Optimization of the Conditions for Preservation of Induced Sputum*
Influence of Freezing on Cellular Analysis

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**Background:** Induced sputum (IS) analysis is a noninvasive, valid, and reproducible method for evaluating airway inflammation. It has been suggested that freezing of IS samples in order to delay analysis is feasible. However, the optimal conditions for preservation of IS samples have not been determined.

**Objectives:** To determine optimal freezing conditions of IS samples, ensuring adequate specimen quality for assessment of cell viability, total cell count, and differential cell count.

**Subjects and methods:** Twenty-one subjects were enrolled: 6 healthy control subjects, 5 patients with allergic rhinitis, 5 patients with mild asthma, and 5 patients with severe asthma. Each came to the laboratory once for IS sampling. Cell plugs were homogenized with dithiothreitol and separated into 12 aliquots. Viability and total and differential cell counts were determined for each aliquot. Bovine serum albumin (BSA) with dimethylsulfoxide (DMSO) was added to half of the aliquots, and fetal bovine serum (FBS) with DMSO was added to the other half. One half of the aliquots containing BSA or FBS were frozen at −15°C, and the other half were frozen at −80°C. After 3, 7, or 10 days, samples were thawed and total cell counts, viability, and differential cell counts were assessed.

**Results:** Slide quality and total cell counts did not vary significantly according to freezing duration, temperature, or medium when compared to nonfrozen control samples. With FBS at −80°C, cell viability did not vary significantly between control samples and freezing for 3, 7, and 10 days (59% vs 54%, 59% vs 54%, and 58% vs 54%, respectively; p > 0.05), whereas every other condition showed a significant decrease. Freezing did not affect the eosinophil percentage significantly.

**Conclusion:** Freezing of IS samples in FBS with DMSO at −80°C allows adequate preservation of IS specimens. Samples can be kept for at least 10 days in those conditions without significantly altering total cell counts, viability, and eosinophil percentage.

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**Key words:** airway inflammation; asthma; eosinophil; freezing induced sputum; preservation

**Abbreviations:** BSA = bovine serum albumin; DMSO = dimethylsulfoxide; DTT = dithiothreitol; IS = induced sputum; FBS = fetal bovine serum

Induced sputum (IS) analysis is a valid and reproducible technique that is increasingly used for the noninvasive assessment of airway inflammation, particularly in conditions such as asthma.¹⁻⁴ Current recommendations indicate that the specimen should be processed within 2 h following induction⁵ (or within 9 h if kept at 4°C)⁶ to ensure adequate quality. Those could be limitations for delayed analysis or clinical use. Freezing of IS samples could offer a solution to this problem, making it possible to delay...
the analysis. However, limited data describing such methods for adequate IS specimen preservation are available.

Holz et al.\(^7\) have suggested that sputum samples can be frozen without significant alteration of slide quality and cellular composition. In their study, samples were frozen at \(-20^\circ C\) in phosphate-buffered saline solution containing 0.5% bovine serum albumin (BSA) and 15% dimethylsulfoxide (DMSO). Sputum samples were frozen at two different stages of processing: following homogenization with dithiothreitol (DTT) [the first method], or immediately before cytospin preparation (the second method). With the first method, the slide quality was slightly better than with the second method.

Following these observations, we aimed to determine the optimal conditions for freezing IS samples. We hypothesized that fetal bovine serum (FBS) would be a better freezing medium for conservation of IS cells, as it is commonly used in laboratories to increase the survival rate of cells that are difficult to preserve. DMSO is routinely used as a cryoprotective agent to minimize the damage that occurs to cells during the freezing process, primarily by avoiding the development of ice crystals.\(^5\) We also hypothesized that samples frozen at \(-80^\circ C\) would be of better quality for cell analysis than those preserved at \(-20^\circ C\), since it was reported\(^\#\) that the optimum recovery of viable cells following freezing is best accomplished by freezing the cells at \(\leq -70^\circ C\).

**Materials and Methods**

In order to determine the optimal freezing temperature as well as the best freezing medium, we assessed the quality of cytospins according to cell morphology, distribution of cells on the slide and staining (on a scale from 0 to 5, with 0 being the lowest score and 5 being the highest), cell differential, total cell count, and viability of cells at day 0 and after freezing. Viability was determined by the Trypan blue exclusion method using a Neubauer hemacytometer chamber (Reichert; Buffalo, NY). Two freezing media, BSA and FBS, and two different temperatures, \(-20^\circ C\) and \(-80^\circ C\), were studied. We also evaluated the influence of the duration of freezing (3, 7, and 10 days) on those parameters. We used the second method described by Holz et al\(^7\) (freezing before preparation of slides) to allow for possible mediator measurements in medium-free supernatants.

**Subjects and Study Design**

Twenty-one nonsmoking subjects (age range, 18 to 45 years) were recruited for this study: 6 healthy control subjects, 5 nonasthmatic subjects with allergic rhinitis, 5 patients with mild asthma, and 5 patients with severe asthma. Results from allergy skin-prick tests, spirometry, and methacholine inhalation were available for each subject from a previous study (performed within 1 year prior to this study). Each subject signed an informed consent form, approved by the local Ethics Committee. The study included only one visit, during which spirometry was performed and an IS sample was collected and processed according to the freezing protocol.

**Spirometry**

Spirometry was performed according to standard methods, and FEV\(_1\) was measured according to the American Thoracic Society specifications.\(^9\) The predicted values for FEV\(_1\) were obtained from Knudson et al.\(^10\) The baseline FEV\(_1\) was calculated as the best of three reproducible curves. The test was done with a spirometer approved by the American Thoracic Society (Vitalograph Medical; Lenexa, KS).

**Sputum Induction**

Sputum was induced and processed according to the method described by Pin et al.\(^3\) and modified by Pizzichini et al.\(^2\) Briefly, subjects were pretreated with 200 \(\mu g\) of salbutamol before inhaling increasing concentrations of hypertonic saline solution (3%, 4%, and 5%) for 7 min each (for a maximum of 21 min) with an electronic nebulizer (Medix; Cathamor, UK) without a valve or nose clip. After each inhalation, subjects were instructed to blow their nose, rinse their mouth, and swallow the water to minimize postnasal drip and squamous epithelial cell contamination, respectively, before trying to expectorate in a sterile container.

**Sputum Processing**

Schematic representation of the IS processing method is shown in Figure 1. Mucus was separated from saliva using forceps, weighed, and rocked with four times its volume of dithiothreitol (DTT) [Sputolysin; Calbiochem; La Jolla, CA] during 15 min. The reaction was stopped by adding an equal volume of Dulbecco phosphate-buffered saline solution (Invitrogen; Burlington, ON, Canada). The cellular suspension was centrifuged at 800 \(g\) for 4 min, and the supernatant was collected and frozen at \(-80^\circ C\). The cells were then resuspended in Dulbecco phosphate-buffered saline solution and separated equally into 12 aliquots (day 0). For each aliquot, data were collected in terms of total cell count, viability, slide quality (0 to 5), and differential cell count, the two latters obtained from a slide prepared with a Cytospin 3 (Shandon Scientific; Astmoor, UK) and colored with Diff-Quik solutions (Dade Diagnostics; Aguada, Puerto Rico) for a count of 400 cells. The aliquots were then diluted 1:1 in FBS/30% DMSO (Sigma-Aldrich; Oakville, ON, Canada) [six aliquots] or in 1% BSA/30% DMSO (six aliquots). Another differential slide was prepared to assess the effect of FBS and BSA on slide quality. Half of the aliquots for either FBS or BSA were frozen at \(-20^\circ C\), and the other half were frozen at \(-80^\circ C\). Therefore, there was one aliquot for each medium, each temperature, and each freezing length (3, 7, and 10 days). Samples were placed in a freezer between two styrofoam supports to minimize thermal shock. At 3, 7, and 10 days, the aliquots were thawed quickly at 37°C and then total cell counts and viability were determined and compared to those of the corresponding nonfrozen control (day 0). A slide for differential cell count and quality assessment was prepared after centrifugation and withdrawal of supernatant. All counts and analyses were performed on coded slides by qualified staff.

**Statistical Analysis**

Data were expressed as mean \pm SD. The parameters studied included total cell count, viability and quality of cytospins, as well as percentages of eosinophils, neutrophils, lymphocytes, macrophages, bronchial cells, and squamous epithelial...
cells (to eliminate the contamination from the mouth) before and after freezing. A factorial design was used to analyze six experimental factors: one associated to the comparison among the four groups (factor group); one linked to the subjects (nested factor in group); one associated to the comparison between media (factor media: FBS or BSA); one associated to the comparison among the length of freezing (factor day: 3, 7 or 10 days); one associated with the comparison of freezing temperature (factor temperature: \(-20^\circ C\) or \(-80^\circ C\)); and one linked to the comparison before and after freezing (factor period). This last factor was analyzed as a repeated-measures factor. A mixed-model analysis was performed with interaction terms between the fixed factors. Different statistical models were involved to obtain the better-fitting model, and likelihood ratio tests were carried out between models contained in the others. Comparisons of the adjusted Akaike information criterion\(^1\)\(^1\) for the different models were obtained. An exponential correlation structure for unequally spaced observations was used. To proceed with the analysis, we used a model with homogeneity between fixed factors (similar covariance structures). For variables expressed in percentage, to improve the precision of measurements obtained at \(-20^\circ C\), we used the value obtained at \(-20^\circ C\) as covariate. These analyses of covariance did not reduce the error mean square significantly nor change the statistical results. The univariate normality assumptions were verified with the Shapiro-Wilk tests, and multivariate normality assumptions were verified with Mardia tests.\(^1\)\(^2\) Posteriori comparisons between fixed factors were done using contrasts. The results were considered significant with \(p\) values \(< 0.05\). All analyses were conducted using statistical software package (SAS version 8.2; SAS Institute; Cary, NC).

**RESULTS**

Subject characteristics are shown in Table 1. Each subject was able to produce enough expectorate for all analyses. There were no differences among the four groups for viability, cell differential, total cell counts, and slide quality for each freezing condition, nor was there any difference in quality of slides and differential cell counts before and after addition of...
the medium in the sample. Consequently, the four groups were analyzed together.

Cell viability was determined after thawing for each freezing condition and duration, and compared with its corresponding nonfrozen control (day 0). Each condition had its own day 0 control. Figure 2 shows the variations of sputum viability in FBS for each condition studied. When using FBS at $-80^\circ$C, cell viability did not vary significantly between control samples and frozen samples for 3, 7, and 10 days ($59\%$ vs $54\%$, $59\%$ vs $54\%$, and $58\%$ vs $54\%$, respectively; $p > 0.05$), while with FBS at $-20^\circ$C and BSA at either temperatures, we observed a statistically significant decrease in viability compared to control samples at each time point. As an example, for BSA at $-20^\circ$C, viability decreased from $59\%$, $59\%$, and $60\%$, to $25\%$, $16\%$, and $11\%$ for days 3, 7, and 10, respectively ($p < 0.0001$; data not shown).

When comparing viability over time (day 3 vs day 7 vs day 10), we observed a significant decrease in FBS at $-20^\circ$C, and at day 10 vs day 3 in BSA at $-20^\circ$C ($p < 0.05$). Viability did not significantly decrease over time in FBS at $-80^\circ$C nor in any other freezing condition. Slide quality and differential cell counts did not vary significantly before vs after the addition of the medium, while slide quality and total cell counts did not vary significantly between day 0 and any freezing condition as to duration, temperature, or medium used.

Results for differential cell counts are shown in Figure 3. The mean bronchial epithelial cell (data not shown) and eosinophil percentages did not vary significantly between control and frozen samples for any condition. Percentages of neutrophils decreased significantly after freezing for all conditions except in FBS at $-80^\circ$C for 10 days ($p = 0.2$). Lymphocyte percentages decreased at 7 days ($p < 0.0001$) and 10 days ($p = 0.02$) with BSA at $-20^\circ$C, and at 10 days with FBS at $-20^\circ$C ($p < 0.0001$). Consequently, macrophage percentages increased for all conditions except in FBS at $-80^\circ$C after 10 days ($p > 0.05$).

Discussion

This study shows that it is possible to freeze sputum samples for at least 10 days without altering cell viability, total cell count, eosinophil percentages, and slide quality, allowing for delayed processing.

Table 1—Subject Characteristics*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age, yr</th>
<th>Male/Female</th>
<th>Gender, No.</th>
<th>FEV₁, L</th>
<th>FEV₁, % Predicted</th>
<th>FEV₁/FVC, %</th>
<th>PC₂₀, mg/mL†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control patients</td>
<td>26 ± 3</td>
<td>2/4</td>
<td>3/4</td>
<td>3.8 ± 0.6</td>
<td>105 ± 11</td>
<td>84 ± 8</td>
<td>71.4</td>
</tr>
<tr>
<td>Allergic rhinitis</td>
<td>27 ± 6</td>
<td>1/4</td>
<td>3/2</td>
<td>3.4 ± 0.6</td>
<td>110 ± 5</td>
<td>89 ± 2</td>
<td>42.4</td>
</tr>
<tr>
<td>Mild asthma</td>
<td>24 ± 1</td>
<td>3/2</td>
<td>3/2</td>
<td>3.8 ± 1.0</td>
<td>95 ± 7</td>
<td>80 ± 6</td>
<td>2.0</td>
</tr>
<tr>
<td>Severe asthma</td>
<td>41 ± 18</td>
<td>4/1</td>
<td>4/1</td>
<td>2.7 ± 0.8</td>
<td>73 ± 25</td>
<td>67 ± 18</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Data are represented as mean ± SD unless otherwise indicated. PC₂₀ = provocative concentration of methacholine causing a 20% fall in FEV₁; ND = not done.
†Data are presented as geometric mean.
Among the conditions studied, freezing in FBS at −80°C was the best to maintain cell viability and integrity. At either temperature, FBS is better than BSA for conservation of cell viability, while freezing at −80°C would be more suitable whatever medium is used. Viability is an important parameter since it has been reported that poor cell viability reduces the accuracy of differential cell counts in IS.13 FBS is routinely used in the laboratory as a cell preservation and freezing medium. DMSO is also widely used as a cryoprotective agent for cell storage. We chose to freeze our samples at −20°C and −80°C since those temperatures are commonly used in laboratories.

We observed that viability decreased in frozen samples (except in FBS at −80°C) compared to control samples, but it did not decrease over time, suggesting that decreased viability may be more associated with the freezing/thawing cycle than with the freezing duration. To verify this hypothesis, we kept one sample frozen for 21 days in all conditions; viability did not vary significantly when compared to days 3, 7, and 10 (data not shown).

Our results suggest that neutrophils may be more sensitive to freezing than other cell types. It is reported in the literature14–17 that neutrophils are very sensitive to the osmotic stress caused by freezing. This could explain why we observed a decrease in neutrophil percentages with freezing. Holz et al7 also observed a neutrophil loss in their study, while macrophage and eosinophil counts were stable. We also demonstrated that freezing did not affect eosinophil percentages, a key cell in asthma studies using the sputum analysis technique.

We decided to freeze our samples just before cytospin preparation to be able to collect medium-free supernatant, in order to perform mediator measurements often assessed in studies. We did not try the first method described by Holz et al7 (freezing immediately after the addition of DTT) for that reason. Nevertheless, it could be interesting to look at the effect of freezing in FBS at −80°C immediately after cell homogenization in DTT, since this method would require fewer manipulations for those collecting the samples.

Kelly and coworkers18 published a method for

![Figure 3](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/22028/ on 06/26/2017)
sputum preservation with paraformaldehyde, demonstrating that sputum samples could be preserved for transportation to a specialized center for processing without cell loss. However, they observed an increase in neutrophil and eosinophil counts (absolute cell count and percentage) compared to controls with this preservation method; this may have been due to the rapid inactivation of cellular enzymes and stabilization of the cell membranes by paraformaldehyde. However, this technique is less suitable for routine analyses because it does not allow the determination of cell viability and the measurement of mediators in supernatant. Our method does not induce changes in eosinophil percentages, and it allows for mediator measurements and determination of cell viability. However, the efficiency of our method for mediator preservation has still to be verified.

CONCLUSION

Our study demonstrates that freezing of IS samples is feasible and that the optimal conditions are in FBS with DMSO at −80°C. Under those conditions, samples can be kept frozen for at least 10 days without significantly altering cell quality, viability, or cell counts, allowing delayed sputum analysis for possible clinical purposes or functional studies. Further investigation should be done to determine the effects of freezing on mediator measurements in inflammatory diseases and on markers of other diseases including lung cancer, interstitial lung diseases, and pulmonary tuberculosis.

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