An International Randomized Multicenter Comparison of Nasal Potential Difference Techniques

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Background: The transepithelial nasal potential difference (NPD) is used to assess cystic fibrosis transmembrane conductance regulator (CFTR) activity. Unreliability, excessive artifacts, and lack of standardization of current testing systems can compromise its use as a diagnostic test and outcome measure for clinical trials.

Methods: To determine whether a nonperfusing (agar gel) nasal catheter for NPD measurement is more reliable and less susceptible to artifacts than a continuously perfusing nasal catheter, we performed a multicenter, randomized, crossover trial comparing a standardized NPD protocol using an agar nasal catheter with the same protocol using a continuously perfusing catheter. The data capture technique was identical in both protocols. A total of 26 normal adult subjects underwent NPD testing at six different centers.

Results: Artifact frequency was reduced by 75% ($P < .001$), and duration was less pronounced using the agar catheter. The measurement of sodium conductance was similar between the two catheter methods, but the agar catheter demonstrated significantly greater CFTR-dependent hyperpolarization, because Δ zero Cl⁻ + isoproterenol measurements were significantly more hyperpolarized with the agar catheter (-24.2 ± 12.9 mV with agar vs 18.2 ± 9.1 mV with perfusion, $P < .05$).

Conclusions: The agar nasal catheter approach demonstrates superior reliability compared with the perfusion nasal catheter method for measurement of NPD. This nonperfusion catheter method should be considered for adoption as a standardized protocol to monitor CFTR activity in clinical trials.

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Abbreviations: ATP = adenosine triphosphate; CF = cystic fibrosis; CFTR = cystic fibrosis transmembrane conductance regulator; NIH = National Institutes of Health; NPD = nasal potential difference; PD = potential difference; SOP = standard operating procedure; TDN = Therapeutics Development Network

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR), resulting in disruption of chloride and bicarbonate transport across epithelial surfaces.¹ In the respiratory tract, deficient anion transport is accompanied by enhanced sodium absorption.² These abnormalities, which are the ion transport hallmarks of CF, can be evaluated by measuring the transepithelial nasal potential difference (NPD).^{2,3} NPD serves as one of the diagnostic criteria for CF^{4,6} and, as the only in vivo measure capable of isolating CFTR activity and epithelial sodium channel func-

tion, it is a frequently used biomarker to test the efficacy of novel therapies directed at the basic CF ion transport defect.^{7–12} Although the methods currently used to measure NPD generate reproducible results in individual laboratories,¹³ recent blinded reviews of tracings conducted as part of randomized controlled trials have revealed significant interlaboratory variability in the stability and quality of NPD tracings and a high frequency of recording artifacts.¹⁴

Many methods for the measurement of NPD, including the standard operating procedure (SOP) used by the Cystic Fibrosis Therapeutics Development

Network, use a catheter filled with a slow and continuously perfusing Ringer electrolyte solution to establish the interface between the nasal mucosa and the exploring electrode.^{15,16} The small diameter of the tubing, in conjunction with a slow flow rate (12 mL/h), make this system prone to bubble formation because of the Bernoulli principle, a characteristic that can interrupt the mucosal connection and create artifacts in the NPD recording. Agar dissolved in electrolyte solution forms a semisolid gel and can be used to form a stable electrical connection. Agar connections are used frequently in lieu of continuously perfusing catheters in many electrophysiologic applications. Because the interface with the mucosa is a likely source of artifacts and variability, we developed a method for measurement of NPD using an agar gel interface for the nasal catheter. We compared the technique with the standard continuously perfusing catheter in an international, multicenter, randomized, crossover trial in normal adult subjects. Some of the results of these studies have been reported previously in the form of abstracts.^{14,17,18}

MATERIALS AND METHODS

Study Design

Each institution obtained approval for the NPD comparison in human subjects from their respective institutional review board or ethics committee, and written informed consent was obtained from all study subjects. The University of Alabama at Birmingham's institutional review board approved the central analysis of the data. The subjects then underwent NPD testing using the

other methodology at least 1 day later, a period sufficient to wash out the effects of amiloride and isoproterenol. Normal subjects were randomly assigned to undergo NPD testing using either the perfusion method specified in the Therapeutics Development Network (TDN) SOP, or the agar catheter method on day 1. The TDN SOP at the time of this study mandated a continuously perfusing nasal catheter using either an Ag-AgCl pellet or KCl calomel electrode pair, per center-specific practices. Inclusion criteria for this study included adult subjects (aged ≥ 18 years) with no diagnosis of CF who were otherwise able to perform NPD testing twice. Exclusion criteria included moderate to severe nasal rhinitis or polyposis (which can interfere with repeat NPD measurements) or a medical condition that, in the opinion of the investigator, might have affected the NPD results.

Catheter Designs and Procedures

The agar nasal catheter was constructed by filling a two-lumen, end-hole, single-use, 28-cm-long, polyvinyl chloride, sterile catheter with an external diameter of 2.5 mm specially designed for NPD in collaboration with a specialized manufacturer (EU certificate 0337/B5/02; Marquat Génie Médical; Boissy Saint Leger, France). Ringer solution with 3% agar was prepared and autoclaved to remove bubbles and sterilize. The agar catheter and subcutaneous bridges were constructed immediately prior to use by warming the 3% agar gel and injecting the solution into the catheter and were then attached to 3M KCl/calomel reference electrodes (Baxter; Deerfield, IL). The agar catheter used in the multicenter clinical trial was constructed and used at each center according to the instructions provided.

The TDN SOP was used for all NPD measurements, including reagent conditions, sequence, and perfusion times, except for the difference in nasal catheter method and reference electrodes described above (Fig 1). The initial potential difference (PD) was measured at the anterior tip of the inferior meatus; then, the basal PD was measured at various distances within the inferior meatus in each nostril. The nasal catheter was fixed at the most negative potential in the right nostril, and the test was initiated using superperfusion with Ringer solutions for a minimum of 1 min and up to 5 min, until a stable value (± 0.5 mV over 30 s) was obtained. Superperfusion of subsequent solutions was 3 min in duration and included Ringer solution with 100 μ M amiloride, zero-chloride gluconate solution with amiloride, and zero-chloride gluconate solution with amiloride and 10 μ M isoproterenol. The temperature of the solutions used for superperfusion was 32°C to 35°C. Adenosine triphosphate (ATP) in zero-chloride with isoproterenol and amiloride was superperfused as a positive control at the end of the test. The PDs were recorded continuously using an electronic bioamplifier system with integrated data output/analysis software optimized for use (AD Instruments; Colorado Springs, CO). The procedure was then repeated in the left nostril and average values were determined.¹⁹

Assessment of Artifact Frequency and Tracing Stability

All tracings were analyzed by a single investigator using a computer-assisted scoring program (LabChart, version 6.0; AD Instruments). The potential difference after each perfusion solution was calculated as the mean value of a 10-s scoring interval obtained at the end of the solution perfusion. Each tracing was scored after 15-Hz low-pass filtering at a data capture rate of 1,000/s. Artifacts were counted manually by a single investigator and considered present if the potential difference value shifted > 5 mV, was not sustained, or was otherwise considered reliable. Tracing stability was assessed as the SD of the filtered potential difference during the 10-s scoring interval.

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FIGURE 1. Schematic of the nasal potential difference apparatus. Nonperfusion catheter (agar) (A). Perfusion catheter (B).

Descriptive statistics (mean, SD, and SEM) were compared using paired Student *t* test or analysis of variance, as appropriate. All statistical tests were two sided and were performed at a 5% significance level (ie, $\alpha = 0.05$) using SPSS software (SPSS Inc; Chicago, IL) and Microsoft Excel (Microsoft; Seattle, WA).

RESULTS

A total of 26 subjects from six centers underwent randomization and completed the protocol. The mean age of the subjects was 39 (± 12) years, and 78% were women (Table 1). Representative tracings are provided in Figure 2 and include examples of transient excursions and sustained breaks (Fig 2A). Comparison of tracings obtained using the agar catheter method with those obtained using the perfusion method in the same nostril of the same subject revealed that tracings using the agar catheter were more stable and exhibited a marked reduction in the frequency of artifacts throughout the study. Both transient excursions and sustained breaks in the tracings were less common with the agar catheter method (Figs 2A, 2B). Although a high-frequency (60 Hz or 50 Hz, the wall current frequency in the United States and Europe, respectively) interference signal in tracings of the unfiltered signal was observed using both methods, the amplitude of this signal was consistently greater in the tracings generated using the perfusion method, indicating the presence of a larger electrical interference signature with the perfusion method (Figs 2C, 2D). This suggested that the catheter constructed with the agar gel interface is less susceptible to the acquisition of electrical noise signatures that originate from the environment (ie, wall current frequency) than the electrode with the continuous perfusion interface. The remaining electrical noise in tracings following a 15-Hz low-pass filter obtained using the catheter with the agar gel interface could be attributed to the underlying ECG signature of the study subject (Fig 2E).

Quantitative analysis of the artifact frequency in tracings generated following a 15-Hz low-pass filter

FIGURE 2. Representative nasal PD tracings generated in the multicenter, randomized, crossover trial from the same nostril on the same subject. Representative tracing generated using the continuous perfusion probe (A) and the agar nasal catheter (B), each following 15-Hz low-pass filtering of original data acquired at a sampling rate of 1,000/s. An example of a transient excursion is indicated by the white arrow, and an example of a sustained break in the tracing is indicated by the black arrow. Unfiltered PD tracings obtained from the same nostril of the same subject during a stable period of the filtered tracing (C, D). PD tracing using the agar method following 15-Hz low-pass filtering after establishment of a steady baseline (E). PD = potential difference.

Table 1—Demographic Features of Subjects in the Multicenter Trial

Center	No. Subjects	Mean Age, y (SD)	Median Age, y (Range)	No. Women (%)
All	26	39 (12)	33 (23-59)	78
Center 1	6	36 (14)	32 (23-59)	50
Center 2	4	43 (12)	44 (31-54)	100
Center 3	5	45 (11)	48 (32-58)	80
Center 4	5	42 (13)	39 (29-59)	60
Center 5	2	38 (8)	34 (32-47)	100
Center 6	4	28 (1)	29 (28-31)	100

showed a lower frequency of both transient and sustained artifacts when the electrode with the agar catheter was used. The frequency of these artifacts was lower during superperfusion of Ringer and amiloride solutions used to measure sodium conductance (Fig 3A) and during superperfusion of zero-chloride gluconate and isoproterenol perfusion used to estimate chloride conductance (Fig 3B). The stability of the tracings following 15-Hz low-pass filtering was also compared by determining the variability of the measure (1,000 measures/s) over the 10-s scoring interval following perfusion with each test solution. A graphic representation of the scoring interval used in this analysis is shown in Figure 3C. Stability was consistently greater for the tracings generated using the agar catheter in both the right (first) nostril and left (second) nostril (Fig 3D), and for the portions of the tracings related to measures of either sodium or chloride transport (Fig 3E). The overall stability of the tracings submitted by each center indicated that improvements in tracing stability were seen across all centers (Fig 3F). The overall mean artifact frequency was reduced by 75% (5 per tracing for the agar catheter vs 20 per tracing for the perfusion method, $P < .001$) with the use of the agar catheter, and the tracings were significantly more stable than with the continuous perfusion method (SD reduced by 40%, $P < .001$), confirming that the agar method was more stable in the measurement of NPD.

As shown in Figure 4A, comparison of aggregate NPD values (each tracing represented the average of both nostrils) indicated that a more polarized PD was obtained when the agar catheter method was used for measurement; this included the NPD with Ringer superperfusion (-15.9 mV for agar vs -14.0 mV for perfusion, $P < .05$), the PD obtained at the end of the zero chloride superperfusion (-19.0 mV for agar vs -14.4 mV for perfusion, $P < .05$), the PD obtained at the end of the isoproterenol superperfusion (-31.2 mV for agar vs -24.8 mV for perfusion, $P < .05$), and the maximally negative ATP response (-34.0 mV for agar vs -25.3 mV for perfusion, $P < .005$). In contrast, the difference in PD obtained at the end of the amiloride superperfusion was not significantly different (-5.7 mV for agar vs -4.6 mV for perfusion, $P = \text{NS}$). These results suggest that the agar catheter method exhibits a greater dynamic range than the perfusion catheter method for detecting PD polarization-produced chloride transport (CFTR) and, to a lesser extent, sodium transport (eg, Ringer solution). Because this may translate into greater values of sodium or chloride conductance, we compared the derived values obtained by the difference in PD in response to perfusion with the test solutions. No significant differences were

observed in either the Δ amiloride values (10.2 mV for agar vs 9.4 mV for perfusion) or the Δ % amiloride values (64% for agar vs 71% for perfusion), indicating that the two methods were comparable in terms of assessment of amiloride-sensitive sodium conductance, other than the slight difference in end Ringer PD (Fig 4B). In contrast, as shown in Figure 4C, the Δ zero chloride (-13.3 mV for agar vs -9.9 for perfusion, $P = .05$) and Δ zero chloride + isoproterenol (-25.5 mV for agar vs -20.3 mV for perfusion, $P < .05$) were significantly more polarized when the agar catheter was used, although the Δ isoproterenol alone was not significantly different (-12.2 mV for agar vs -10.4 mV for perfusion, $P = \text{NS}$). The average difference between measures is shown in Figure 4D. When the Δ zero chloride + isoproterenol measurements were analyzed on an individual subject basis, the majority of subjects demonstrated hyperpolarization with the agar catheter (Fig 4E). Evaluation of the agar catheter in CF subjects is provided in the online supplement, as is a pilot comparison of normal subjects with the agar catheter and an alternative nonperfusion method.

An alternative nonperfusion method previously described is an ECG cream-filled catheter design.²⁰⁻²² To confirm that the benefits of the nonperfusion approach using the agar catheter could also be observed using an alternative nonperfusion approach, one separate center compared the agar nasal catheter with the ECG cream-filled nasal catheter, which were analyzed separately. The ECG catheters were fashioned similarly except that the catheter tubing was filled with a 1:1 slurry of ECG conducting cream (Signa; New York, NY) and Ringer solution and connected to an Ag/AgCl reference electrode system (Warner Instruments; Hamden, CT).²³ Enrollment criteria were otherwise the same. The aggregate NPD for the five subjects who completed NPD testing with each method is shown in Figure 5A. In this analysis, only differences in zero chloride measurements approached statistical significance (-18.4 mV for agar vs -25.3 mV for ECG, $P = .054$). When analyzed for values of sodium conductance (Fig 5B) or chloride conductance (Figs 5C, 5D), no differences were observed, suggesting that the ECG-cream nonperfusion method also confers the benefits noted with the agar catheter. Variability associated with each method is shown in e-Figure 1.

DISCUSSION

To our knowledge, this study represents the first rigorous, randomized, controlled, multicenter comparison of methods for measurement of the NPD. Our results demonstrate that an agar catheter yields

significantly enhanced data quality compared with the perfusion catheter. The improvements in signal quality and tracing stability address important limitations of the NPD assay that have adversely affected reproducibility in previous studies.^{19,24} These findings were observed both in centers with operators experienced in using KCl/calomel electrodes and in centers in which operators had little previous experience in the use of these electrodes.

Although we anticipated the use of the agar catheter would improve signal quality and reduce the frequency and severity of artifacts, the ~20% increase in chloride conductance observed using the nonperfusion method was not fully anticipated. The lack of continuous perfusion with Ringer electrolyte solution in the probe electrode results in a more potent chloride secretory gradient at the site of measurement, and this at least partially explains the enhanced CFTR-dependent chloride transport observed with the agar catheter. For example, the slow perfusion of Ringer in the standard perfusion technique results in a 5.6-mM concentration of chloride at the contact point with the nasal mucosa during zero-chloride glucuronate superperfusion, as opposed to ~0 mM with the agar catheter. A 6-mM increase in chloride gradient was reported to induce a 2-mV hyperpolarization in normal subjects in a study by House and Middleton.²⁵ This explanation would also account for the enhanced chloride conductance measurements observed in our study (including with the ECG nonperfusion method), compared with the relative absence of a change in sodium transport measurements. Use of the agar catheter also avoids the accumulation of microbubbles in the nasal catheter that can occur during the course of the continuous perfusion method, which can induce an electrical resistance in the circuit and reduce the PD magnitude, and could account for the slightly diminished PD seen with Ringer perfusion using the perfusion method.

The techniques for performing NPD have historically varied widely among sites proficient in its conduct and have included differences in solution content, perfusate temperature, nasal interface, reference interface, electrodes, and method of data capture. The results of our study provide an evidence basis for use of a nonperfusion catheter, and the protocol described here also addresses other aspects of

variable methodology. The differences in tracing stability among centers also point to the importance of attention to detail in performing the procedure, including accounting for the unique anatomic features of individual subjects. Based on our observations derived from tracings submitted from a large cross section of CF and normal subjects submitted by TDN centers undergoing a standardized qualification process, the benefits of this approach appear generalizable to NPD centers outside those included in this study. In tracings of CF subjects submitted from 15 centers participating in a quality assurance program, the mean chloride conductance observed was 1.3 ± 4.1 mV (agar method, $n = 34$) vs 3.6 ± 4.2 mV (perfusion method; $n = 18$; $P = .057$). We have also observed a trend toward enhanced Δ zero chloride + isoproterenol in qualification tracings submitted from normal subjects (-22.0 ± 10.1 vs -18.6 ± 10.7 mV with agar [$n = 42$] and perfusion [$n = 23$], respectively; $P = .22$).

CONCLUSIONS

A number of CFTR modulators that can be used potentially to improve the underlying defect in CFTR activity are being evaluated in clinical testing, including potentiators of surface-localized CFTR,¹¹ correctors of $\Delta F508$ CFTR processing (clinicaltrials.gov, NCT00865904), and suppressors of premature termination codons.^{8-10,23} The development of each of these approaches has used proof-of-concept studies based on NPD measurements. Given the importance of this biomarker in the measurement of CFTR function, a fully standardized assay that can be performed worldwide is required. The nonperfusion catheter represents a significant advance in the execution of the assay that confers improved signal intensity, ease of use, and cost, and can be rapidly taught to both experienced and naïve NPD operators. As such, use of the agar catheter and the NPD method described here provides standardized test conditions, type of electrode, and data capture, which could facilitate comparison of data among centers and among different clinical studies.^{15,19} Given its ease of use and wide acceptance by operators, the nonperfusion catheter should be considered for use in trials using the NPD end point.

FIGURE 3. Frequency of artifacts encountered during nasal PD tracings obtained in the multicenter, randomized, crossover trial. Each two-nostril tracing was scored for artifacts following 15-Hz low-pass filtering. Artifacts were considered present if the PD tracing shifted >5 mV and was not sustained or otherwise considered reliable. All artifacts, artifacts sustained for >10 s, and artifacts sustained for >30 s were tabulated (A-B, D-F). Results shown are the mean $\pm$ SD. Number of artifacts during portion of tracing associated with Na conductance measurements (A). Number of artifacts during portion of tracing associated with Cl conductance measurements (B). Representative PD tracing to demonstrate methodology used to quantify tracing stability (C). Open boxes represent the 10-s scoring interval used to quantify tracing stability. SDs of PD measurements were obtained during the 10-s scoring interval (D-F). Tracings were scored following 15-Hz low-pass filtering for the right and left nostril (D), the Na and Cl conductance measurements for both nostrils (E), and for all the two-nostril tracings stratified by each individual center (F). Cl = chloride; Na = sodium. See Figure 2 legend for expansion of the other abbreviation.

FIGURE 4. Comparison of nasal PD measurements obtained in the multicenter, randomized, crossover trial. Results shown represent the mean $\pm$ SD for all subjects ($N = 26$) (A-C). Each data point represents the PD obtained 3 min after superfusion with Ringer (labeled Ringers), Ringer + amiloride ($100 \mu\text{M}$) (labeled Amil), zero chloride gluconate with amiloride ($100 \mu\text{M}$) (labeled Zero Cl⁻), zero chloride Ringer gluconate + amiloride ($100 \mu\text{M}$) + isoproterenol ($10 \mu\text{M}$) (labeled Iso), and zero chloride gluconate + amiloride ($100 \mu\text{M}$) + isoproterenol ($10 \mu\text{M}$) + adenosine triphosphate ($10 \mu\text{M}$) (labeled ATP), all at 32°C to 35°C (A). Within tracing measures of sodium transport (B). PD at end of Ringer perfusion, change in PD on perfusion with amiloride (Δ Amil), and percentage change on perfusion with amiloride (Δ %Amil). Within tracing measures of chloride conductance (C). Changes in PD on perfusion with zero chloride gluconate (Δ Zero Cl⁻), perfusion with isoproterenol (Δ Iso), and perfusion with zero chloride gluconate + isoproterenol (Zero Cl⁻ + Iso). $*P < .05$; $**P < .005$. Differences in nasal PD values obtained using the agar and continuous perfusion methods (D). $*P < .05$; $**P = .05$; $\pm 95\%$ CIs. The total chloride conductance value (Δ Zero Cl⁻ + Iso) as determined using the standardized agar catheter and the continuous perfusion method is plotted for each individual subject (E). The graph is color coded to indicate the results from the six different centers participating in the trial. $*P < .05$. See Figure 2 legend for expansion of the other abbreviation.

FIGURE 5. Comparison of nasal PD measurements obtained using two nonperfusion methods (agar gel interface and ECG cream interface) at a single center experienced in both techniques. The results shown represent the mean $\pm$ SD, $N = 5$ (A-C). Each point represents the PD obtained 1 min after superfusion with Ringer, and 3 min after superfusion with Ringer + amiloride (100 μ M) zero chloride Ringer with amiloride (100 μ M), zero chloride gluconate + amiloride (100 μ M) + isoproterenol (10 μ M), and zero chloride gluconate + amiloride (100 μ M) + isoproterenol (10 μ M) + ATP (10 μ M), all at 32°C to 35°C (A). Within tracing measures of sodium transport (B). PD at end of Ringer superfusion, change in PD on superfusion with amiloride, and percentage change on superfusion with amiloride. Within tracing measures of chloride conductance (C). Changes in PD on superfusion with zero chloride gluconate, superfusion with isoproterenol (Δ Iso), and superfusion with zero chloride gluconate + isoproterenol. The total chloride conductance value as determined using the standardized agar gel catheter and the ECG cream method is plotted for each individual subject (D). See Figure 2 and 4 legends for expansion of the abbreviations.

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Dr Solomon: contributed to the development and pilot testing of the agar catheter, the performance of NPDs, the supervision of statistical analysis, and the writing of the manuscript, and was one of the overall principal investigators.

Dr Konstan: contributed as principal investigator at his site.

Dr Wilschanski: contributed as principal investigator at his site.

Dr Billings: contributed as principal investigator at her site.

Dr Sermet-Gaudelus: contributed as principal investigator at her site.

Dr Accurso: contributed as principal investigator at his site.

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Dr Clancy: contributed to the development of the agar catheter, assistance with the study design, and the revision of the manuscript.

Dr Rowe: contributed to the development and pilot testing of the agar catheter, the performance of NPDs, the supervision of statistical analysis, and the writing of the manuscript, and was one of the overall principal investigators.

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Additional Information: The e-Figure can be found in the Online Supplement at <http://chestjournal.chestpubs.org/content/138/4/919/suppl/DC1>.

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