete cell death by RNA quantification in those exposed to EPO-ARG and ARG, suggesting the direct effect of high pH solution on airway tissue results in cell death, although other mechanisms of cell death cannot be completely ruled out.

The absence of LDH in the EPO-ARG and ARG group was unanticipated, especially since complete cell death was verified in this group by RNA quantification and LDH release indicates necrotic cell death. If cells are rapidly lysed instead of undergoing necrosis, LDH may not be released. Since the EPO-ARG and ARG have an even higher pH than the EPO-GLY and GLY (12.9 versus 12, respectively), this suggests that the higher pH causes rapid cell lysis. We also observed complete cessation of ciliary beat frequency after exposure to these compounds. Mucociliary function is important in those patients recovering from airway infection and/or respiratory failure. Decreasing mucociliary clearance and increasing cell death may worsen pulmonary system function; patients with ARDS or other critical pulmonary illness, who are often the recipients of inhaled vasodilators, may be even more susceptible to the effects we observed[35].

This study had several limitations. As with all *in vitro* studies, the findings may be subclinical or not representative of clinical effect. However, these results suggest consideration of unwanted effects of aerosolized EPO with high pH diluents is important. In order to control a uniform amount of drug exposure in each group, we elected to introduce the EPO and diluents to the airway epithelia by volumetric pipette instead of a nebulizer. This likely simulates the deposition, rainout, and collection of aerosolized medication in the larger airways more accurately than the entire delivery of aerosolized compounds to the targeted lower airways. In the future, it may be helpful to design an experiment that includes nebulization as a mechanism of introducing the agents. The experiments were performed on bronchial epithelial cells from healthy though many recipients of the tested compounds *in vivo* have ARDS or other airway disease. The effects we observed in healthy airway cells may differ from what would be

observed in diseased cells or tissue that may be more acidic[1, 33]. Cell death occurred very quickly in the EPO-GLY/GLY groups and virtually instantaneously in the EPO-ARG/ARG groups. While this supported the hypothesis and previously reported findings, it confounded the ciliary videos and rendered the findings binary (motion present before treatment and absent afterwards). Finally, the method of ciliary motion measurement by digital video file analysis requires further optimization. Although ciliary motion was verified by direct visualization, the video files were unable to capture this movement in the ARG group. Higher resolution cameras may make this technique more robust.

More work is needed to better understand the clinical effect of high pH preparations has on airway tissue and relevant clinical outcomes. Studies designed to evaluate local inflammation, alterations in airway epithelial function, or technical errors related to delivery of inhaled pulmonary vasodilators may help elucidate clinical effects of these compounds. There is some debate in the literature as to the most effective aerosolized pulmonary vasodilator. It is recommended that nebulization of prostacyclin analogs approved and available in inhalational preparations should be considered for administration via the respiratory tract as a first line aerosolized therapy for pulmonary hypertension or hypoxic respiratory failure[1, 2]. Agents such as iloprost, which is pharmacologically similar to EPO with lower viscosity, greater stability, a longer half-life, and more physiologic pH may be a more suitable consideration[36].

Conclusion:

To our knowledge, this is the first report of airway epithelium exposure to EPO-GLY and EPO-ARG which was observed to result in rapid cell death and cessation of ciliary movement *in vitro.*

These results were observed even in the absence of EPO, suggesting they are likely due to the high pH of the GLY and ARG diluents. Future clinical studies are indicated to assess the clinical significance of these findings.

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Figure Legends:

Figure 1 – Ciliary beat frequency analysis.

Example of the calculation of ciliary beat frequency. First a region of interest is manually recorded based on observed cell motion (indicated by *). The motion of the cells is analyzed by plotting the grayscale intensity over time. Ciliary beat frequency is calculated by summing the number of peaks detected and dividing by the total time of the recording.

Figure 2 – LDH and Total RNA results.

Cells exposed to EPO-GLY and GLY released 200% more LDH than the control group (A). No LDH is measured from cells exposed to EPO-ARG or ARG. However, no RNA is detectable in the EPO-ARG and ARG cells after exposure, indicating complete death and lysis of cells (B).

Figure 3 – Ciliary beat frequency before and after exposure to EPO-GLY.

Ciliary beat frequency decreased to zero after exposure to EPO-GLY.

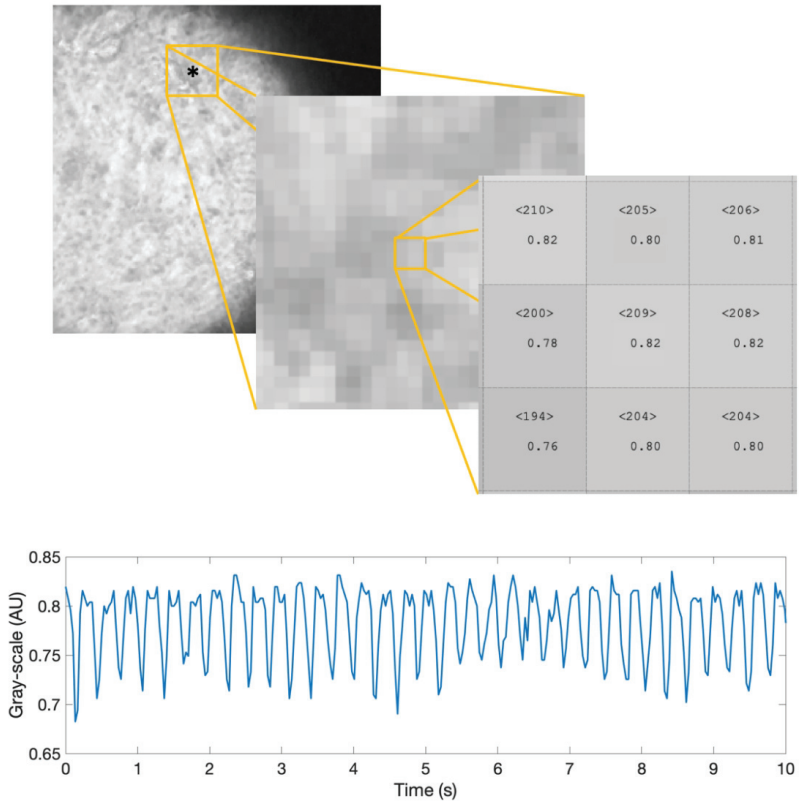

Figure 1 – Ciliary beat frequency analysis.

310x300mm (96 x 96 DPI)

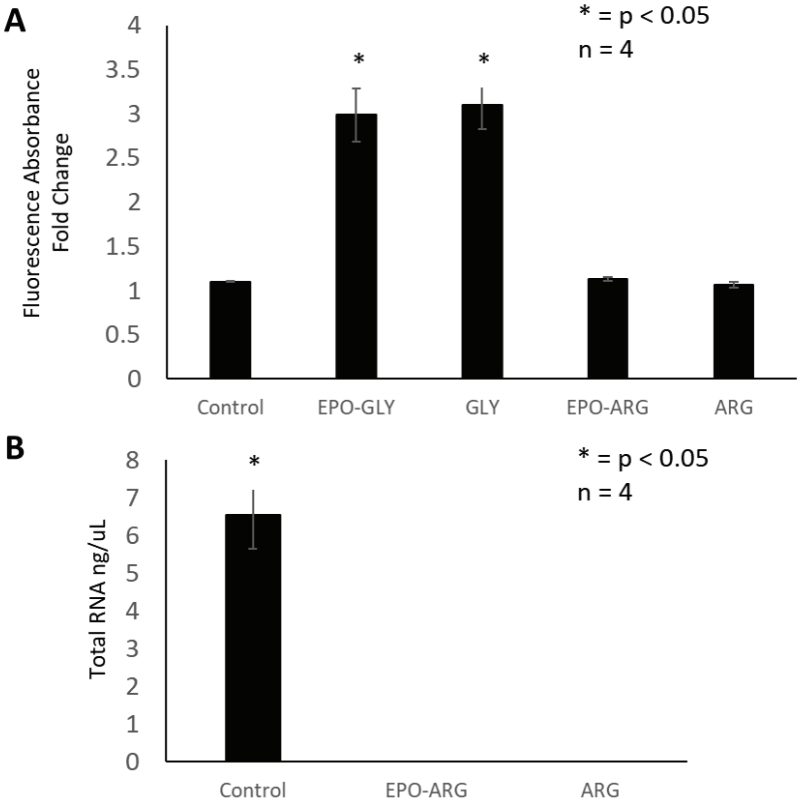

Figure 2 – LDH and Total RNA results.

146x143mm (150 x 150 DPI)

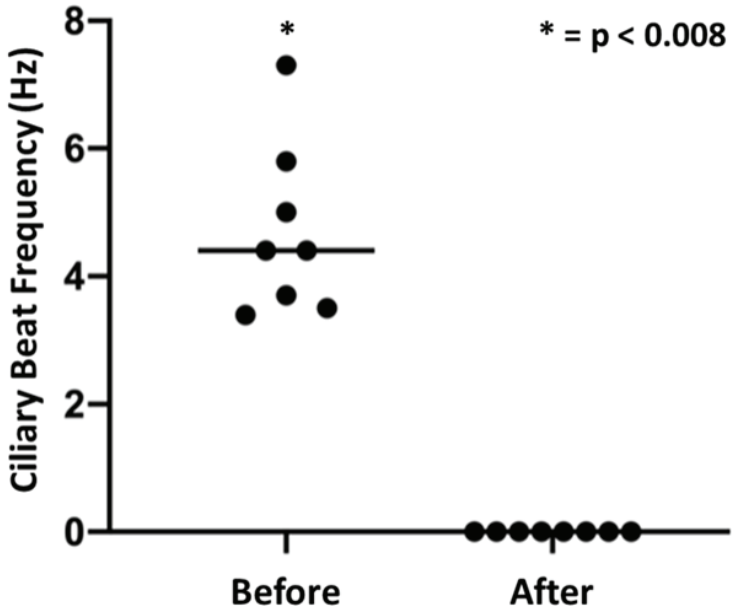

Figure 3 – Ciliary beat frequency before and after exposure to EPO-GLY.

115x90mm (150 x 150 DPI)