Thoracoscopic Findings and Pharmacokinetics of Inhaled Fluorescein in a Pig Model

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Abstract

Background: Fluorescein-enhanced autofluorescence thoracoscopy (FEAT) reveals regions of abnormal fluorescence in patients with primary spontaneous pneumothorax and in normal subjects. Some of these lesions are undetectable by white light thoracoscopy and it has been hypothesized that they represent underlying pleural and/or parenchymal abnormalities. Objectives: In order to standardize and evaluate this novel technique, we developed an animal model. Methods: Six pigs underwent thoracoscopy after the inhalation of nebulized sodium fluorescein by either volume-controlled mechanical ventilation or spontaneous ventilation. Pleural cavity and lung surface were inspected by white light thoracoscopy and FEAT during a period of 90 min. Fluorescence intensities were quantified in pleura and in blood. Regions of interest were examined postmortem for a histological assessment of the lesions. Results: FEAT lesions were observed in all animals, with a maximum intensity of the lesions 20–30 min after the onset of fluorescein administration. The plasma concentrations of sodium fluorescein reached a maximum after approximately 20 min. The microscopic findings suggest that fluorescein accumulates in the subpleural space of better ventilated lung areas. Conclusions: This is the first animal model using FEAT. Valuable information has been gathered but further investigations are required to explain the phenomena observed in humans and pigs.

Key Words
Sodium fluorescein · Thoracoscopy · Pig model · Fluorescein quantification

Introduction

Fluorescein-enhanced autofluorescence thoracoscopy (FEAT) allows the visualization of lung regions with abnormal subpleural fluorescence [1, 2]. In a study conducted by the senior author of the current paper, patients with primary spontaneous pneumothorax inhaled an aerosolized fluorescein solution prior to medical thoracoscopy [2]. During FEAT we found that: (1) macroscopically abnormal lung regions in white light conditions such as blebs and bullae could also be detected in blue light, and (2) normal lung regions in white light often seemed ab-
normal’ in blue light conditions. These observations seemed to confirm an association between those ‘abnormal’ regions and the disease process in pneumothorax, but the finding of abnormal fluorescence at macroscopically normal parts of the lung was intriguing. Moreover, in a group of control subjects undergoing bilateral thoracoscopy, FEAT revealed significant regions of abnormal fluorescence in contrast to normal lung inspection in white light. It was hypothesized that lung regions with abnormal fluorescence reflect an underlying pathological process which cannot be detected in white light, but the anatomopathological correlation of these observations remained unclear.

Disodium fluorescein \((\text{C}_2\text{H}_8\text{O}_3\text{Na}_2)\) is a relatively low-molecular-weight, highly water-soluble compound which, when exposed to light of wavelengths between 465 and 490 nm (blue), emits light at a wavelength of 520 to 530 nm (yellow-green). The pharmacokinetics of fluorescein have been shown to be linear after intravenous administration. It is rapidly metabolized by the liver with a half-life in plasma of 1.7 min and is excreted in bile and in urine [3]. Only 1 of the 3 metabolites formed in the liver, fluorescein monoglucuronide, is fluorescent. The emission peak is the same as that of fluorescein, but fluorescein glucuronide has been shown to be much more hydrophilic at physiological pH. This means that when using fluorescence intensity as a measurement of fluorescein concentration, fluorescein glucuronide may contribute to the measured intensity [4]. Therefore, plasma concentrations of disodium fluorescein were measured in order to correlate them with the fluorescence intensities observed during thoracoscopy, and to follow its course in time.

Aerosol administration of solid disodium fluorescein has been used in dogs to study the pulmonary and systemic deposition of drug-containing aerosols [5–9]. These studies showed that even a lipophobic compound such as fluorescein is absorbed rapidly via the lung when administered as an inhalation aerosol.

Pleural disease is not uncommon and differential diagnosis sometimes difficult, hence the search for improved technologies to detect pleural abnormalities by thoracoscopy [10, 11]. Autofluorescence bronchoscopy has already been shown to be an interesting and effective tool in the diagnosis of both malignant and nonmalignant lesions [12, 13]. Fluorescein administration by inhalation has been used under white light thoracoscopy conditions in the past but, thus far, with few clinical applications [14], which stands in sharp contrast to the extensive use of fluorescein by intravenous injection in ophthalmology and gastroenterology [15–17]. The aims of this study were: (1) to evaluate and standardize fluorescein administration in an animal thoracoscopy model, and (2) to study the pharmacokinetics of nebulized fluorescein in blood and pleura.

Methods

Animals and Preparation

The experiments, approved by the Institutional Animal Care and Use Committee of the Centre Hospitalier Régional Universitaire de Lille, France, were conducted using 6 experimental animals (pigs). The pigs were each 3 months old, of either sex, and weighed 28 kg on average. All animals had been fasted for 48 h prior to the experiments but were allowed free access to water. The extubation of the tracheal intubation was performed after a 1-cm incision on the animal’s lateral chest. After blunt dissection of the intercostal space, the parietal pleura was penetrated to create a pneumothorax. A 7-mm-diameter pleural trocar was then inserted to inspect the pleural space and lung using a DAFE autofluorescence endoscopy unit (Richard Wolf, Knittlingen, Germany). After an initial thorough inspection of the pleural cavity, the pigs received an aerosol (10 ml) containing either 500 mg (2 pigs) or 250 mg (4 pigs) of fluorescein in approximately 15 min, maximum 18 min.

Aerosol Generation

The apparatus used for the nebulization was an Atomisor® MegaHertz ultrasonic nebulizer (Diffusion Technique Française, Saint-Etienne, France) running with a frequency of 2.4 MHz. According to previous studies, the mass median aerodynamic diameter at the outlet of the nebulizer is 4.2 ± 0.2 μm (mean ± SD) [9]. In the mechanically ventilated pigs, the nebulizer was positioned in the inspiratory limb of the ventilatory circuit, 40 cm proximal to the Y-piece, and filled with a 10% sodium fluorescein solution (5 ml; Novartis Pharma, Vilvoorde, Belgium).

Study Design and Thoracoscopy

After the instrumentation and onset of mechanical ventilation, a stabilization period of 5 min was allowed before exploratory thoracoscopy was started. Thoracoscopy was performed through a 1-cm incision in the animal’s lateral chest. After blunt dissection of the intercostal space, the parietal pleura was penetrated to create a pneumothorax. A 7-mm-diameter pleural trocar was then inserted to inspect the pleural space and lung using a DAFE autofluorescence endoscopy unit (Richard Wolf, Knittlingen, Germany). After an initial thorough inspection of the pleural cavity, the pigs received an aerosol (10 ml) containing either 500 mg (2 pigs) or 250 mg (4 pigs) of fluorescein in approximately 15 min, maximum 18 min.
Venous blood samples were taken at baseline and at 1, 5, 10, 20, 30 and 60 min. The plasma concentrations of sodium fluorescein were determined by spectrofluorometry, and fluorescence intensities were measured as counts per pixel in arbitrary units (AU).

Analysis of Pleural Fluorescence
Video recordings of the visceral pleura were scheduled at regular time intervals using the onset of fluorescein inhalation as reference time point: 0, 2, 5, 10, 15, 30, 45, 60 and 90 min after the onset of nebulization. Video recordings and still images were taken and stored for further image processing. Images were processed using V paint, SigmaScan Pro® (Systat, version 5, trace measurement mode) for fluorescence quantification as described previously [20]. Five fluorescent lesions were analyzed per pig. For each lesion, fluorescence intensities were measured at the center of the lesion (region of interest, ROI) and at surrounding areas with no macroscopic evidence of fluorescence (normal area, NA). Fluorescence intensities were measured as counts per pixel in arbitrary units.

Euthanasia and Histological Assessment
After each experiment, the animals were sacrificed by an injection of potassium chloride, whereas exsanguination was performed through direct cardiac puncture, and the lungs were exposed through a cervicothoracic incision. ROIs and macroscopically normal areas were marked ex vivo under blue light inspection with needles placed 0.5 cm away from the periphery of the area. The lungs were preserved in a fixative (4% formaldehyde) and transported to the Pathology Department where the marked regions were examined by a blinded pathologist.

Statistical Analysis
Data are expressed either as median (25th–75th percentile or the interquartile range, IQR) for continuous data, or as n (%) for categorical data. Differences between groups were determined using the nonparametric Wilcoxon signed rank test for paired data and the Mann-Whitney test for unpaired data. SPSS 13® for Windows (SPSS Inc., Chicago, Ill., USA) was used for the analysis, and p < 0.05 was accepted as significant.

Results
Clinical Findings
All but one pig had a completely normal lung during white light inspection. There were no blebs/bullae, air leakage or anthracotic lesions present. FEAT revealed different types of lesions: small areas of 1–2 mm diameter with intense fluorescence (hot spots) and larger areas with diffuse fluorescence (table 1). Subpleural accumulation of fluorescein was either discrete (+) or intense (+++) as shown in figure 1. Lesions appeared within 5 min after the onset of inhalation, and most lesions were seen at the upper lobe and apex of the pig lungs. Throughout the experiments, SpO2 remained within the normal range in each animal and no unexpected death occurred.

Fig. 1. Examples of FEAT lesions (a, b) and their corresponding white light images (c, d). Hot spots and areas with diffuse accumulation can be seen. a, c Discrete subpleural fluorescein accumulation. b, d Intense subpleural fluorescein accumulation.
Plasma Concentration of Fluorescein

The median time profile of the fluorescein plasma concentration in AU is presented in figure 2. At 15 min, the end of aerosol administration, the median (25th–75th percentile) plasma concentration reached 220 AU (IQR: 199–253), while 30 and 60 min after the onset of nebulization, the concentrations were 282 AU (IQR: 230–380) and 158 AU (IQR: 133–183), respectively.

Pleural Fluorescence

The time profiles of the pleural fluorescein concentration in pigs before and after fluorescein administration are shown in figure 3. The administration of sodium fluorescein by aerosol caused a progressive increase of fluorescence intensity in the visceral pleura (fig. 4). At the end of the administration of fluorescein, the concentration both in the ROI and surrounding areas was significantly higher compared to baseline (Mann-Whitney U test, p < 0.001). The overall fluorescein concentration reached 62.2 AU (IQR: 50.4–71.5) at the ROI and 39.5 AU (IQR: 31.3–40.7) at areas with no macroscopic evidence of fluorescein. An example of fluorescence intensity changes observed in time is shown in figure 5. Time profiles for the ROI and NA are presented in figure 6. The highest concentration at the ROI was observed in mechanically ventilated pigs receiving 5 ml of fluorescein solution.

Table 1. Thoracoscopic white light thoracoscopy and FEAT findings in 6 pigs receiving fluorescein as aerosol by mechanical or spontaneous ventilation

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Ventilation mode</th>
<th>Dose, ml</th>
<th>White light thoracoscopy</th>
<th>FEAT hot spots</th>
<th>diffuse lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VC</td>
<td>5</td>
<td>normal</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>VC</td>
<td>5</td>
<td>normal</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>VC</td>
<td>2.5</td>
<td>normal</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>VC</td>
<td>2.5</td>
<td>normal</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>SV</td>
<td>2.5</td>
<td>adhesions</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>SV</td>
<td>2.5</td>
<td>normal</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

Pleural adhesions were seen in one pig.

VC = Volume-controlled mechanical ventilation; SV = spontaneous ventilation; 0 = absence of visible fluorescein accumulation; + = discrete fluorescein accumulation; ++ = intense fluorescein accumulation.

Fig. 2. Mean plasma concentration of sodium fluorescein after the inhalation of fluorescein solution in pigs. Data are medians and IQR.

Fig. 3. Fluorescence intensity in pleura before and after the administration of sodium fluorescein in mechanically ventilated pigs. Fluorescein concentration in different lung areas are shown. a Mechanical ventilation + 500 mg sodium fluorescein. b Mechanical ventilation + 250 mg sodium fluorescein. c Pigs spontaneously ventilated + 250 mg sodium fluorescein.

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whereas the lowest concentration at the ROI was observed in spontaneously ventilated pigs receiving 2.5 ml of fluorescein.

**Histology**

Twenty-six abnormal ‘FEAT-positive’ areas and 13 macroscopically normal ‘FEAT-negative’ areas were examined microscopically. All tissue samples (n = 39) showed a certain degree of inflammation but a difference was found in microatelectasis (table 2). Significantly more microatelectasis was found in the subpleural space of FEAT-negative samples ($\chi^2$ test, p = 0.045).

**Discussion**

This is the first report of an animal model using FEAT. We compared white light thoracoscopy and FEAT in 6 pigs after the administration of nebulized sodium fluorescein and performed fluorescence quantification. In contrast to a normal lung appearance in white light, FEAT revealed an accumulation of fluorescein in several lung areas in all animals. Fluorescent lesions were visible within 5 min after initiating fluorescein administration and were not associated with blebs, bullae or air leakage. Some of these FEAT lesions were still visible at the time of euthanasia, 90 min after aerosol onset.

FEAT data interpretation depends both on the local concentration of the fluorophore and on the depth of penetration through the lung tissue by the fluorophore [21, 22]. Therefore, FEAT lesions are quantified macroscopically in a semiquantitative fashion as: absence of subpleural fluorescence (0), discrete subpleural glow (+), spots or larger areas with intense fluorescence (++ or +++), and areas with visible fluorophore leakage from the lung (+++ or ++++), as described previously for humans [2]. In this study, however, FEAT lesions were also analyzed by using a method for the quantification of fluorescence intensity [20]. This method revealed different intensities at the center of FEAT lesions (ROI) and in the surrounding, macroscopically normal areas. It confirms that fluorescein diffuses widely into the visceral pleura, but the achieved concentrations are different within lung regions.

The histological findings of the present study suggest that fluorescein accumulates in the visceral subpleural space. Fluorescein is localized extracellularly since it is mainly a hydrophilic molecule. However, under certain circumstances it can be diffused through cellular membranes, either passively or by active transport [23–25].

<table>
<thead>
<tr>
<th>Table 2. Histology of FEAT-positive and FEAT-negative tissue samples</th>
</tr>
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<tbody>
<tr>
<td>Inflammation</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Positive areas (n = 26)</td>
</tr>
<tr>
<td>Negative areas (n = 13)</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

Lung inflammation was classified by the pathologist as: + = minimal; ++ = important; +++ = major. Microatelectasis was either absent or present.

* $p = 0.045$, $\chi^2$ test for atelectasis in FEAT-positive and FEAT-negative areas.

![Fig. 4. Fluorescence intensity changes during FEAT in 6 pigs. At time 0, administration was started. VC = Volume-controlled mechanical ventilation; SV = spontaneous ventilation. SV versus VC: $p = 0.4$, Wilcoxon paired test.](image-url)
Therefore, an increased accumulation of inhaled fluorescent dye in subpleural regions may represent an alteration of physiological barriers which could include the alveolar-endothelial membrane and bronchial-epithelial cell-to-cell junctions.

A histological analysis of the pig lungs showed alveolar inflammation of variable severity in all tissue samples. The only difference found in the histological pattern of FEAT-positive and FEAT-negative areas was in the presence of subpleural microatelectasis. Macroscopically normal areas presented with a significantly higher percent-

**Fig. 5.** Changes of fluorescence intensity in time observed in the paracardiac region of a right upper lobe during FEAT. From top left to bottom right, images were collected at the 5th, 15th, 30th, 45th, 60th and 90th minute.

**Fig. 6.** Fluorescein concentration time profiles in the visceral pleura. Center of FEAT lesions: hot spot (a), area with diffuse accumulation (b). (c) Macroscopically normal surrounding tissue. Fluorescein concentrations at different time points are shown. Data are medians and IQR.
the absolute values of fluorescein concentration in different types of FEAT lesions, no significant differences were observed in the elimination time of fluorescein between hot spots and areas with diffuse accumulation of fluorescein. At 60 min after inhalation, the fluorescein concentration for both was comparable (fig. 6). From a practical perspective this could be useful information, considering that this time interval (15–60 min) after fluorescein administration might be the optimal time period for assessing pleural abnormalities when using inhaled sodium fluorescein during thoracoscopy.

The merit of the current study lies in evaluating a technology that might turn out to have clinical applications, not only pertaining to primary spontaneous pneumothorax but also in the assessment of other visceral pleural diseases such as malignant pleuritis [26]. Furthermore, the fluorescence intensity quantification of ROI and NA by digital image analysis is an improvement compared to the semiquantitative scoring system previously used for FEAT data interpretation. Its negativity, regarding specific underlying pathology, does not diminish its importance in the systematic evaluation of this novel technique called FEAT. After all, from the published human studies it is not clear if all regions that fluoresce represent a clinically significant abnormality, hence the need for an animal study.

Limitations of the current paper include the presence of substantial, widespread evidence of chronic lung inflammation, a common finding in otherwise healthy laboratory pigs that certainly weakens the validity of our model. The pig was chosen as a model for this project because the anatomy and physiology of its lungs can be compared to human lungs. Our study lacks a systematic examination of mechanism, for example by pathological verification of the depth of deposition. Indeed, deeper deposition may scatter the fluorescent light explaining the more diffuse areas. Despite these limitations, our experimental study shows that the pig is a valid animal model for the study of FEAT, and the observed time course profile of inhaled fluorescein can be used in further human studies.

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References


