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Bronchoscopic mucosal cryobiopsies as a method for studying airway disease

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Abstract

Background

Investigating disease mechanisms and treatment responses in obstructive airway diseases with invasive sampling is hampered by the small size and mechanical artefacts that conventional forceps biopsies suffer from. Endoscopic cryobiopsies are larger and more intact and are being increasingly used. However, the technique has not yet been explored for obtaining mucosa biopsies.

Objective

To investigate differences in size and quality of endobronchial mucosal biopsies obtained with cryotechnique and forceps. Further, to check for eligibility of cryobiopsies to be evaluated with immunohistochemistry and in situ hybridization and to investigate tolerability and safety of the technique.

Methods

Endobronchial mucosal biopsies were obtained with cryotechnique and forceps from patients with haemoptysis undergoing bronchoscopy and evaluated by quantitative morphometry, automated immunohistochemistry and in situ hybridization.

Results

A total of 40 biopsies were obtained from 10 patients. Cross-sectional areas were three-fold larger in cryobiopsies (median: 3.08mm² (IQR: 1.79) vs. 1.03mm² (IQR: 1.10), P<0.001). Stretches of intact epithelium were 8-fold longer (median: 4.61mm (IQR: 4.50) vs. 0.55mm (IQR: 1.23), P=0.001). Content of glands (median: 0.095mm² (IQR: 0.30) vs. 0.00mm² (IQR: 0.01), P=0.002) and airway smooth muscle (median: 0.25mm² (IQR: 0.30) vs. 0.060mm² (IQR: 0.11), P=0.02) was higher in the cryobiopsies compared with forceps biopsies. Further, the cryobiopsies had well-preserved protein antigens and mRNA. Mild to moderate bleeding was the only complication observed.

Conclusion and clinical relevance

By yielding significantly larger and more intact biopsies, the cryotechnique represents a valuable new research tool to explore the bronchi in airway disease. Ultimately with the potential to create better understanding of underlying disease mechanisms and improvement of treatments.

Introduction

Patients with obstructive airway diseases, asthma and COPD, form a heterogeneous group where understanding of different underlying disease mechanisms remains a challenge. Further, the necessity of precise phenotyping for patient-targeted treatment of airway disease is evident: Both to ensure correct treatment of patients with severe disease and to optimize use of available resources as costly new pharmacologic agents appear.¹

Obtainment of endobronchial biopsies for research purposes in patients with airway disease is safe and widely used.^{2,3} It enables researchers to obtain *in vivo* tissue material from the airways and has played a key role in both basic and clinical research.⁴⁻⁶ However, the ability to investigate important disease mechanisms, such as immunologic pathways, spatial distribution of immunologic cells and smooth muscle remodelling, is with today's forceps technique limited due to small biopsy size and mechanical crushing artefacts.⁷ Mechanical exfoliation of epithelial cells makes it difficult to examine epithelial integrity and may disturb the analysis of epithelial parameters. When studying airway smooth muscle function, the usefulness of forceps biopsies is also limited, as forceps biopsies often contain little or no smooth muscle. Hence, most studies on airway smooth muscle function rely on animal models and post mortem samples from fatal asthma.⁸

Obtainment of transbronchial lung cryobiopsies has been shown to be useful in diagnosing interstitial lung disease.⁹ The primary advantage of the cryo-technique is that it enables obtainment of larger biopsies with fewer crushing artefacts compared with conventional transbronchial biopsies.^{9–11} Cryobiopsies may therefore replace surgical biopsies in the diagnostic assessment of interstitial lung disease.¹² Additionally, the method is used for endobronchial tissue sampling of tumours and has been shown to increase the diagnostic yield in suspected lung cancer.^{13,14}

Despite these promising results, sampling of the airway wall with the cryotechnique for the examination of airway disease remains unexplored. The ability to obtain bigger and better biopsies with this technique will likely open up for substantial new achievements in research and personalized medicine in airway diseases such as asthma and COPD.

In this pilot study, we compared overall size, morphology and intactness of endobronchial mucosa biopsies obtained with standard forceps and cryo-technique and we evaluated the cryobiopsies for suitability for immunohistochemistry and in situ hybridization. Further, we assessed the tolerability and safety of the cryo-technique.

Methods

Population and ethics

This pilot study was conducted with patients undergoing bronchoscopy as part of a diagnostic work-up for haemoptysis: Patients referred to the respiratory outpatient clinic at Bispebjerg Hospital, Copenhagen, due to haemoptysis and an American Society of Anaesthesiologists physical status (ASA score) ≤ 2 ¹⁵ who were booked for bronchoscopy were given oral as well as written information on the aims, contents and risks regarding the study. Each subject gave oral and written consent to participate in the study. All patients had normal chest X-ray and blood coagulation parameters.

The study was approved by the Danish National Committee on Health Research Ethics (H-16023219) and the Danish Data protection Agency (2012-58-0004).

Bronchoscopy and tissue sampling

During bronchoscopy, with a flexible bronchoscope (Olympus BF-1TQ180/BF-1TH190, Olympus, Hamburg, Germany), four mucosal biopsies were obtained: two with forceps (Endojaw™, Ø 1.9mm, Olympus, Hamburg, Germany) and two with cryo-technique (Erbecryo® 2, Ø 1.9mm, Erbe, Tübingen, Germany). Biopsies were obtained from segmental subcarinae at the discretion of the operator. Order of biopsy method, cryotechnique or forceps, was randomized prior to the procedure. The cryoprobe was placed onto the sampling site initiating a freeze cycle of 2-3 seconds where after the probe and bronchoscope was retracted with the biopsy still frozen and attached to the probe tip. The biopsy was then released from the probe by thawing in a saline bath. After release, all biopsies were immediately subjected to overnight fixation in 4% buffered formaldehyde, dehydration and paraffin embedment until histological analysis.

Bronchoscopy was performed according to the general standard procedure.¹⁶ For sedation, midazolam and fentanyl were administered according to the operator in doses ranging from 5-15 mg and 50-150 µg respectively. A dose of 500-1000 mg tranexamic acid was administered as bleeding prophylaxis. Oral access with bronchoscopic guided placement of an orotracheal tube (Rüsch BronchoFlex® inner Ø 7.5mm) was used for easy access and retrieval of the bronchoscope as well as for protection of the vocal chords from the cryo probe. Supplemental oxygen was administered through a canal in the tube if necessary. During the procedure all complications were registered by the operator. Bleeding was scored as: none, mild, moderate or severe according to British Thoracic Society guidelines.¹⁶

During the procedure patients were monitored with continuous oxygen saturation and repeated vital signs measurements. After ended procedure patients were transferred to the recovery ward for post procedural observation until discharge the same day. Sedation and monitoring parameters are outlined in Table 1.

Histological Analyses

Standard histological techniques were used to evaluate biopsy size and to explore the morphology, the mucosal structural integrity and architecture as well as preservation of proteins/antigens and mRNA for routine molecular analyses. Multiple 3 µm tissue sections were generated from the

paraffin-embedded biopsies. Standard haematoxylin-eosin sections were digitized by an Aperio slide scanner and used for morphometric analyses. Separate sections were tested for suitability to perform routine protein visualization by immunohistochemistry (IHC) as well as visualization of specific gene expression (mRNA) by in situ hybridization (ISH).

Following antigen retrieval in a PT-Link machine (DakoCytomation, Glostrup, Denmark), tissue sections for IHC were subjected to single and double immunostaining via an automated immunohistochemistry robot (Autostainer Plus, DakoCytomation, Glostrup, Denmark) and polymer horseradish peroxidase (HRP)-linked secondary antibodies (EnVision™ G|2 Doublestain System Rabbit/Mouse, K5361, Dako) using DAB and Vina green as detection chromogens.¹⁷ The following primary antibodies were used to test staining for selected structural and immunological cells: anti-alpha smooth muscle actin (clone 1A4, Dako, dilution 1:200), anti-human CD31 (clone JC70A, Dako; dilution 1:50), anti-human IL-33 (clone Nussy-1, Alexis; dilution 1:500), anti-human mast cell tryptase (clone Mab 1222A, Millipore; dilution 1:10 000), mouse anti-human CD3 (clone 2.38, Dako; dilution 1:50), anti-human CD207 (langerin, Leica; dilution 1:100). All primary and secondary antibodies used have been extensively validated for use in clinical diagnostics and research.

In-Situ Hybridization (ISH)

mRNA probes against the human house-keeping gene UBC and the prokaryote gene DapB were used as positive and negative mRNA controls, respectively. The mRNA probes were visualized using the RNAscope 2.5 formalin-fixed paraffin-embedded assay kit (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer instructions.^{18,19} Sections were incubated with endogenous enzyme block, boiled in pre-treatment buffer, and treated with protease prior to target probe (UBC and DapB) hybridization. The target RNA was then amplified using a series of amplification solutions and visualized with coloured chromogen.^{18,19}

Statistics

Descriptive statistics including demographics, smoking and alcohol consumption, baseline spirometry as well as perioperative medication, oxygen supplement and pulse oximetry were performed. Results are presented as means with standard deviations and numbers with percentages. For comparison of size and histological quality parameters of biopsies, medians with range are presented and tests for differences were performed using Mann-Whitney tests.

All analyses were performed using IBM® SPSS statistics version 22 (IBM Software, IL, US).

Results

Population

Out of 15 patients screened, 10 entered the study and a total of 40 biopsies, 20 with cryotechnique and 20 with forceps, were obtained. Patient characteristics are presented in Table 1.

Biopsy size

Representative microscopic images of biopsies obtained with forceps and cryotechnique are presented in figure 1. An overview of biopsy size and quality is outlined in Table 2 with individual measures of each biopsy shown in Figure 2. Overall biopsy sizes were measured as a mean area of biopsy sections using computerized image analysis (Scanscope, Aperio, US). Median tissue cross section area was higher ($P<0.001$) for cryobiopsies: 3.08 mm^2 (IQR: 1.79) than for forceps: 1.03 mm^2 (IQR: 1.10).

Tissue preservation and biopsy content of key bronchial tissue compartments

To give a measurement of tissue preservation, the length of intact epithelium stretches was measured by visual inspection and manual cursor tracing on digitalized section images. The median length of intact epithelium was 4.61 mm (IQR: 4.50) and 0.55 mm (IQR: 1.23) for biopsies obtained with cryotechnique and forceps respectively ($P=0.001$).

Median area of submucosal glands was 0.095 mm^2 (IQR: 0.30) for cryobiopsies and 0.00 mm^2 (IQR: 0.01) for forceps biopsies, respectively ($P=0.002$). Airway smooth muscle median areas were: 0.25 mm^2 (IQR: 0.30) and 0.060 mm^2 (IQR: 0.11) for cryobiopsies and forceps biopsies, respectively ($P=0.02$).

Suitability for Immunohistochemistry and In Situ Hybridization

To investigate for suitability for IHC, tissue sections were immunostained for a selection of cell markers representing antigens on structural cells as well as tissue infiltrating immune cells. The

antigens included: alpha smooth muscle actin (smooth muscle marker), CD31 (blood vessel endothelium marker), CD3 (pan T-cell marker), IL-33 (a nuclear immune-regulatory alarmin), tryptase (mast cell marker), as well as for langerin (CD207, a marker of Langerhans dendritic cells). All tested antibodies yielded the same high-quality staining as produced by standard IHC protocols on routine paraffin material. Representative example images of the IHC-stained sections are shown in figure 3.

Suitability for paraffin-compatible ISH was evaluated by staining for the standard positive control gene ubiquitin C (UBC) mRNA, which showed well defined staining, and the negative control gene DapB mRNA, which showed no staining. Images of positive and negative gene control probes are shown in figure 4.

Safety

Bronchoscopy was performed at an outpatient setting with standard doses of sedation and was well tolerated without significant oxygen desaturation or deteriorations in blood pressure. Procedural time was slightly increased mainly due to time spent placing the orotracheal tube. Bleeding from the biopsy site was the only complication seen. Out of the 10 patients, nine experienced mild bleeding, while one patient had an intermediate bleeding needing local adrenaline. In the latter case, bleeding was equally severe from both cryo and forceps biopsy-sites. We experienced no increase in post procedural observation time.

Discussion

The most important findings of the present study are that cryobiopsies are bigger, have a higher content of submucosal tissue and better preservation of epithelial morphology than forceps biopsies. Further, antigens and mRNA for immunohistochemistry and in situ hybridization are well conserved in the cryobiopsies. The cryobiopsies were obtained during bronchoscopy at a standard outpatient setting with mild to moderate bleeding as the only complication with no difference in complications related to the biopsy technique.

Endobronchial biopsies for research have many advantages compared with other methods for tissue sampling e.g. brush biopsy and bronchoalveolar lavage, as the structural morphology and spatial relationship of tissues including immunologic cells are preserved. Forceps biopsies are, however; limited by their small size, crushing artefacts and lack of peribronchial tissue, including submucosa and airway smooth muscle. A validation study by Labonté et al. found that in forceps biopsies, obtained in a controlled setting by experienced staff, only 62% were scored as of “good” or “excellent” quality and 24% did not contain smooth muscle. In addition, only 41% of the biopsies presented intact epithelium.⁷ To our knowledge, the present study is first to examine the quality of mucosal biopsies from the airway wall obtained with the cryotechnique.

We found that cryobiopsies were on average larger by a factor of approximately 3 and contained higher amounts of smooth muscle and submucosal glands when compared with forceps biopsies. This suggests that the biopsies obtained with cryotechnique are not only bigger with regards to surface area, but also deeper, containing more submucosal tissue, making them more representative of the entire airway wall. Further, the cryobiopsies did not share the same degree of crushing artefacts as the forceps biopsies; stretches of intact epithelium in this study were almost 6 times longer in the cryobiopsies. This may be a particular advantage, as the airway epithelium is attracting increasing attention as a key immunological orchestrator in obstructive airways disease.²⁰

The bigger size also allows for manifold slicing with the opportunity to perform multiple analyses on the same biopsy; an important feature in the era of omics, where bigger samples can reduce the number of biopsies needed to be obtained.²¹

Disruptions of cell integrity, due to the freezing process were a potential concern in relation to the staining of the samples, but were nevertheless not apparent: Both immunohistochemistry and in situ hybridization were performed on the cryobiopsies with good results, suggesting applicability for a variety of stainings.

In suspected lung cancer, forceps as a tool for endobronchial sampling share the same limitations; most importantly small size and crushing artefacts.²³ The cryotechnique, which has been used in many years for debulking of tumours, has been shown to increase diagnostic yield from 85% with

forceps to 95% in a head to head trial. The cryotechnique is therefore believed to be able to decrease the need for multiple investigations and re-bronchoscopies in the diagnostic work-up of cancer.¹⁴ Likewise, in suspected interstitial lung disease, transbronchial cryobiopsies increase diagnostic confidence and can thereby reduce the need of the more invasive video assisted thorascopy.^{9,11,24}

Overall the current study conforms with prior findings evaluating endobronchial biopsies for use in cancer diagnostics. One major difference, though, was that the mucosal cryobiopsies, even though bigger than forceps biopsies, were significantly smaller compared with cryobiopsies described in earlier reports sampling from endobronchial lesions.²² This, we believe, is due to the different nature of the tissue sampled. Cancer lesions may very likely be different from tightly attached mucosa when it comes to thermal transfer and ability to detach as the cryoprobe is retracted. It is our experience that freezing cycles exceeding 3 seconds causes the cryoprobe to become undetachable, possibly due to freezing of the underlying cartilage, and we have thus limited each freezing cycle to 2-3 seconds.

As a consequence of the inadequacy of the currently available methods for endobronchial sampling, obtainment of lung biopsies in severe asthma has been proposed.²⁵ As seen with transbronchial cryobiopsies in interstitial lung disease, we believe the cryotechnique can be used for obtainment of both trans- and endobronchial biopsies to support new findings to better understand pathophysiological changes in the airways as was the case with lung biopsies.²⁶ However, this calls for further studies.

In general, endobronchial sampling in patients with asthma and COPD is considered a safe procedure,^{2,3,27,28} but the safety of mucosal cryobiopsies has not previously been reported. The present study included only 10 patients and was not powered as a safety study; however, the only complications seen were mild to moderate bleeding with no differences related to sampling technique. In cancer diagnostics, endobronchial sampling with the cryotechnique has not only been shown to increase diagnostic yield but also found to have safety equal to forceps: In one study with 600 patients randomized to either forceps or cryobiopsy, an increase in overall bleeding was seen among the cryobiopsy patients but no differences in bleeding needing intervention or time needed for subsequent bleeding control were detected among the two groups.¹⁴ In another study with 41

patients, undergoing both forceps and cryobiopsies, similar results were found with bleeding as only complication with no differences regarding technique.¹³

In interstitial lung disease, use of the cryotechnique for transbronchial sampling has increased diagnostic yield. In this setting complications are common with bleeding and pneumothorax being the two most frequent. Bleeding is most commonly controlled with a Fogarty balloon, whereas pneumothorax is seen in up to 25% of cases.⁹⁻¹¹ Despite this high risk of procedural complications, the risk is still lower than what is seen in relation to surgical biopsies where the rate of pneumothorax is 100%. Thus, the cryotechnique could save a proportion of these patients from undergoing surgery.

The present study was a pilot study investigating the feasibility of using the cryotechnique for obtainment of endobronchial mucosal biopsies in the investigation of obstructive airway disease. We chose to do this in patients who already, due to haemoptysis, were to undergo bronchoscopy. For further consolidation of the safety and applicability of this method, studies including patients with obstructive airway disease are warranted.

In airway disease, bigger biopsies containing deeper lying structures, could pave the way for new studies increasing our understanding of disease mechanisms in obstructive airways disease. One gain is better visualization of spatial distribution of immunologic cells in the different compartments of the airway wall. An earlier study by Brightling et al which found that asthma patients have mast cell infiltration in their airway smooth muscle, has not since been reproduced.²⁹ This could be due to the difficulty in obtaining representative samples. In the original study eligible biopsies were obtained only in 60% of the patients, as 40% did not contain a sufficient amount of airway smooth muscle²⁹. A further advantage is that, better preservation of the tissue sampled with longer intact stretches of airway epithelium, may enhance our capability to investigate the disruption of airway epithelial function, such as release of the epithelial cytokines including IL-33 and TSLP.³⁰ This is of current interest given the ongoing development of monoclonal antibody treatments targeting both IL-33 and TSLP.³¹

Further, this method could be applicable for future diagnostic purposes as the need of precise pheno- and endotyping increases alongside better understanding of underlying disease mechanisms and evolvement of new targeted treatments. However, before the method can be incorporated into everyday practice, a more thorough safety profile needs to be established. We are currently undertaking a larger study that investigates the usefulness and safety of the method in patients with asthma.

Conclusion

The present study shows that the cryo-technique yields bigger and more intact bronchial biopsies with a higher content of deeper lying structures than conventional biopsies obtained with forceps. Further, the cryotechnique ensures better tissue preservation making them applicable for a variety of analyses including immunohistochemistry and in situ hybridization. We observed no increase in procedural complications. Hence, the cryotechnique seems to be an appropriate research tool for studying disease mechanisms in the airway wall. Mucosal cryobiopsies thus appears to be a superior and safe alternative to conventional forceps biopsies in studying pathophysiological mechanisms of airway disease. Studies examining the usefulness of the cryo-technique in patients with obstructive airway disease are warranted.

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Tables

Table 1

Patient characteristics	
Age (years)	60 (± 19.0)
Sex (female)	3 (30%)
BMI (kg/m^2)	25.2 (± 5.2)
Smoker (former or current)	7 (70%)
Pack years	26.2 (± 24.7)
FEV1 (% of predicted)	88.0 (± 24.1)
Alcohol consumption (units/week)	8.0 (± 11.4)
ASA score	1.7 (± 0.5)
Sedation and monitoring during bronchoscopy	
Midazolam dose (mg)	10.25 (± 4.2)
Fentanyl dose (mg)	82.5 (± 33.4)
Highest oxygen supplement (l/s)	6.8 (± 4.1)
Lowest saturation (%)	93.8 (± 3.3)

Table 2

	Forceps Biopsies	Cryobiopsies	Ratio	P-value
Overall size (mm^2)	1.03 (1.10)	3.08 (1.79)	2.99	<0.001
Length of intact epithelium stretches (mm)	0.55 (1.23)	4.61 (4.50)	8.38	0.001
Area of submucosal glands (mm^2)	0.00 (0.01)	0.10 (0.30)	-	0.002
Area of airway smooth muscle (mm^2)	0.06 (0.11)	0.25 (0.30)	4.17	0.024

Legends

Table 1. Patient characteristics at inclusion. Doses used for sedation and vital signs monitoring during bronchoscopy. Presented as means with standard deviation for longitudinal data and numbers with percentages for categorical data. BMI: Body Mass Index. FEV1: Forced Expiratory Volume in the first second. ASA score: American Society of Anaesthesiologists score.

Table 1. Comparison of size and quality of forceps biopsies and cryobiopsies presented as medians with interquartile range, ratio and Mann-Whitney test for difference.

Figure 1. Bright field micrographs exemplifying haematoxylin-eosin stained section from representative conventional forceps biopsies (A) and a larger bronchial cryobiopsy (B). Arrowheads point at intact epithelium. Scale bar = 400 μ m.

Figure 2. Scatter grams showing cross sectional biopsy areas of total tissue, intact airway epithelium, subepithelial glands, and smooth muscle bundles. Horizontal dashes represent median values. P values denote significance levels for test for difference (Mann-Whitney test).

Figure 3. Micrographs from immunohistochemistry (IHC) tests in bronchial cryobiopsies and positive visualization of single stained CD3-positive T lymphocytes (A), double stained brown smooth muscle and CD31-positive endothelial cells (B), and double staining for langerin (CD207)-positive dendritic cells and green IL-33+ cell nuclei (C). Positive immunoreactivity was visualized DAB (brown) and VINA green (green) chromogen. Scale bars: A=140 μ m, B=90 μ m, C=100 μ m.

Figure 4. Representative micrographic images of gene probe visualization of cryobiopsies by in situ mRNA hybridization and detection of positive (A,C) and negative (B, D) control probes, respectively. The house keeping gene ubiquitin C (UBC) mRNA was used as positive control, whereas the prokaryote gene DapB mRNA was used as negative control (B,D). After amplification positive hybridization was visualized by DAB chromogen. Scale bars: A-B =120 μ m, C=80 μ m, D= 50 μ m. High magnification in D to reveal absence of small singular mRNA dots in the negative control.







