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Effect of storage temperatures on the stability of cytokines in cervical mucous

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Abstract

Cytokines have progressively come to serve as indicators for the presence or severity of a disease. But accurate measurement of cytokine levels can be deterred by lack of proper handling and storage of the samples. In this study, we attempted to measure the effect of snap-freezing and refrigeration at the time of collection on cervical mucous. Luminex analyses of the frozen and refrigerated pairs exhibited no significant differences in levels for 7 out of the 10 cytokines measured simultaneously. The cytokines TNF-α, IFN-γ and IL-1β, were significantly different between the pairs with the refrigerated samples showing higher levels for each of these cytokines. The results suggest that refrigeration of mucous samples immediately after collection would allow for better conservation of the cytokines in cervical mucous.

Keywords

Cervical mucous; Cytokines; Luminex

1. Introduction

Intercellular mediators like cytokines, that modulate immune function, serve as precursors to effector mechanisms that are selected and propagated in an immune response. Therefore they are also indicators of the type of immune response elicited and their levels in biologic fluids can be used in monitoring and characterizing disease [1].

Cytokines are known to have a short half-life in-vivo and are also subject to rapid degradation in-vitro following sample collection if appropriate storage and handling procedures are not adopted. In several cytokine studies, these same factors involving sample collection, processing and storage have been shown to be critical for achieving accurate and reproducible results [2–4]. But, most of these published reports on cytokine stability and storage are related to serum or plasma samples [5–8]. Several studies of cytokines in cervical mucous have been performed to investigate the local immune response to human papillomavirus (HPV), in hopes of determining immune responses associated with clearance or disease progression. To date, because of uncertainties of the stability of cytokines in cervical mucous, studies have relied on

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snap freezing at the time of collection. For ease of implementation into clinical settings, simply refrigerating the sample until transferring to the lab for long-term storage and assay would have significant advantages [9,10]. In the present study, the effect of refrigeration or immediate freezing on the levels of ten cytokines in cervical mucosal samples was determined using Luminex xMap technology. Data generated from this study helped determine the appropriate conditions or storage of patient samples to aid in preservation of the cytokines for future assays.

2. Methods

2.1. Sample collection

Samples were collected from 35 women attending urban public health hospital colposcopy clinics who were enrolled as part of an ongoing study of cervical neoplasia [11]. After visualization of the cervix, two Weck-Cel sponges (Xomed Surgical Products, Jacksonville, FL) were placed into the cervical os for a minimum of 1 minute, or until the sponges appeared saturated. Each sponge was placed in a labeled microcentrifuge tube; one was placed on wet ice and refrigerated in the clinic, while the other was immediately snap-frozen on dry ice. Specimens were transferred to the laboratory within eight hours of collection and then stored at −80°C until further analysis.

2.2. Protein extraction from Weck-Cel

We used the protein extraction protocol described [12]. Briefly, the sponges were equilibrated in 300 l of extraction buffer consisting of PBS (pH 7.0), 0.25M NaCl and 10% fetal calf serum, for 30 min at 4°C. The diluted samples were then separated from the sponge using a Spin-X filter unit (Corning Inc., Acton, MA) by centrifugation at 16, 000 X *g* for 15 min at 4°C. The sponge was then washed immediately with an additional 300 l of extraction buffer and collected as before. Total protein content was measured using the Coomasie Plus™ kit (Pierce technologies) as per the manufacturer's protocol.

2.3. Multiple cytokine testing using Bio-Plex™ protein array system

The cytokine concentrations were determined using the Bio-Plex protein array system and Bio-Plex multiple-cytokine assay kits (Bio-Rad, Hercules, CA) as per manufacturer's protocols. The Bio-Plex 10-plex kits were used to determine the concentration of 10 cytokines in each sample being tested [IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, GM-CSF, IFN-gamma, TNF-alpha, and IL-1beta]. A wide range (1.95 pg/ml-32000 pg/ml) of assay standards re-suspended in standard diluent was used to plot the standard curves for the 10 cytokines. All reagents needed for the assays were provided in the kits. Calibration of the instrument was performed daily, along with the monthly-recommended system validation. The mucosal samples were diluted 1:2 in Bio-Plex serum diluent prior to analysis and assayed in duplicate. The assay technique was specific and sensitive for simultaneous detection of multiple cytokines with a coefficient of variation ≤ 10%.

Data was obtained using Bio-Plex Manager software program (Bio-Rad) for standardization and standard curve acquisition followed by conversion to Excel™ (Microsoft Corporation, Seattle, WA) for further analysis. Optimal standard curve fits, either 4-parameter or 5-parameter, were selected for every assay run as well as for every cytokine within a given assay. Conversion of the optimal data to Excel ™ (Microsoft Corporation) facilitated the comparative analysis of cytokines between frozen and refrigerated patient samples.

2.4. Statistical Analysis

Statistical analysis of the data was performed using the Wilcoxon signed rank test to determine whether the median difference in parameter values between frozen and refrigerated mucosal

samples was equal to 0. Statistical significance was determined if p-values were <0.05. All analyses were performed using the SAS software.

3. Results

3.1. Extraction of proteins from Weck-Cel sponge

The optimized protein extraction protocol from the Weck-Cel sponge was successfully applied to the 35 cervical mucus samples collected. The total protein content measured in these samples was similar for snap frozen (mean 4.41 ± 1.21 mg/ml; range 1.67–7.18 mg/ml) and refrigerated samples (mean 4.37 ± 1.08 mg/ml; range 1.62–6.27 mg/ml).

3.2. Comparison of Cytokine levels in refrigerated and frozen samples

The cytokine levels obtained from the frozen and refrigerated sample sets are shown in Fig. 1. Samples were run over the course of 4 months and frozen and refrigerated pairs were included in the same run. The intra-assay and inter-assay variabilities measured in 8 frozen and refrigerated samples was ≤ 10% in 8 out of the 10 cytokines. But, in the case of IFN-γ and IL-4, the interassay variability was 33% and 24% respectively. For several cytokines including, IL-2, IL-4, IL-6, IL-8, IL-12, IL-10 and GM-CSF, the data showed no significant differences in cytokine levels with a p-value of ≥ 0.3 (Table 1, Fig. 1). But, in the case of TNF-α, IFN-γ and IL-1β, there was a significant loss of cytokine in the samples that were frozen immediately after collection (p-value ≤ 0.05) (Table 1, Fig. 1).

4. Discussion

This study was conducted to determine if snap-freezing or refrigeration of cervical mucous samples at the time of collection would affect measured cytokine levels. We found that only TNF-α, IFN-γ and IL-1β, exhibited significant differences in measured levels with refrigerated mucosal samples having higher levels of these cytokines as compared to their snap-frozen samples.

Not much is known as to how the biological structure of the cytokine is affected in these situations, but several reports on serum and plasma cytokine levels have indicated that most cytokines are prone to degradation if blood samples are not processed correctly and stored at −70°C. TNF-α, measured in serum or plasma is commonly cited as very susceptible to changes in collection and storage methods [13–15], and seems to possess the similar vulnerability in mucous samples. The levels of the cytokines TNF-α, IFN-γ and IL-1β, measured in our sample set were well above the detection limits of our assay, and therefore the differences seen were not assay artifacts. The results also imply that the time (8 h) by which the refrigerated samples were transferred to freezers did not affect cytokine levels, therefore making it very convenient for collection of these samples when conducting large clinical studies where storage facilities are not readily accessible.

Thus, in this study we have shown that refrigeration of mucous samples instead of snap-freezing them immediately after collection would allow for better conservation of the cytokines in cervical mucous. Cytokines exist in complex tertiary and sometimes quarternary structures that could be susceptible to structural damage when exposed to improper handling and storage conditions, and therefore it is critical to maintain an optimized protocol for collection, handling, storage and assay of the samples that are to be used for cytokine measurement.

This study has several limitations. As both the refrigerated and snap frozen samples were stored prior to extraction and testing, we were unable to measure the impact of storage. To conserve reagents we needed to perform the Luminex assay on batched samples, so immediate extraction and testing was not possible in our hands, nor is it likely to be possible in most epidemiologic

studies. In addition, the number of samples tested was small, and the differences in TNF-α, IFN-γ and IL-1β, between refrigerated and snap frozen samples, while statistically significant, may not be biologically significant.

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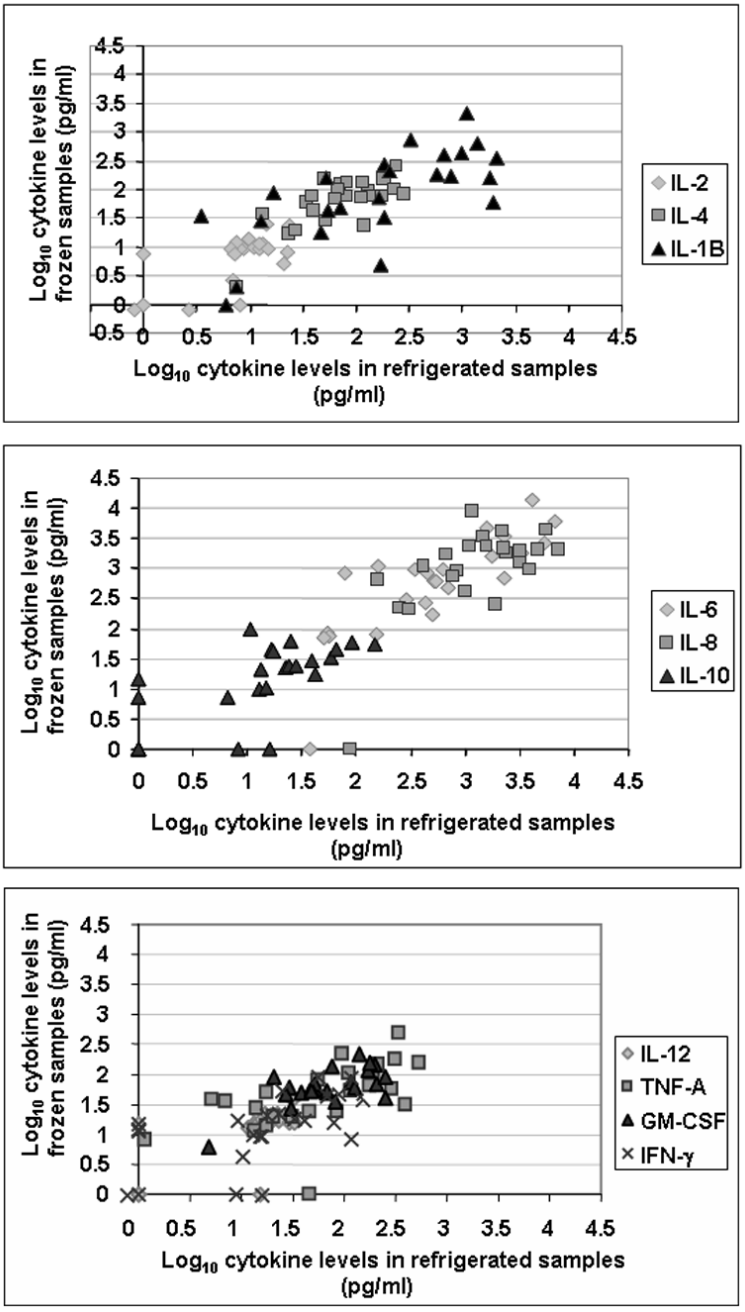

Fig 1.

Scatter plots showing the correlation between frozen and refrigerated samples for ten cytokines as determined by the Bio-Plex assay.

Table 1

Mean, median and p-values determined by the Wilcoxon signed rank test for the different cytokines in cervical mucus samples that were either refrigerated or frozen

Cytokines	Difference between Frozen and Refrigerated samples[a]		Significance (p-value)
	Mean value	Median value	
IL-1β	−298.45	−25.02	0.01
IL-2	−0.32	0.0	0.89
IL-4	−12.93	−3.5	0.36
IL-6	391.63	18.64	0.31
IL-8	−359.38	−82.91	0.29
IL-10	−2.20	−0.0	0.85
IL-12	−0.47	0.0	0.94
IFN-γ	−13.060	−4.68	0.03
TNF-α	−43.01	−15.36	0.02
GM-CSF	−16.35	−1.86	0.30

[a]The difference in cytokine levels between refrigerated and frozen samples is considered the variable in Wilcoxon signed rank test.