ONLINE SUPPLEMENT

Adult Cystic Fibrosis (CF) Protocol

Twenty adult CF subjects were enrolled. CF subjects ≥18 years old with FEV_1 ≥40% of predicted, and clinically stable as determined by the investigating pulmonologist were eligible for inclusion. CF diagnosis was ascertained through clinical manifestations consistent with CF and a sweat test and/or two cystic fibrosis mutations on genotyping. Subjects were not eligible if they were intolerant to hypertonic saline (HS), actively nursing, had a positive urine pregnancy test, regularly smoked cigarettes within 6 months of the study, or were unwilling to discontinue clinical use of inhaled HS for 72 h prior to testing days. Adult CF subjects performed two study days: one in which they inhaled isotonic saline [(IS), 0.9% NaCl] and the other in which they inhaled HS (7% NaCl) during the intervention period (t=10-20 min). The order of the treatments was randomized. Subjects who had not previously utilized inhaled HS performed a screening treatment prior to the study.

Adult Control Protocol

The adult control protocol enrolled 10 subjects ≥18 years old with an FEV_1 ≥80% of predicted. Exclusion criteria for this group included regular cigarette smoking (within 6 months), history of lung disease, a positive pregnancy test or actively nursing. Subjects performed a single study and were given isotonic saline during the intervention period (t=10-20 min), matching the IS treatment day procedures for the CF adult study.

Pediatric CF Protocol

Ten pediatric CF subjects were enrolled. Pediatric CF patients were eligible if they were 6-14 years old with an FEV_1≥40% of predicted, were able to lie recumbent for 80 minutes, demonstrated repeatable pulmonary function testing results in the past, and were clinically stable. CF diagnosis was ascertained through clinical manifestations consistent with CF and a sweat test and/or two cystic fibrosis mutations on genotyping. Exclusion criteria were identical to those described above for the adult CF protocol. The imaging procedure designed for this group was identical to the adult control protocol with the exception of the radioaerosol delivery time which was set to 2 minutes. Subjects performed a single study and were given isotonic saline during the intervention period (t=10-20 min).

Inhalation of Radioaerosol

Subjects were seated to perform radioaerosol inhalation. The test aerosol consisted of 55.5 MBq of Indium 111 (In-DTPA) and 296 MBq of unfiltered Technetium 99m-sulfur colloid (Tc-SC) in 3-4 mL of normal saline which was loaded into a DeVilbiss 646 jet nebulizer. The nebulizer was connected to a dosimeter system (Spira, Hameenlinnan, Finland) driven by a DeVilbiss 8650D compressor (DeVilbiss, Somerset, PA) set to 10 L/min. During inhalation, the dosimeter pulsed compressed air to the nebulizer for 0.7 seconds after an inhalation volume of 100 mL was detected. Subjects maintained a consistent inhalation flow rate (0.5 L/s) and tidal volume (adult: 400-500 mL; pediatric: ~300-500 mL) using visual feedback from the Spira dosimeter. A metronome set the breathing rate at 30 breaths per minute. This delivery strategy was designed to maximize inter-subject dosing uniformity and to concentrate deposition in the large airways[1]. Aerosol delivery time in the adult CF protocol was increased from 2 to 4 min at mid-protocol based on low deposited doses in some subjects. Subjects with Tc count rates <500 counts/minutes were not included in the analysis. All adult controls inhaled the aerosol for 4 min. Pediatric subjects inhaled until they had completed 2 minutes of effective inhalation. Simulation studies performed in our lab showed that approximately 4.1 ± 0.7% (± SD) of total radioactivity loaded into the nebulizer will be delivered to the patient during a 4 minute inhalation. Laser diffraction studies determined the size of the aerosol to be 5.4 ± 0.1 μm (volume median diameter) with a geometric standard deviation of 1.8 ± 0.03.

Imaging Protocol

Prior to aerosol delivery, adult subjects were placed in the supine position on the imaging table of a Siemens Symbia S dual-head γ-camera (Siemens Medical Solutions USA) fitted with medium energy collimators. A General Electric Hawkeye camera was used with similar positioning for all pediatric studies. Following a background image acquisition, a transmission image was acquired by briefly placing a large ^{57}Co sheet source (370 MBq) over the subject's chest. Aerosol delivery was then performed with the subjects seated. Within 5-7 minutes after radioaerosol inhalation, subjects were returned to the imaging table in their original position. A dynamic series of posterior and anterior lung projections were recorded (1 frame/min) on a 256 x 256 imaging

matrix for 80 minutes. At the beginning of the 11th frame (t=10 min), while still lying recumbent, subjects were administered either IS or HS (one study day - adult CF only) delivered via a Sidestream nebulizer. A special tubing arrangement was used to allow for aerosol inhalation while recumbent. Dynamic imaging continued during the 10 minute treatment. At the conclusion of the saline treatment, imaging continued for an additional 60 minutes. Subjects remained on camera throughout the imaging period.

The different energy levels associated with Tc-99m and In-111, commonly used clinically to differentiate solid and liquid transit in gastric emptying scans [2], allowed these isotopes to be mostly differentiated during scintigraphy acquisition. Tc-99m has a single gamma ray emission peak at 140 keV and In-111 has two emissions peaks at 173 and 247 keV. Due to photon scattering there will be a contribution of the higher energy In-111 gamma rays into the lower energy window used to image Tc-99m. Therefore, imaging Tc-99m and In-111 together requires the correction of the In-111 downscatter into the Tc-99m window. The spillover correction method used in the present study requires the acquisition of a "middle" image centered at 210 keV, which is the energy midpoint between the 247 and 173 keV In-111 energy peaks. The radioactive counts measured in this "middle" image can be measured and subsequently scaled to provide an estimate of the overall scatter contamination of In-111 in the Tc-99m image. We empirically determined this scaling factor to be 2.74 through phantom studies. The contribution of Tc-99m into the In-111 energy window was approximately 1% of the overall Tc-99m counts and therefore was considered insignificant in this study. Only the upper energy level window (247 keV) was utilized for In-111 imaging.

Image analysis

A region of interest (ROI) was drawn around the boundary of the entire right lung using the ^{57}Co transmission image for anatomical reference, which was then transferred to the emission image data for further analysis. Only the right lung was analyzed to avoid possible contamination from gastric radioactivity. The spatial position of the right lung ROI was adjusted on a frame-by-frame basis whenever patient movement was detected visually. ROI-derived counts for each probe were corrected for physical decay, spillover contamination, and background activity to produce retention curves. The starting point of the retention curves was set to the time of scan initiation; no attempt was made to correct for slight intersubject time differences from the end of aerosol delivery to scan initiation.

A fitting routine was employed to fit the normalized whole lung Tc-SC and In-DTPA retention curves to the following model types: single exponential, double exponential, and single exponential with a constant offset. For each subject, the Akaike Information Criterion [3] was used to identify the model that achieved the best fit. From the fitted curves, the rate of In-DTPA absorption was calculated for each subject by computing the difference between the curves (total DTPA clearance – mucociliary clearance) at 80 minutes. An image-derived count rate of 500 counts/min measured in the right lung on the initial Tc-99m image was considered to be the minimum deposition required to produce retention curves that could be fitted reliably. Images that produced rates below this amount were excluded from the study.

Central lung zone derived retention curves were also fitted using the same model types and evaluation criteria. However, in addition to correcting these curves for physical decay, spillover contamination, and background activity, the curves were also corrected for the influx of radioactivity entering the central lung zone from the surrounding peripheral lung over the 80 minute imaging period. The peripheral zone was defined as the area within the whole lung ROI not including the central lung box. As the basis for this correction, it was assumed that a reduction of Tc-SC-associated peripheral counts by mucociliary transport over consecutive frames caused an equivalent increase in counts in the central zone. Therefore, the change in counts associated with Tc-SC in the peripheral lung zone was computed for each pair of consecutive frames and subtracted from the central lung zone retention curve at the corresponding frame. Similarly, the central lung-derived In-DTPA retention curve was corrected for peripheral lung influx. This was accomplished in two steps. First, the percent change in counts associated with Tc-SC in the peripheral lung zone for each pair of consecutive frames was computed. Second, this percent change was assumed to also describe the influx of In-DTPA from the peripheral zone to the central zone. Therefore, each data point of the In-DTPA central lung retention curve was adjusted by the frame-by-frame percent change in Tc-SC counts computed from step 1 to obtain a corrected In-DTPA central lung retention curve.

The percent clearance at 80 minutes for Tc-SC (referred to as MCC) and total In-DTPA was calculated from fitted whole right lung and central zone retention curves. The difference between MCC and total DTPA

clearance, calculated for each zone, is our estimate of DTPA clearance by absorption (ABS). As an additional metric for comparison, the area above the right lung retention curves was calculated for Tc-SC (AAC_{Tc}) and In-DTPA (AAC_{In}) by integration over 80 minutes using all 80 frames. The difference between AAC_{Tc} and AAC_{In} is the calculation of the AAC for DTPA absorption (AAC_{ABS}).

The initial distribution of the inhaled radioaerosol was quantified on the initial posterior image by computing the counts per pixel in a central rectangular ROI drawn to 50% of height and of width of the whole right lung ROI and placed along the left lung margin and centered vertically. The area outside of this ROI but within the subject's right lung was considered the peripheral lung zone. The ratio of counts per pixel measured in these two zones on the first frame of the emission scan provided our central-to-peripheral ratio (C/P) deposition ratio. The analysis was performed using a custom analysis script written in MATLAB (Mathworks, Natick, MA).

Radiochemical purity, appearance, and physiochemical integrity of the admixture of Tc-SC and In-DTPA

The Tc-SC and In-DTPA mixture was tested for radiopharmaceutical purity and physiochemical integrity through a series of *in vitro* studies on two separate testing days. Measurements of particle size distribution and radiochemical purity were performed using a serial filtration technique and paper chromatography, respectively. Serial filter tests demonstrated that the radioactivity associated with the Tc-SC particles distributed approximately in thirds into 0.8 μm and 0.22 μm filters and a filtered solution vial. In contrast, all of the activity associated with In-DTPA passed through the filters into the solution vial. The calculated sum of these distributions matched the radioactivity distribution measured from the mixture of Tc-SC and In-DTPA and there was no significant change in distribution over time following the mixture of the solutions.

Radiochemical purity tests conducted using paper chromatography yielded expected results when the components of the mixture were assessed. Upon combination, no increases in free pertechnetate were detected using an acetone mobile phase. The relative ratio of saline mobilized activity (associated with In-DTPA) to origin activity (associated with Tc-SC) from the Tc-SC/In-DTPA mixture appeared to increase slightly over the 75 minutes following combination but the changes were not statistically significant. These studies provided evidence that Tc-SC and In-DTPA can be combined without causing any significant changes in the radiochemical purity or physiochemical integrity of the individual components over at least a 75 minute period after combination.

Primary Human Airway Epithelial Cell Culture Model

Primary human bronchial epithelial cells (HBE) were isolated from excess airway tissue dissected from lungs removed for transplantation under protocols approved by the University of Pittsburgh Investigational Review Board. The details of these methods have been previously described [4]. Briefly, the airway sections were digested overnight in a protease solution, suspended in epithelial growth media, and seeded onto sterile tissue culture flasks pre-coated with human placental collagen. After 5 to 6 days, the cells were detached and seeded onto 0.33-cm^2 collagen-coated transwell filters (0.4 μm pore size, Corning-Costar Transwell Collagen T-cols, Acton, MA, USA) at a density of approximately $2 \times 10^6/cm^2$. When confluent, the cells were maintained at an air-liquid interface, and the basolateral media was changed to differentiation media. All cultures utilized in these experiments were fully differentiated, determined when a mucociliary phenotype was apparent on phase-contrast microscopy. Each cell line described herein is from a unique donor.

DTPA Absorption Response to Inhibition of Transepithelial Water Transport

Radioactivity absorption through CF-HBE cell cultures was measured serially from 2 to 12 h after the apical addition of 0.37 MBq/mL Tc-DTPA (10 μL) in either normal saline or osmolarity–matched mannitol (300 mM) in water to inhibit ion-mediated transepithelial fluid flow. Radioactivity was measured in each filter containing the epithelial cells and the airway surface liquid (ASL) by removing it from the media and placing it in a well counter. Serial radioactivity counts were decay corrected and normalized. Immediately following the radioactivity measurement, ASL volume was also measured for each filter using an optical method established in our laboratory [5]. The details of this method are as follows: plates containing 12 HBE cultures were placed into an optical scanner (Epson V500, Epson Corporation, Long Beach, CA, USA) to image the pattern of transmitted light that passes through the meniscus which forms at the edge of cultured primary cells. The graded light intensity variation from the center to the edge of each filter was fitted by a series of radial spokes using a script written in ImageJ (NIH, Bethesda, MD, USA). The fitted spokes were then used to estimate the area under the curve, which was shown to robustly correlate with ASL volume following a calibration procedure.

The normalized radioactivity and apical liquid volume clearance data were processed using a MATLAB smoothing algorithm (loess, span 5%) and the percent change from 2 to 12 h was computed. Three different CF cell lines were used for this study (six cultures per case per line). (CF cell line genotypes: F508del/G551S, F508del/Q39X, F508del/N1303K).

DTPA Absorption Response to Mucus

ASL and DTPA absorption rates were serially measured in CF HBE cell cultures under four different conditions: with or without apical mucus and normal basolateral media, and with or without apical mucus and hyperosmotic basolateral media. For this study, airway surface fluid was carefully collected from differentiated, non-CF HBE cell cultures and centrifuged at 12,000 g for 1 minute to isolate mucus. The mucus was re-hydrated with water and applied to the apical surface of CF-HBE cell cultures (10 µL). Eighteen hours after mucus addition, DTPA in normal saline was applied to the cells in the manner described above. The addition of mannitol to basolateral media created an osmotic gradient favoring apical to basolateral movement of fluid. Two different CF cell lines were used for this study (six cultures per case per line). (CF cell line genotypes: F508del/F508del, F508del/N1303K).

Table S1. PEDIATRIC CF SUBJECTS

Subject	Gender	Age	Mutation 1	Mutation 2
1	M	13	ΔF508	ΔF508
2	F	9	ΔF508	ΔF508
3	F	9	ΔF508	ΔF508
4	F	12	ΔF508	ΔF508
5	M	11	ΔF508	ΔF508
6	M	10	ΔF508	ΔF508
7	F	9	ΔF508	ΔF508
8	M	12	ΔF508	R1162X
9	M	11	ΔF508	ΔF508

Table S2. ADULT CF SUBJECTS

Subject	Gender	Age	Mutation 1	Mutation 2
2	F	19	ΔF508	ΔF508
3	F	21	ΔF508	ΔF508
4	F	19	ΔF508	ΔF508
11	M	33	ΔF508	ΔF508
12	F	20	ΔF508	ΔF508
13	M	34	ΔF508	ΔF508
14	M	26	G551D	3659ΔC
15	M	18	ΔF508	ΔF508
16	M	21	ΔF508	ΔF508
17	M	25	2711Δt	5T
18	F	29	ΔF508	ΔF508
19	M	29	ΔF508	ΔF508
20	M	23	ΔF508	G551D
21	M	18	ΔF508	G551D

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