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"Safety of an alkalinizing buffer designed for inhaled medications in humans"

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Conflicts of Interest: JH is a cofounder of Respiratory Research, Inc, which manufactures exhaled breath condensate collection equipment. JH and the University of Virginia have intellectual property interest in airway pH diagnosis and therapy.

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Abstract:

BACKGROUND: Airway acidification plays a role in disorders of the pulmonary tract. We hypothesized that the inhalation of alkalinized glycine buffer would measurably alkalinize the airways without compromising lung function or causing adverse events. OBJECTIVE: To evaluate the safety of an inhaled alkaline glycine buffer in both healthy subjects and in subjects with stable obstructive airway disease. METHODS: This work includes two open-label safety studies. The healthy controls were part of a Phase 1 safety study of multiple inhalations of low-dose alkaline glycine buffer; nebulized saline was used as a comparator in 8 of the healthy controls. Subsequently, a Phase 2 study in subjects with stable obstructive airway disease was completed using a single nebulized higher-dose strategy of the alkaline inhalation. We studied 20 non-smoking adults (10 healthy controls and 10 subjects with obstructive airway disease) both at baseline and after inhalation of alkaline buffer. We used spirometry and vital signs as markers of clinical safety. We used changes in exhaled nitric oxide (eNO) and exhaled breath condensate pH (EBC pH) as surrogate markers of airway pH modification. RESULTS: Alkaline glycine inhalation was tolerated by all subjects in both studies with no adverse effects on spirometric parameters or vital signs. Airway alkalinization was confirmed by a median increase in EBC pH of 0.235 pH units (IQR = 0.56-0.03, p = 0.031) in subjects after inhalation of the higher-dose alkaline buffer (2.5 ml of 100 mmol/L glycine).. CONCLUSIONS: Alkalinization of airway lining fluid (ALF) is accomplished with inhalation of alkaline glycine buffer and causes no adverse effects on pulmonary function or vital signs.

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Introduction:

The regulation of airway pH plays a role in the pathogenesis of obstructive lung diseases. Airway acidification, caused by both intrinsic and extrinsic factors, is associated with neutrophilic and eosinophilic inflammation, bronchospasm, bronchial hyperreactivity, ciliary dysfunction, epithelial dysfunction, augmented oxidative damage, abnormal fluid transport, inhibition of transport of cationic drugs such as albuterol, and alteration of cellular death pathways, including inhibition of apoptosis[1]. Knowledge of the role of airway pH in pulmonary health, along with the development of devices and techniques to measure it, has created interest in treatment of airway pH disturbances. Improved ability to treat or potentially reverse acidic airway pathology by means of therapeutic alteration of airway pH could have an impact in respiratory medicine. The ability to normalize airway pH via inhalation[2] may allow introduction of new pulmonary therapeutics.

Airway lining fluid (ALF) acidity can be qualitatively determined non-invasively via the collection of exhaled breath condensate (EBC) and the measurement of its pH[3]. Assays for the measurement of EBC pH have been developed for patients of all ages and sizes, including those receiving mechanical ventilation[4-7]. EBC pH normally lies within a mildly alkaline range of 7.5-8.2[7-19]. EBC has a minimal buffer capacity, which allows EBC to assess the presence of volatile acids in ALF as indicated by a change in its pH[20, 21]. Although a normal EBC pH does not exclude airway acidity at some level, a low EBC pH value is highly specific for acidity somewhere within the airway. Using EBC methods, several studies have shown that patients with COPD[13], asthma[7, 9] ,

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bronchiectasis[13], cystic fibrosis[9], and chronic cough[15] have airway acidification. We incorporated EBC into this study as a non-invasive safety measure to assess for the possibility of excessive alkalinization from our intervention.

Airway pH also affects exhaled nitric oxide (eNO) levels by simple chemistry. As the pH of ALF decreases, commonly present nitrite becomes protonated to nitrous acid, which decomposes to nitric oxide [22], which is then in part exhaled. eNO analyzers may be used qualitatively to longitudinally assess the alkalinizing effects of alkaline inhalation therapy through monitoring decreases in eNO levels[2, 23]. The first aim of the present study was to evaluate the safety of an inhaled aerosol of alkaline glycine buffer in healthy controls; we also investigated the potential of this inhaled, aerosolized buffer to alkalinize the ALF pH in both healthy subjects and those with stable obstructive airway disease.

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Methods:

Protocol: Two open-label safety studies were approved by the University of Virginia Institutional Review Board (UVA IRB) under an Investigational New Drug Application from the United States Food and Drug Administration. Approval and initiation of the second study by the UVA IRB was dependent upon successful completion of the first study and results indicating safety of the intervention. We obtained informed consent from all subjects for both studies. The first study recruited ten healthy volunteers via direct approach from the study team. The study was performed in the General Clinical Research Center (GCRC). No reimbursement was provided. The second study recruited ten subjects with stable obstructive lung disease via referral from their Allergist and was performed in the Allergy/Asthma/Immunology Clinic. Subjects were reimbursed $100.00 for participation. An independent medical safety monitor reviewed the results for each subject daily during the studies. Both studies were executed between 1000 and 1400 hours Eastern Standard Time.

For the first study (Study 1), we acquired (in the following order) baseline eNO levels, EBC samples, spirometry (FEV1, FVC, FEV1/FVC, FEF25/75), and vital signs (heart rate, respiratory rate, oxygen saturation and breath sounds) from the subjects. The order these measurements were obtained was kept consistent throughout all points of the study with the exception of EBC collection, which was only collected before the first inhalation and immediately after the third inhalation. All of the subjects were within healthy limits for spirometry (baseline >80% predicted for all observed values) and vital signs, and had not ingested anything except water for 8 hours prior to the study. Exclusion criteria included a > 5 pack-year smoking history, current pregnancy (all

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female subjects were either abstinent, post-menopausal, or practicing adequate contraception), a history of pulmonary disease (verified through verbal medical history), or acute illness (verified by clinical history or reported by the subject) within five days of study.

The Study 1 treatment preparation was an isotonic solution of a sodium chloride diluent mixed with glycine to a concentration of 17.8 mmol/L at pH of 10.5, equating to 44.5 micromoles/dose. The patients received this through a small volume nebulizer in 2.5 mL increments every twenty minutes for a total of three nebulizations and total delivered quantity of 133.5 micromoles of glycine (Figure 1). Each nebulization lasted for ten minutes, with a ten minute break in between, during which vital signs, eNO, and spirometry levels were obtained. A final set of physiologic measurements was performed twenty minutes after the completion of the study.

After determining safety in healthy volunteers of serial incremental inhalations of glycine, a second study (Study 2) was conducted in stable subjects with known obstructive lung disease defined as a documented history of asthma or COPD and an FEV1 less than 75% predicted on the day of the study. Exclusion criteria included cigarette smoking in the past six months, any acute illness within five days of the study (verified by clinical history or reported by the subject), or an FEV1 < 50% predicted on the day of the study. Eight of these subjects had a current diagnosis of asthma, one had a current diagnosis of chronic bronchitis, and one had diagnoses of both asthma and chronic bronchitis. The ages of enrolled subjects ranged from 24-62 years old, with a mean of 44 years. This study protocol only varied from the Study 1 protocol by using a single 2.5mL dose of alkaline diluent with 100 mmol/L of glycine (total glycine dose of

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250 micromoles) instead of the three lower-concentrations nebulizer treatments used in Study 1. Vital signs (heart rate, respiratory rate, oxygen saturation and breath sounds) were obtained prior, during, and after the nebulization. eNO levels, EBC samples, and spirometry were obtained before and after the nebulization. All measurements were obtained in the same order as in Study 1.

Instrumentation: eNO levels were measured at an expiratory flow rate of 50ml/s using the Niox Mino (Aerocrine AB, Solna, Sweden).

Spirometry was measured in triplicate at each collection point using the SDI SBG spirometer (Queset Medical, Brockton, MA).

EBC was collected orally during tidal breathing for 7 minutes at initial temperature of -20 Celsius using the RTube (Respiratory Research, Inc, Austin, TX) without wearing nose clips. All samples underwent gas-standardization with research-grade oxygen for ten minutes at 300 ml/min prior to pH measurement.

EBC pH was measured using the Orion 8220BNWP PerpHecT® ROSS® Combination pH Micro Electrode (Thermo Scientific, Waltham, Ma). The probe was calibrated in standard pH 4 and 7 buffers, and then verified in low ionic-strength buffers of the same pH, and then pH of samples was measured immediately after gas-standardization.

Nebulizations were administered for ten minutes using the Hudson RCI Micromist nebulizer with a mouthpiece. The nebulizer was powered by 7 liters per minute of room air, a rate at which it averages an output mass median aerodynamic diameter (MMAD) of 2.1 microns, according to manufacturer. The nebulizer cup was

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percussed periodically during medication delivery to minimize residual volume. The residual volume was removed at the end of each ten-minute inhalation.

Oxygen saturation was obtained using a Masimo fingertip pulse oximeter.

Study Medication Specifications: The alkaline glycine buffer was formulated by a compounding pharmacy under sterile conditions. Sodium chloride and sodium hydroxide were added to a solution of double deionized water and alkaline glycine in order to attain appropriate isotonicity (0.9% w/v), osmolarity (338 mOsmoles), and pH (9.8) to prevent cough upon nebulization. After receipt of each batch of medication from the pharmacy, one vial was used to confirm pH and osmolarity.

Statistical Analyses:

Outcome parameters post-treatment were compared with a pre-treatment baseline using Wilcoxon rank-sum measures. Data are presented as median and interquartile range (IQR). A p-value of less than 0.05 was considered significant. P-values considered to be significant were not modified in the setting of multiple comparisons.

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Results:

Ten subjects (7 males, 3 females, mean age 33.2 years) performed the glycine inhalation into the study 1. Eight subjects (6 males, 2 females, mean age 35.4 years) repeated the experiment six weeks later, inhaling standard normal saline instead of alkaline glycine. Two subjects had moved away during this time and were unable to complete this portion of the study. Ten subjects (4 males, 6 females, mean age 43.9 years) performed the 2nd study. All of the subjects tolerated the inhaled glycine buffers. There were no adverse events of any kind. The changes in measurements for the studied groups from baseline throughout the studies are reported in Table 1. The median change in eNO from baseline after both glycine buffer and normal saline inhalation is reported in Figure 2. Vital signs were unchanged in all subjects throughout both studies. eNO levels decreased by a median of 27.1% (IQR = -26.1-28.5, p = 0.004) after Study 1 glycine inhalation, but there was no significant change in eNO in study 2. The largest decreases in eNO from baseline occurred in subjects with clinically elevated eNO levels in absolute terms at baseline (> 40 ppb). Spirometry levels remained within normal clinical ranges for both studies (Table 1). There was no adverse effect on our primary safety outcome variable, FEV1. There was a slight decrease of 5.3% in FEF 25/75 after inhalation of Study 1 glycine (IQR = 0.292-12.579, p = 0.037), which was not seen in Study 2, and also of 4.1% in FVC after inhalation of Study 2 glycine (IQR = -2.16-6.17, p = 0.035, Table 1), which was not seen in study 1.

EBC pH increased by a median of 0.235 pH units after inhalation of the Study 2 alkaline glycine buffer (IQR = 0.56-0.03, p = 0.031, Figure 3), but had no statistically significant change after the Study 1 treatments.

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Discussion:

Our results provide additional data supporting the safety of inhaled alkaline glycine in healthy subjects and those with obstructive lung disease. It is possible to achieve increases in airway pH by means of inhaled alkaline glycine, and inhalation of alkaline glycine in healthy subjects and in subjects with obstructive lung disease causes no adverse effects on spirometric parameters. EBC pH did increase, especially after inhalation of the higher-dose Study 2 alkaline glycine, indicating the potential for efficacy as an airway alkalinization method. Subjects reported no subjective adverse effects after treatment with alkaline glycine. The EBC pH of one subject in the Study 1 Glycine group dropped below normal (7.18 after the study), and this subject had witnessed belching during the study (the sensitivity of EBC pH to gastric acid reflux is well recognized [22]).

As shown in Figure 1, changes in eNO after inhalation of Study 1 alkaline glycine were pronounced compared to the Study 1 saline group. Although it is possible that levels of eNO can be lowered by spirometry [24], the statistically insignificant change in eNO seen after saline inhalation effectively assures that the alkaline glycine was the primary cause of the eNO decline. Although the EBC pH did not change significantly in the study 1 subjects, this does not preclude that alkalinization occurred. In many letters to editors, Effros et al contend that oral contamination of EBC by ammonia eliminates the ability of EBC to function as an indicator of ALF pH[25]. While the weight of peer-reviewed published original research refutes that contention and supports the use of EBC pH as one indicator of ALF pH[4, 7, 12, 19, 26], it seems certain to us that the sensitivity of

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EBC pH for identifying airway acidity is indeed *decreased* when high levels of oral ammonia are present to neutralize exhaled airway acids. Thus, a low EBC pH is highly specific for airway acidification, but when oral ammonia release is high, EBC pH is not as sensitive for airway acidification. This phenomenon could explain the lack of change in EBC pH in Study 1. It is noted that in Study 1 the baseline eNO was higher, and EBC pH and FEV1 were lower prior to the glycine inhalation compared to the saline inhalation intervention given six weeks later. We speculate that this may result from the timing of the study in relation to allergy seasons, as the glycine intervention was performed in September (high allergy season in our region).

After inhaling alkaline glycine, the subjects in Study 2 did not experience a statistically significant change in eNO, but in contrast did demonstrate a marked increase in EBC pH. This finding supports the use EBC pH to detect acidic ALF pH, as several of the subjects in this study demonstrated acidic EBC pH at baseline, consistent with known findings of low EBC pH in patients with disease (median 7.71, IQR = **7.17**, 8.27). These subjects had known obstructive airway disease and many were receiving inhaled corticosteroid therapy, which is known to decrease nitric oxide synthase activity and net nitrogen oxide production. Corticosteroid therapy decreases eNO at baseline, which likely explains the lack of an observed eNO effect in Study 2[27].

Our studies had several limitations. The primary focus was on safety of the inhaled alkaline glycine buffer, therefore several pieces of data that would have strengthened the secondary endpoints were not measured. In Study 1, the healthy controls were not screened for atopy prior to enrollment. This could explain the differences noted in baseline eNO, EBC pH, and spirometry levels in the same subjects

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between the glycine treatment and the saline treatment. In Study 1, EBC pH was collected only at baseline and after the final nebulization of alkaline glycine buffer. This was done to minimize delays between the three nebulizations. We used change in eNO after inhalation as a surrogate detector of airway pH changes at these timepoints due to the rapidity of the testing (~ eleven seconds for eNO compared to ~ 7 minutes for EBC collection)). Since study 1 was the first human trial of this alkaline glycine buffer, it was necessary to deliver the compound through serial small doses in order to evaluate its safety and detect potential adverse reactions prior to delivery of a larger dose. Given the full tolerability of the inhaled buffer, we increased the concentration of buffer so that the next phase would receive only one treatment.

In Study 2, we wished to evaluate the effects of inhaled alkaline glycine on patients with obstructive lung function – specifically, on the physiologic issue of airflow limitation. Although heterogeneous, all enrolled population had documented airway obstruction on the study day. We did not determine their degree of airway hyperresponsiveness nor responsiveness to beta-agonists at the time of the study. We did not test the patients for atopy, immunologic disease, unrecognized environmental/occupational illness, or for any of the other numerous factors that could contribute to their obstruction. This current study was simply examining safety in patients with airflow obstruction. In future studies, we will be evaluating the efficacy of inhaled alkaline glycine buffer in patients with obstructed lower airways, and will use classification schemes and semi-specific disease names such as “asthma” and “COPD” to the extent that they are not misleading.

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Also in study 2, we did not screen out subjects based on corticosteroid use. This may explain the lack of significant change in eNO results since corticosteroid therapy decreases eNO at baseline[27]. We also did not serially measure eNO and EBC pH for time points after inhalation of the alkaline glycine buffer. In previous studies, the greatest reduction in eNO following inhalation of alkaline buffer occurred 15-60 minutes after inhalation[2, 23]. When using eNO to detect changes in airway pH, we recommend documenting and/or withholding corticosteroids prior to the study and measuring eNO at several time points in the hour following the study intervention.

Although the inhaled medication evaluated in this study was a solution (not a colloid), the formulation was different from the physiologic saline used by the manufacturer to determine the 2.1 micron MMAD output of the nebulizer that was used in this study. It is possible that the MMAD output of the nebulizer of the study medication may be different from that of physiologic saline. This will be evaluated in future studies.

Conclusions

In conclusion, we report that the inhalation of isotonic alkaline glycine is safe in humans with or without obstructive airway disease and that both EBC pH and eNO levels may be useful to indicate effective alkalinization of the airways. Future studies should evaluate the effects of alkaline therapy on subjects with known acute airway pH disturbances. Perhaps the most interesting near-term use of inhaled alkaline glycine is to improve the absorption across the airway epithelium of certain therapeutic agents that carry a charge at acidic but not alkaline pH. Such therapeutics (including most beta-

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agonists and anticholinergics) achieve better passive and active transfer through the airway epithelium—and therefore access to their smooth muscle target—when the airway lining fluid is alkaline [28]. Because these medications are currently delivered at low pH, and they are mostly used during acute respiratory illnesses when the airways are most likely to be acidic, the possibility of improving drug delivery by means of alkalinization with glycine is undergoing clinical investigation currently.

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Figure Legends

Figure 1. Timeline of events for Study 1

Figure 2. Comparison of alkaline glycine to normal saline inhalation (Study 1). Median % change in exhaled NO from baseline at 5 time points during the study, saline compared to glycine. Time 0 is baseline, followed by an eNO measurement after each nebulization (alkaline glycine is represent by white box plots; normal saline is represented by gray box plots). The final (recovery) measurement was taken 30 minutes later, prior to discharge from the clinical research unit. Box plots represent data minimum/maximum (whiskers), upper and lower quartiles (top and bottom of the boxes, respectively), and median (line inside of the box).

Figure 3. EBC pH results from Study 1 and Study 2

Increase in median EBC pH by 0.235 pH units ($p = 0.031$) after inhalation of 100 mmol/L alkaline glycine, compared to no statistically significant changes after low-dose alkaline glycine and normal saline.

Table 1. Median changes in physiologic outcome measurements, compared to baseline measurements. Statistically significant values are in bold. p-values have not been adjusted for multiple comparisons.

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Changes in Physiologic Measurements from Baseline Throughout Studies

Initial Study - Saline Group

	Median Pre	IQR Pre	Median Post	IQR Post	% Δ	p value
FEV1	4.08	4.53, 3.74	4.24	4.56, 3.68	3.93	0.192
FVC	5.06	5.58, 4.88	5.12	5.51, 4.9	1.19	0.812
FEV1/FVC	80.06	82.18, 76.38	81.69	81.90, 78.62	2.03	0.432
FEF 25/75	3.59	4.48, 3.36	4.04	4.62, 3.25	12.69	0.847
eNO	15	52, 12	15.5	43.5, 10.5	3.33	0.094
EBC pH	8.62	8.86, 6.95	8.59	8.83, 8.37	NA	0.945

Initial Study - Glycine Group

	Median Pre	IQR Pre	Median Post	IQR Post	% Δ	p value
FEV1	3.97	4.47, 3.46	3.99	4.25, 3.52	0.38	0.232
FVC	4.96	5.51, 4.61	5.04	5.45, 4.54	1.61	0.622
FEV1/FVC	78.55	83.19, 72.82	79.44	81.68, 72.08	1.14	0.323
FEF 25/75	3.72	4.89, 2.83	3.53	4.58, 2.84	-5.11	**0.037**
eNO	18.5	32, 14	14.5	32, 10	-21.62	**0.004**
EBC pH	7.75	8.48, 7.47	7.85	8.40, 7.56	NA	0.849

Second study - Glycine

	Median Pre	IQR Pre	Median Post	IQR Post	% Δ	p value
FEV1	2.76	3.48, 2.58	2.79	3.31, 2.49	1.09	0.361
FVC	3.7	4.7, 3.24	3.47	4.41, 3.17	-6.22	**0.035**
FEV1/FVC	78.6	83.1, 74.0	80.35	82.1, 75.6	2.23	0.499
FEF 25/75	2.6	3.14, 2.32	2.84	3.25, 2.17	9.23	0.881
eNO	23	43, 16	21.5	38, 18	-6.52	0.38
EBC pH	7.71	8.27, 7.17	8.34	8.62, 7.36	NA	**0.031**

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Figure 1

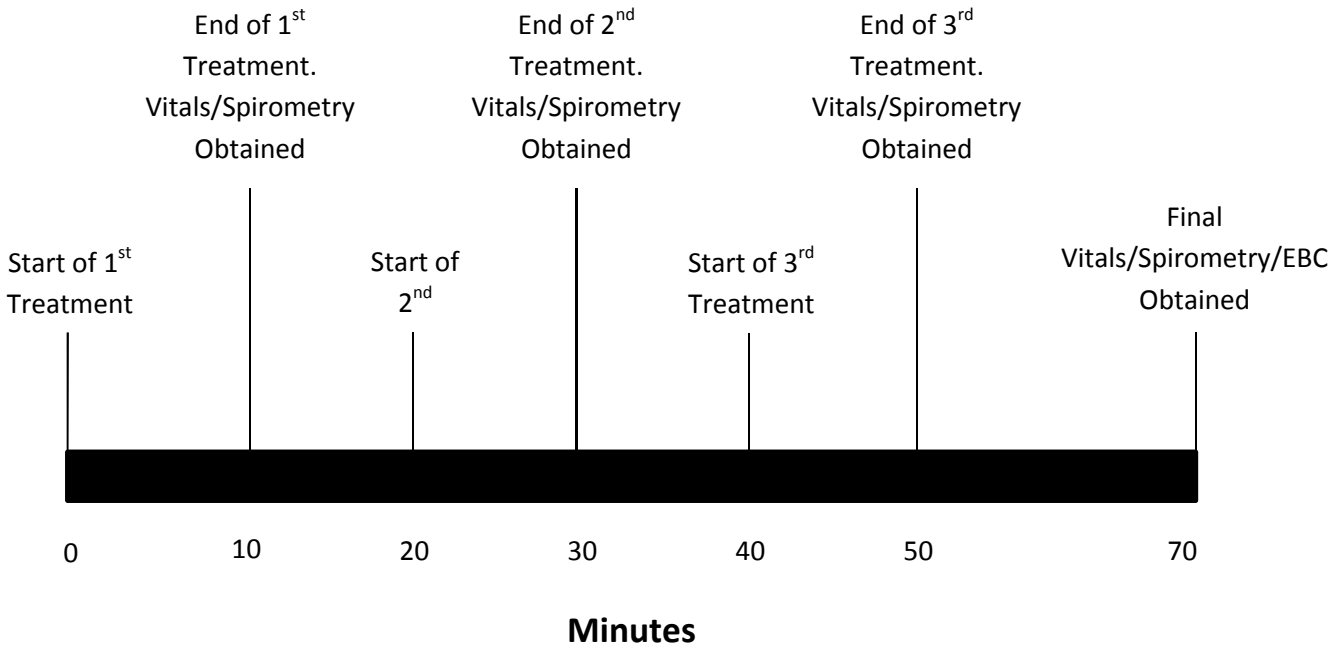

Figure 2

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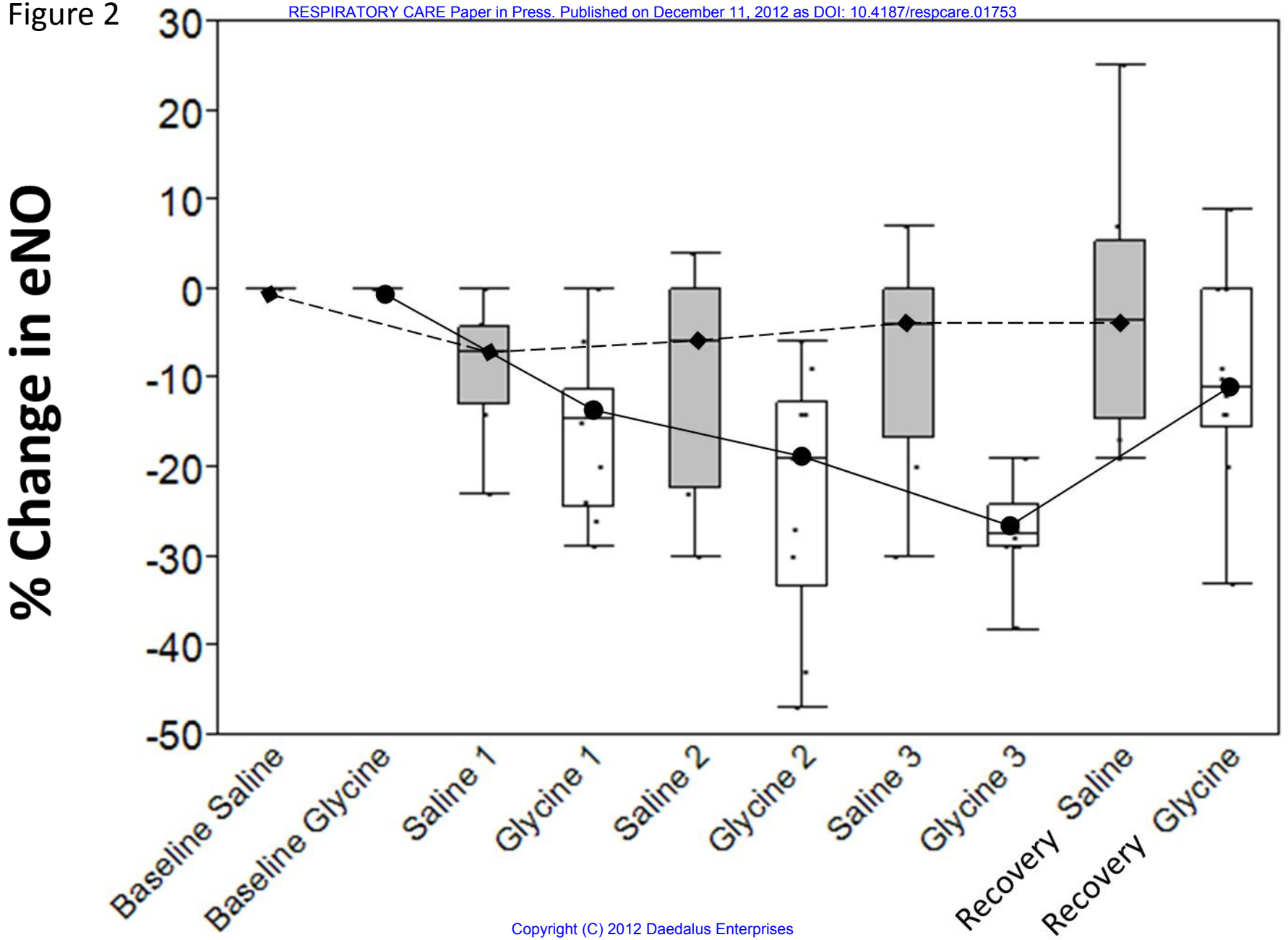

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Figure 3