

Freezing of homogenized sputum samples for intermittent storage

O. HOLZ, M. MÜCKE, P. ZARZA, D. LOPPOW, R. A. JÖRRES and H. MAGNUSSEN

Krankenhaus Grosshansdorf, Zentrum für Pneumologie und Thoraxchirurgie, Grosshansdorf, Germany

Summary

Background Among the reasons that restrict the application of sputum induction in outpatient settings is the need for processing of samples within 2 h after induction.

Objective The aim of our study was to assess whether freezing is suitable for intermediate storage of sputum samples before processing.

Methods We compared differential cell counts between two sputum aliquots derived from the same sample. One aliquot was processed within 2 h after production and one, after it had been frozen under addition of dimethyl-sulfoxid (DMSO) and stored up to 10 days at -20°C . Thirty-five samples were frozen immediately prior to preparation of cytopins, and 10 samples were frozen at an even earlier stage, directly after homogenization.

Results In both sets of experiments we observed a significant relationship between frozen and native samples regarding macrophages, neutrophils and eosinophils, as indicated by respective intraclass correlation coefficients of 0.96, 0.96, and 0.93 in the first, and of 0.92, 0.96 and 0.77 in the second experiments.

Conclusion Our results indicate that the freezing of sputum samples at different stages of processing does not alter sputum morphology to an extent that affects the results of differential cell counts.

Keywords: Differential cell counts, induced sputum, methodology

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Introduction

Recent recommendations claim that the processing of samples of induced sputum should be performed within 2 h after production to avoid noticeable losses in cytopsin quality and changes in sputum composition [1]. Indeed, storage of sputum samples for 24 h at 4°C results in a selective loss of neutrophils [2]. The requirement for immediate processing as well as the lack of adequate equipment appear to be the major reasons why sputum examinations are rarely used in clinical practice. Recently, it has been suggested that the freezing of sputum samples is feasible for longer storage and transport [3]. However this

study did not assess the potential effect of freezing on cellular composition, since that would have required a comparison of frozen and native samples under identical conditions.

Based on these considerations, we analysed in a prospective study whether the freezing of sputum samples noticeably affects cellular morphology or composition. In the first approach we selectively studied the effect of freezing and compared two aliquots that were obtained from the same processed sputum sample. One aliquot was used to prepare a cytopsin slide immediately, the second one was first stored at -20°C for 2–10 days. In a second set of experiments we studied whether it was possible to freeze sputum aliquots at an earlier stage of processing, i.e. immediately after homogenization, in order to further reduce processing prior to freezing.

Correspondence: Olaf Holz, Krankenhaus Grosshansdorf, Zentrum für Pneumologie und Thoraxchirurgie, D-22927 Grosshansdorf, Germany.
E-mail: o.holz@pulmoresearch.de

Table 1 Median values (quartiles) of the percentages of sputum cells

	Native	Frozen
First part of the study (<i>n</i> = 35)		
Macrophages	28.0 (18.8; 72.1)	36.2 (21.0; 67.5)**
Neutrophils	56.8 (9.4; 70.2)	49.2 (12.7; 66.9)*
Eosinophils	6.4 (0.8; 15.1)	4.5 (0.8; 14.8)
Lymphocytes	2.3 (0.8; 4.4)	2.0 (0.8; 4.9)
Second part of the study (<i>n</i> = 10)		
Macrophages	49.5 (42.2; 68.7)	54.9 (18.0; 60.9)
Neutrophils	37.6 (16.5; 48.0)	34.9 (22.9; 72.7)
Eosinophils	2.7 (0.3; 5.4)	4.9 (0.3; 8.6)
Lymphocytes	2.7 (1.0; 4.0)	2.3 (0.4; 3.3)

P* < 0.05. *P* < 0.01 Wilcoxon matched-pairs test

Materials and methods

In the first part of the study we analysed 35 sputum samples from 21 patients with mild to moderate asthma (10f/11m, mean (SD) age 45 ± 17 year, FEV₁% pred $83 \pm 21\%$) and from 4 subjects with rhinitis (4 m, age 25 ± 4 year, FEV₁% pred $108 \pm 10\%$) after a previous allergen challenge. The diagnosis of bronchial asthma followed internationally accepted criteria [4]. In the second part of the study we included a total of 10 sputum samples from three healthy subjects (3 m, age 42 ± 10 year, FEV₁% pred $118 \pm 8\%$), five patients with mild to moderate asthma (2f/3m, age 38 ± 18 year, FEV₁% pred $79 \pm 5\%$) and two patients with chronic bronchitis (1f/1m, age 59 ± 4 year, FEV₁% pred $63 \pm 21\%$). Sputum examinations had been approved by the Ethical Committee of the Medical Board of the State of Schleswig-Holstein.

Inductions were performed with increasing concentrations of nebulized hypertonic saline (3–5%) [5] or with 3% hypertonic saline only. In the first part of the study sputum was homogenized (37 °C) with sputolysin (6.5 mM dithiothreitol and 100 mM phosphate buffer, pH 7.0, Calbiochem, Munich, Germany) for 15 min (gently vortically spun every 5 min) and diluted with phosphat-buffered saline (PBS, final dilution 1 : 20) [5]. Then cells and supernatants were separated by centrifugation (10 min, 600 g) and cells were resuspended in 200 µL PBS (1% bovine serum albumin, BSA). One aliquot was used to prepare a cytospin slide immediately. A second aliquot was diluted 1 : 1 with 30% dimethylsulfoxid (DMSO) (Sigma, Munich, Germany) in PBS, resulting in a final concentration of 15% DMSO. The aliquot was gently mixed and stored frozen at –20 °C.

In the second part of the study the sputum was separated

into 2 aliquots immediately after homogenization with sputolysin (15 min at 37 °C). Again, one aliquot was mixed 1 : 1 with PBS (1% BSA) containing 30% DMSO before it was stored frozen, the other aliquot was processed immediately. Frozen samples were thawed quickly at 37 °C, and after very gentle mixing, taken to prepare the cytospin. Samples were Giemsa stained [5] and at least 400 non-squamous cells were evaluated by an observer blinded to the origin and handling of the sample. All samples were rated with a half-point quality score ranging from 0 (not sufficient) to 2 (good).

Comparisons were made by the paired *t*-test or the Wilcoxon matched-pairs test, depending on the data distribution. Linear correlation coefficients were computed for differential cell counts. Reproducibility was expressed by intraclass correlation coefficients that were derived from one-way ANOVA as the ratio of variance among subjects to total variance. Statistical significance was assumed at *P* < 0.05.

Results

In the first part of the study, mean (SD) cell number was $10.4 (7.5) \times 10^6$ cells/mL sputum, squamous cell contamination 4.2 (6.5)% and viability 85.2 (11.2)%. The median storage time examined was 4 (range 2–10) days. The median (quartiles) quality score of the frozen samples was 1.0 (0.5; 1.5) and lower (*P* = 0.006) than the score of the native samples (1.5 (1.0; 2.0)). Quality scores did not depend on storage time. Median percentages of sputum cells are presented in Table 1.

In the second part of the study, mean cell number was found to be $5.7 (4.3) \times 10^6$ cells/mL sputum, squamous cell contamination 5.3 (6.1)% and viability 89.8 (5.5)%. The median storage time was 5 (range 3–6) days. Quality scores of the frozen samples were 1.5 (1.0; 1.5) and not significantly different from those of the native samples (1.5 (1.0; 1.5)). There were also no significant differences in differential cell counts between frozen and native samples (Table 1).

The intraclass correlation coefficients between frozen and native samples regarding macrophages, neutrophils and eosinophils were 0.96, 0.96, and 0.93 in the first (Fig. 1) and 0.92, 0.96 and 0.77 in the second experiments.

Discussion

Our data indicate that freezing is suitable for intermediate storage of sputum samples after production. This was reflected in the high level of agreement between the percentages of macrophages, neutrophils and eosinophils in native and frozen samples.

We assessed the effect of freezing at different stages of

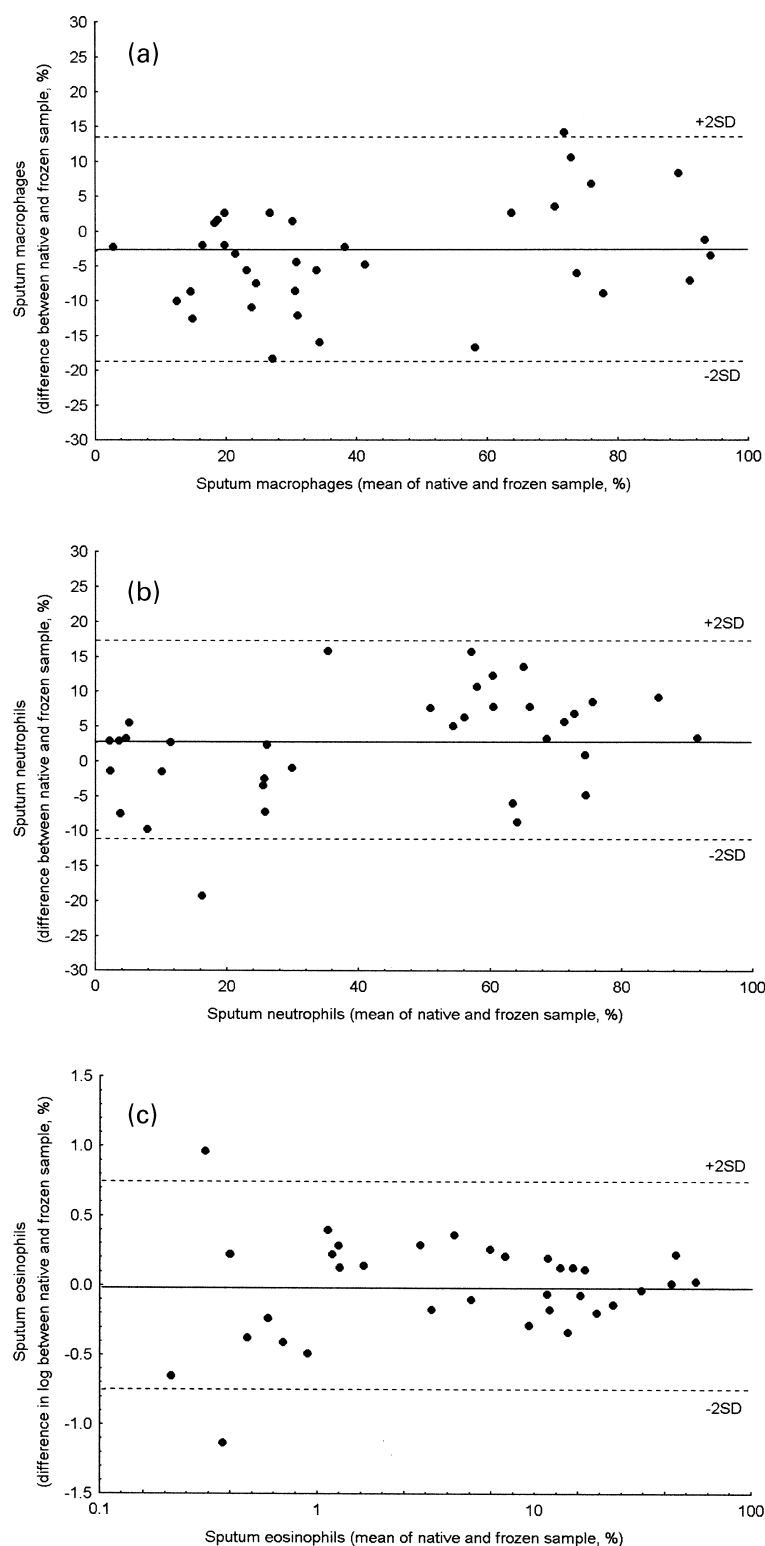


Fig. 1. Differential cell counts (% non-squamous cells) of native and frozen sputum samples. The agreement between native and frozen samples is demonstrated by a plot of differences against mean values for macrophages (a), neutrophils (b), and eosinophils (c) (according to [6]). The solid line represents the mean difference, the broken lines indicate ± 2 SD from the mean.

processing to accommodate for potential differences in equipment and available time. As freezing at deeper temperatures is not commonly available, the storage temperature was chosen to be -20°C . Our data show that freezing could even be performed immediately after homogenization, i.e. in the presence of dithiothreitol (DTT), without detrimental effects on cell morphology.

Since frozen cells are fragile, we handled all samples as gently as possible during thawing and mixing. Despite this, quality scores were often lower in the frozen samples, owing to a minor deterioration of cell morphology, which rendered the identification of cells slightly more difficult. Also, the loss in neutrophils after freezing, which is suggested by the comparison of the percentages of macrophages and neutrophils, might be explained by the higher sensitivity of these cells. However the effect was small in both sets of experiments. Whether freezing affects other parameters than differential cell counts to any significant extent, has to be determined in future studies.

In summary, our study demonstrates that freezing is suitable to store sputum samples at different stages of processing. As the procedure of freezing significantly reduces technical and time requirements, it might help to spread the use of sputum induction into clinical practice.

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