Computer assisted measurement of airway gland secretions by the Hillocks technique

Jonathan E. Phillips *, John A. Hey, Michel R. Corboz

Schering-Plough Research Institute, Allergy (M/S 1600), 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

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Abstract

We describe a computer-based image acquisition and analysis system designed for the quantitation of airway submucosal gland fluid flux by the hillocks technique. This technique is based on the detection of the increase in surface area of hillocks formed above gland duct openings by secretions captured under a thin layer of tantalum powder. The advantages of the system are: (a) ability to detect and measure individual submucosal gland secretions, (b) computer assisted data acquisition and analysis, and (c) decreased propagated error in hillock volume measurements. Test results of a practical implementation of the system demonstrate a swine tracheal submucosal gland flux induced by the muscarinic agonist acetylcholine (50 μM) of 0.65 μl/min/cm². © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

In healthy airways, mucus comprises approximately 1% of the thin layer of fluid, which protects the surface epithelium. The airway epithelium is composed of many different cell types and although all are capable of contributing to the total airway secretion, the volumetric contribution of the glands is most important in airway hypersecretory disease [1]. In diseased airways, changes in the quantity and physiochemical properties of the airway lining fluid contribute to the morbidity of diseases associated with mucus abnormalities including cystic fibrosis, asthma, and chronic bronchitis. These diseases to a varying extent are characterized by excessive airway secretion due to submucosal gland hypertrophy and hyperplasia.

The hillocks technique was developed by Davis et al. [2] in anesthetized and paralyzed dogs where they measured the number of hillocks that appeared on the surgically exposed tracheal epithelium per unit time following electrical stimulation. In the hillocks technique, particles of tantalum are sprayed onto the epithelial surface to prevent dispersion of fluid secreted from the glands. Any secretions from the glands induced by chemical or
electrical stimuli cause hillocks to form over the gland duct openings in the epithelial surface and changes in the number and diameter of the hillocks are observed with a microscope. The purpose of this work is to introduce a computer-based image acquisition and analysis system using the computational power of current image analysis software to quantitate airway submucosal gland secretions with improved accuracy and computer assisted analysis of data acquired by the hillocks technique. All previous studies using the hillocks technique have focused on airway submucosal glands while the hillocks technique could also be used to study other glands such as mucus glands in gut tissues, skin sweat glands, and mandibular salivary glands. Two-thirds of the manuscripts only report the change in the number of hillocks per image after challenge with chemical or electrical stimuli and do not measure hillock area [3,4]. The other studies determine hillock areas from measurements of hillock diameters using a video-image shearing monitor [5,6]. This method is time consuming and induces error by not measuring the hillock area directly. Our interactive data acquisition and analysis system not only determines the number of hillocks per image but also calculates the gland fluid flux from measurements of the hillock areas. It is a useful tool to study the glandular contribution to the total airway secretion along with the regulatory control and volume output of individual submucosal glands. To test our acquisition and analysis system, submucosal gland secretions from swine tracheal epithelia were studied before and after stimulation by the muscarinic agonist acetylcholine, a potent mucus gland secretagogue [7]. We have used swine trachea in the present study as this tissue is histologically comparable to human airways in terms of submucosal gland location and organization [8].

2. Materials and methods

2.1. Experimental procedure

Swine tracheae from three (59–91 kg) pigs were obtained from the local abattoir and cut into tubes composed of three or four cartilage rings. The posterior half of the tube was cut away exposing the anterior epithelium. The pieces of anterior epithelium with cartilage attached were placed in a temperature controlled (37°C), water-jacketed, tissue bath (158400; Radnoti, Monrovia, CA) filled with 5 ml of Hanks’ Balance Salt Solution (HBSS) continuously bubbled with 95% O2 and 5% CO2. The composition of HBSS in mM is: NaCl, 136.8; dextrose, 5.6; KCl, 5.4; NaHCO3, 4.2; CaCl2, 1.3; MgSO4, 0.8; KH2PO4, 0.4; and Na2HPO4, 0.3. After a 45 min equilibration period, a piece of trachea was removed from the bath and the epithelial surface was blotted with a tissue wiper (Kimwipes; Kimberly-Clark Co., Roswell, GA) to remove any airway secretions present. The epithelial surface was evenly coated with aerosolized tantalum from an aerosol generator (WDFII; BGI Incorporated, Waltham, MA) and the tissue was replaced in the tissue bath under the microscope for 6 min while images were acquired every 3 min. The first two images taken at \( t = 0 \) (just after application of the tantalum powder) and \( t = 3 \) min (just before addition of acetylcholine) were used to measure baseline gland secretion. The gland secretion was then stimulated by addition of acetylcholine (50 \( \mu \)M) to the HBSS. The images taken at \( t = 3 \) min (just before addition of acetylcholine) and \( t = 6 \) min (3 min after addition of acetylcholine) were used to calculate the acetylcholine-induced gland secretion. The areas of the hillocks within the epithelial surface area digitized by the CCD (Charged Coupled Device) camera (C2400-75, Hamamatsu, Bridgewater, NJ) were measured and converted to volumes assuming the hillocks were hemispheres. The results are expressed as mucus gland flux (\( \mu l/min/cm^2 \)) by dividing the total calculated volume of the hillocks by the 3 min data acquisition time interval and the digitized epithelial surface area (0.042 cm²).

2.2. Application of tantalum powder

The Wright dust feed [9] is a dry powder aerosol generator suitable for deagglomerating and dispersing dry dusts. A biologically inert dust (1–5 \( \mu \)m particle size tantalum; Atlantic Equip-
ment Engineers, Bergenfield, NJ) is aerosolized and applied to the epithelium.

The dust feed (Fig. 1) can be operated after the powder to be aerosolized is packed into a cake, of which the surface is then scraped off in a very thin layer into an air stream. Thirty grams of tantalum powder are compacted with a hammer and ram rod at a time into the dust cylinder (A) of 0.67 cm radius until the cake density is 8.5 g/cm³. The dust cylinder is inverted and placed over the scraper head (B). A motor and gear mechanism spins the dust cylinder at 6 rpm and lowers the dust cylinder 0.18 mm per revolution toward the scraper head (B). The groove (C) in the scraper head collects 1.3 g of tantalum per min.

The tantalum is pushed out of the groove by dry compressed air flowing from a rotometer and needle valve assembly (GF-5321-1502; Barnant Co., Barrington, IL) at 18 l/min into the air inlet (D). This is the lowest air flow rate that provides enough energy to completely disperse the tantalum. The air and tantalum proceed down a small hole (E) in the center of scraper head (B) and through the lumen of tube (F). The dust is then deagglomerated as it passes through the nozzle (G), strikes plate (H), and is finally aerosolized.

2.3. Hillock area measurement

The areas of the hillocks are measured over time from 40 × magnified images digitized by a CCD camera coupled to a microscope (Axiootech-Vario; Carl Zeiss, Thornwood, NY). Two macros (Fig. 2) were written for the KS-300 software package supplied with the microscope.

The image acquisition macro (Fig. 2a) has four parts. The first part deletes any images in the memory of the computer and opens image display windows for the newly acquired images. The second part sets the image acquisition time interval (milliseconds), the total number of images to be digitized, and allows the user to input the root name for the images saved for each experiment. The third part notifies the user to confirm the camera setup, which usually involves adjusting the position of the epithelium under the microscope, the intensity of the light source, and the microscope focus. The final part digitizes and saves to the hard drive in the data acquisition computer three tagged image file format (tif) images; one at \( t = 0 \) (just after application of the tantalum powder), one at \( t = 3 \) min (just before addition of acetylcholine), and one at \( t = 6 \) min (3 min after addition of acetylcholine).

The data analysis macro (Fig. 2b) also has four parts and interactively measures the individual areas of the hillocks on each image, consecutively. The first part of the analysis macro deletes any images from the computer's memory, loads a geometric calibration that allows the software to determine the actual area represented by each pixel, and defines the directory where the images are stored in the computer. The geometric calibration values are determined from interactive measurements of an image of a length scale. Our 3.7 μm pixel size \( (P, the length of the side of a square pixel) \) was determined from a 640 × 480 pixel digitization of an image with 2370 μm by 1780 μm dimensions. The next part sets an approximate value for \( \pi \), the loop counter variable (imgs) to one, and initializes the root name variable (imname) to the string 'exp'. The next section of the macro defines the parameters for a database (spread sheet) that will store the image number along with the measured area and calculated vol-

Fig. 1. Schematic of the Wright dust feed used to evenly coat epithelium with tantalum power aerosol. (A) Dust cylinder, (B) Scraper head shown in more detail in upper left hand corner, (C) Groove, (D) Air inlet, (E) Hole in center of scraper head, (F) Aerosol outlet tube, (G) Nozzle, and (H) Plate. Taken from B.M. Wright, J. Sci. Inst., 2, (1950) p. 14, (with kind permission from the Institute of Physics Publishing) [9].
Fig. 2. Macros written for the KS300 software package to perform (a) image acquisition and (b) analysis of images to interactively measure hillock areas and calculate hillock volumes.

\[ \text{Hemisphere Volume} = \frac{2}{3} \sqrt[3]{\pi (\text{Hillock Area})^{1.5}} \]  

2.4. Calibration

A calibration was performed to determine the accuracy and precision of the area measurements. Seven images of an image analysis micrometer (K53-713; Edmund Industrial Optics, Barrington, NJ) with four circles of known diameter were acquired and analyzed. The accuracy of the area measurements (range $3 \times 10^{-3}$ to 0.2 mm$^2$) is represented by the small deviation of the slope (1.003) and intercept ($-2.5 \times 10^{-8}$ mm$^2$) of the statistically adequate linear model from identity and zero, respectively (Fig. 3). The precision is represented by the 95% confidence intervals.

3. Results

Seven porcine tracheal tissues from three pigs exhibited no detectable baseline airway submucosal gland secretion (Fig. 4a) during the 3 min
measurement period. However, after challenge with acetylcholine (50 μM), the fluid flux from the submucosal glands was stimulated to 0.65 ± 0.15 μl/min/cm² of tissue (n = 7, Fig. 4b and Table 1). The smallest hillock area measured under the present magnification (40 × ) was 0.02 mm² and the largest was 0.35 mm² (Fig. 4b). Assuming these hillocks are of hemispherical shape (Eq. (1)), this corresponds to volume secretions of 1 and 78 nl, respectively.

4. Discussion

In the present study, we have described and demonstrated the utilization of a computer-based image acquisition and analysis system for measurement of airway submucosal gland fluid flux. The use of a computer assisted image analysis macro to measure the area of each hillock reduces the analysis time and the error induced by the original hillock area measurement method which calculated hillock areas from diameter measurements [5]. An improvement in accuracy is realized due to less propagated error from area measurements than from diameter measurements. Indeed, the propagated error increases as a function of the square root of area when area measurements are used to calculate hillock volume, whereas it increases in direct proportion to area when hillock diameter measurements are used.

We used swine tracheal epithelia in the present study because the distribution of the different types of mucin (acidic and neutral glycoprotein) in pig glands are similar in humans [10] and pig submucosal glands are more histologically com-
parable to human submucosal glands than other species [11]. In the present study, we also found that the density of submucosal glands in swine trachea is similar to the adult human trachea (~1 gland/mm²) [12]. Our measured acetylcholine-stimulated submucosal gland flux of 0.65 µl/min/cm² in pig is comparable with the 0.4 µl/min/cm² induced by another muscarinic agonist methacholine (50 µM) in ferret in vitro [13], but smaller than those obtained by mechanical stimulation of larynx (7.4 µl/min/cm²) in dog in vivo [14], and by electric field stimulation (2.5 µl/min/cm²) in ferret in vitro [13]. Volumetrically greater secretions are expected in vivo when compared to in vitro conditions due to intact vasculature and neural regulation. Electrically stimulated secretions in vitro are likely greater than our acetylcholine (cholinergic) induced secretions due to the combined effects of all cholinergic, adrenergic, and non-adrenergic/non-cholinergic (NANC) secretagogues released [7]. It is interesting to note that a baseline (unstimulated) hillock formation was observed in the only other in vitro swine study using the hillocks technique [8], but the gland flux was not calculated in this study. The finding that we did not observe a baseline hillock formation may be due to our different method of application of the tantalum powder or to a difference in the experimental measurement time interval used in the present study.

The current 40 × total magnification allows on average the measurement of four hillocks of 0.15 mm² area per image (Table 1). The ideal magnification for measurement of submucosal gland fluid flux is an optimization between the maximum magnification, where the user starts to image regions with no glands; and the minimum magnification, when area measurement become inaccurate. By decreasing the magnification to 25 ×, the average number of hillocks per image should increase to six giving an improved average value for the submucosal gland flux. However, only a certain level of accuracy can be achieved because pixel-based images represent an approximation to the continuous real scene being represented. An overestimate in hillock area measurement due to the digitized image (digitization error) comes from pixels lying partially outside of the hillock boundary interactively drawn by the data analyst. A hillock is represented as a circle of radius r, and the image pixel size (P) is represented as a square with diagonal length d in Fig. 5. An estimate of the increase in error of the hillock area measurement due to decreasing magnification can be derived from Eq. (2) and Eq. (3).

\[
\%\text{DAI} = \frac{\pi(r + d)^2 - \pi r^2}{\pi r^2} \times 100 \tag{2}
\]

\[
\Delta d = \frac{2P(\sqrt{MR} - 1)}{\sqrt{2}} \tag{3}
\]

Table 1

<table>
<thead>
<tr>
<th>Tracheal tissue</th>
<th>Number of hillocks</th>
<th>Average area per hillock (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>0.21</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.11</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.07</td>
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<tr>
<td>5</td>
<td>2</td>
<td>0.16</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>0.20</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0.21</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>4 ± 1</td>
<td>0.15 ± 0.02</td>
</tr>
</tbody>
</table>

Fig. 5. Representation of a hillock as a circle with radius r. The image pixel size is represented as a square with diagonal length d. The outer (gray) ring represents the maximum increase in hillock area measurement due to digitization.
The maximum possible percentage increase in hillock area measurements due to digitization or percent digitization area increase (%DAI) can be calculated with Eq. (2). In Fig. 5, the %DAI is the area of the gray outer ring divided by the actual hillock area ($\pi r^2$). Eq. (3) represents the change in pixel diagonal length ($\Delta d$) as a function of $P$ and the magnification ratio (MR), which is the magnification used to determine the pixel size divided by the desired magnification.

Eq. (4) is used to calculate an estimate of the maximum percent increase in error of the hillock area measurement due to decreasing magnification ($\Delta%$ error). It is derived by inserting $\Delta d$ from Eq. (3) into a simplified Eq. (2) for $d$, with the assumption that the change in $P$ is small compared to $r$. In our case, $P$ in Eq. (4) is 3.7 $\mu$m at 40 x magnification and the MR (40/25) is the magnification used to determine the pixel size (40 x) divided by the desired magnification (25 x).

$$\Delta% \text{ error} = \frac{4P(\sqrt{\text{MR}} - 1)}{\sqrt{2r}} \cdot 100 \quad (4)$$

Due to digitization at the lower magnification, the smallest hillock area measured (0.02 mm$^2$, $r = 80 \mu$m) would yield an estimated 4% increase in area while the largest hillock (0.35 mm$^2$, $r = 334 \mu$m) measured would realize an estimated 1% increase in area. Eq. (4) calculates an upper limit to the hillock area measurement error induced by increasing pixel size and will always be an overestimate of the actual error. Therefore, the lower (25 x) magnification will yield an improved value for gland flux by averaging over a 60% larger area of epithelium while only inducing a minor loss in accuracy of hillock area measurement. The calibration graph (Fig. 3) incorporates the digitization error along with other experimental errors such as inclusion or exclusion of shadows and reflections in the hillock area measurements.

We would like to emphasize that alternatives to the hardware and software used in this manuscript exist. The traditional hillocks technique with the new data acquisition and analysis methods described in the present study can be implemented by using any microscope that can acquire digital images. The images can be analyzed by the public domain image analysis program ImageJ (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/ij/), a platform independent Java application which has the necessary image analysis capabilities and subroutines to control image acquisition from some CCD cameras. Also, an alternative method to spraying the tantalum power on the epithelium with the Wright dust feed has been described by Steiger et al. [8] where the tantalum powder was sieved through a fine nylon mesh.

In conclusion, a microscope based image analysis system and programs have been assembled to: (a) measure the increase in the area of hillocks due to airway submucosal gland secretions and (b) to calculate gland secretory fluxes. These data were acquired using the classical hillocks technique which is capable of detecting nanoliter changes in the volume of airway gland secretions. We also describe in detail for the first time the method for application of the biologically inert powder to the epithelium. We conclude that our image analysis macros combined with a detailed description and analysis of the experimental parameters involved with the hillocks technique will greatly assist the researcher in obtaining computer assisted and accurate information concerning gland fluid secretion. The method described in this study can be used not only to delineate the glandular contribution to total airway secretion and the regulation of airway submucosal gland secretion but also the regulation of gland secretions from other tissues.

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References


