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ORIGINAL ARTICLE

Effect of Repeated Freezing and Thawing on Biomarker Stability in Plasma and Serum Samples

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Abstract
Objectives: The stability of circulating proteins can be affected by repeated freezing and thawing. The aim of our study was to identify the effect of repeated freezing and thawing on the plasma and serum concentrations of eight proteins [interferon-γ, interleukin (IL)-8, IL-15, IL-17A, matrix metalloproteinase (MMP)-7, tumor necrosis factor-α, vascular endothelial growth factor (VEGF), and VEGF receptor 2 (VEGF-R2)].
Methods: We assessed the concentration changes of these proteins in 30 plasma and serum samples subjected to three, four, or five freeze—thaw cycles, and compared these with the concentration changes in the samples that were subjected to two freeze—thaw cycles before analysis.
Results: Repeated freezing and thawing by up to five cycles did not modify the plasma and serum concentrations of interferon-γ, IL-8, and VEGF-R2, while levels of MMP-7, tumor necrosis factor-α, and VEGF were significantly changed in both plasma and serum samples. Moreover, MMP-7 and VEGF concentrations tended to increase with freeze—thaw cycles. They were more elevated in plasma samples (up to about 15%) than in serum samples (up to about 7%), suggesting that serum is the preferred sample type for the analysis of circulating proteins.
Conclusion: This is the first report on the effect of repeated freezing and thawing on plasma concentrations of MMP-7 and VEGF-R2. Our findings propose that researchers should consider the number of freeze—thaw cycles to select plasma or serum samples, depending on the type of analyte.

1. Introduction

The plasma and serum proteome, released from various cells and tissues, reflect the dynamic health status of human beings [1—4]. Some proteins have been used as biomarkers for disease prognosis or diagnosis, or have been studied as candidates for biomarker discovery. For example, serum vascular endothelial growth

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factor (VEGF) was identified as a prognostic candidate biomarker for various diseases, including cervical cancer and acute ischemic stroke [5,6]. Serum tumor necrosis factor-alpha (TNF-α) was reported as a candidate biomarker of systemic inflammatory response in patients with chronic obstructive pulmonary disease [7].

Many biobanks collect and store plasma and serum samples for future biomedical research, including biomarker discovery. Because these biobanks contain a limited number of aliquots [8], the samples may undergo repeated freezing and thawing, thereby causing denaturation, aggregation, and functional loss of circulating proteins [9,10]. Although biobanks are required to secure and store samples in small aliquots, this is often impossible due to the sample size and limitations of the storage space.

Several scientists have studied the pre-analytical variations of plasma and serum proteins caused by repeated freezing and thawing to establish standard operating procedures for the collection, management, and storage of human biospecimens and to identify quality assessment biomarkers [9−11]. However, not much is known about the effect of repeated freezing and thawing on protein stability. In this study, we assessed plasma and serum concentration changes of various proteins induced by repeated freezing and thawing.

2. Materials and methods

2.1. Sample preparation

The blood samples remaining after medical examination were used for this study. Blood samples were collected in plasma separator tubes (K_2 ethylenediaminetetraacetic acid tubes; Becton Dickinson, NJ, USA) and vacutainer serum separator tubes (Becton Dickinson). Different aliquots of plasma and serum samples, obtained after centrifugation at 2,000*g* for 15 minutes, were pooled (2−3 samples in each pool) to secure a sufficient amount for analysis and then stored at −75°C. Thirty pooled plasma and serum samples were thawed at 37°C and each sample was aliquoted into four tubes. Among them, one aliquot was stored at −75°C, until it was used for analysis. The remaining aliquots were additionally thawed one, two, or three times at 37°C and frozen again.

2.2. Protein measurement

Plasma and serum concentrations of cytokines and VEGF (pg/mL) were assessed using the Milliplex Map Human Cytokine/Chemokine Magnetic Bead Panel kit-Immunology Milliplex Assay (Millipore, Billerica, MA, USA), according to the instructions in the manufacturer's manual. Cytokine analytes included interleukin (IL)-8, IL-15, IL-17A, interferon-γ (IFN-γ), and TNF-α. Plasma and serum levels of matrix metalloproteinase-7 (MMP-7) and VEGF receptor 2 (VEGF-R2) were measured using the Human Total MMP7 Quantikine ELISA kit and the Human VEGFR2/KDR Quantikine ELISA kit (R&D Systems Europe, Lille, France), respectively, in accordance to the manufacturer's protocol.

2.3. Statistical analysis

Plasma and serum concentrations of analytes are shown as the mean ± standard deviation. The variations in the analytes due to repeated freezing and thawing for three, four, or five cycles are expressed as mean percentage changes with a "+" for an increase and a "−" for a decrease compared with two freeze−thaw cycles (baseline). Statistical significance of these variations was assessed through repeated-measures analysis of variance and paired 2-tailed *t*-test using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was regarded as statistically significant.

3. Results

We assessed the concentration variations of eight proteins (IFN-γ, IL-8, IL-15, IL-17A, MMP7, TNF-α, VEGF, and VEGF-R2) in plasma and serum samples that were repeatedly frozen and thawed for three, four, or five cycles, and compared these to the concentration variations in the samples that were subjected to two freeze−thaw cycles before analysis (baseline). As shown in Table 1 and Figure 1, IFN-γ, IL-8, IL-15, IL-17A, and VEGF-R2 were stable in plasma samples throughout repeated freezing and thawing, whereas the concentrations of MMP-7, TNF-α, and VEGF were significantly changed. MMP-7 and VEGF levels increased up to >15% after five freeze−thaw cycles and showed a tendency to increase with the number of freeze−thaw cycles. TNF-α levels significantly decreased (approximately 3%) after five freeze−thaw cycles.

In the case of serum samples, IFN-γ, IL-8, and VEGF-R2 levels did not change significantly during repeated freeze−thaw cycles, whereas the concentrations of IL-15, IL-17A, MMP7, TNF-α, and VEGF increased or decreased significantly during the freezing and thawing process (Table 2, Figure 2). As in plasma samples, MMP-7 and VEGF levels showed a tendency to increase with freeze−thaw cycles in serum samples; however, the increase was more limited than in plasma samples.

4. Discussion

In this study, we assessed whether the plasma and serum concentrations of eight different proteins are affected by repeated freezing and thawing. The levels of IFN-γ, IL-8, and VEGF-R2 were stable in both plasma and serum samples during repeated freeze−thaw cycles in our experimental conditions. There are a few reports

Table 1. Concentration changes of analytes induced by repeated freezing and thawing of plasma samples.

Analyte (pg/mL)	Freeze−thaw cycles				p
	2 cycles (baseline)	3 cycles*	4 cycles*	5 cycles*	
IFN-γ	6.14 ± 8.80	6.07 ± 7.74	5.49 ± 5.17	5.45 ± 6.05	0.205
		(−1.2)	(−10.6)	(−11.3)	
IL-8	96.35 ± 164.46	96.82 ± 164.79	93.96 ± 154.99	94.58 ± 159.41	0.548
		(+0.5)	(−2.5)	(−1.8)	
IL-15	1.70 ± 1.05	1.72 ± 1.00	1.69 ± 1.11	1.67 ± 1.26	0.963
		(+1.0)	(−0.7)	(−1.7)	
IL-17A	1.24 ± 1.59	1.20 ± 1.54	1.13 ± 1.36	1.084 ± 1.21	0.495
		(−3.7)	(−9.4)	(−13.1)	
MMP7	2.34 ± 1.91	2.57 ± 2.11†	2.64 ± 2.18†	2.71 ± 2.03†	<0.001
		(+9.9)	(+12.5)	(+15.6)	
TNF-α	48.55 ± 49.42	47.48 ± 47.41	47.56 ± 47.69	47.02 ± 47.56†	0.072
		(−2.2)	(−2.0)	(−3.2)	
VEGF	134.39 ± 80.64†	144.40 ± 90.81†	148.67 ± 97.15†	154.69 ± 104.38†	0.124
		(+7.4)	(+10.6)	(+15.1)	
VEGFR2	7,292.03 ± 878.64	7,259.46 ± 833.46	7,266.65 ± 866.95	7,278.96 ± 850.16	0.903
		(−0.4)	(−0.3)	(−0.2)	

*Values within"( )" show the percentage change with a "+" for an increase and a "−" for a decrease compared with baseline; †Indicates $p < 0.05$ calculated using paired 2-tailed t test. The p value was measured using repeated-measures analysis of variance. IFN = interferon; IL = interleukin; MMP = matrix metalloproteinase; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor.

on the effect of repeated freeze−thaw cycles on plasma and serum concentrations of IFN-γ, IL-8, and VEGF-R2 [9,12−14], except for the plasma concentration of VEGF-R2. Our findings are concordant with these publications, showing that these proteins are not susceptible to degradation induced by repeated freeze−thaw cycles. In addition, we determined for the first time that VEGF-R2 is also stable in plasma samples during repeated freeze−thaw cycles. IFN-γ, IL-8, and VEGF-R2 have attracted attention as new biomarkers of various diseases such as ovarian carcinoma, urinary bladder cancer, acute pyelonephritis, osteoarthritis, or rheumatoid arthritis [15−20]. Our study shows that these may become stable biomarkers, which can be used for diagnosis or prediction of prognosis, regardless of repeated freezing and thawing of samples.

MMP-7, TNF-α, and VEGF levels were significantly changed in both plasma and serum samples during repeated freeze−thaw cycles. MMP7 and VEGF levels were elevated by approximately 15% in plasma samples and by 7% in serum samples after five freeze−thaw cycles, compared with two freeze−thaw cycles. MMP7 and VEGF levels showed a tendency to increase with freeze−thaw cycles. In a previous study, changes in the MMP-7 level induced by repeated freezing and thawing were determined in serum samples [9], concordant with our results. We identified for the first time that the MMP7 level was elevated by repeated freezing and thawing in serum as well as in plasma samples. MMP7 has attracted attention as a new biomarker for cancer, joint diseases, and liver diseases [21−23]. Concentrations of MMP7 are increased in the serum of rheumatoid arthritis patients with interstitial lung disease [22] and in the plasma of patients with asymptomatic interstitial lung disease [21]. Therefore, we recommend that researchers should consider the number of freeze−thaw cycles to select plasma or serum samples for MMP7 analysis. Azimi-Nezhad et al [24] reported that plasma VEGF levels changed as a result of repeated freezing and thawing. Guo et al [13] identified that concentrations of VEGF did not change in the plasma samples for up to 10 freeze−thaw cycles, compared with unfrozen samples, which was in contrast to our results. Guo et al [13] collected whole blood into lithium heparin tubes, unlike our and other studies, which use ethylenediaminetetraacetic acid tubes. Taken together, these facts indicate that the effect of repeated freezing and thawing on the stability of plasma VEGF may be different depending on the tube type used for sample collection. In the case of serum, it has been reported that VEGF levels are not affected by repeated freezing and thawing [13,24]; however, our study shows that VEGF levels were slightly elevated after five freeze−thaw cycles. We thawed serum samples at 37°C, but in other studies, samples were thawed at room temperature, implying that the thawing temperature of serum samples may influence the stability of circulating proteins. Thus, sample thawing should occur at temperatures as low as possible. In addition, we determined that serum concentrations of MMP-7 and VEGF are less affected than that in plasma samples, suggesting that serum is the preferred sample for the analysis of circulating proteins.

In conclusion, our study shows the different effects of repeated freezing and thawing on the stability of eight

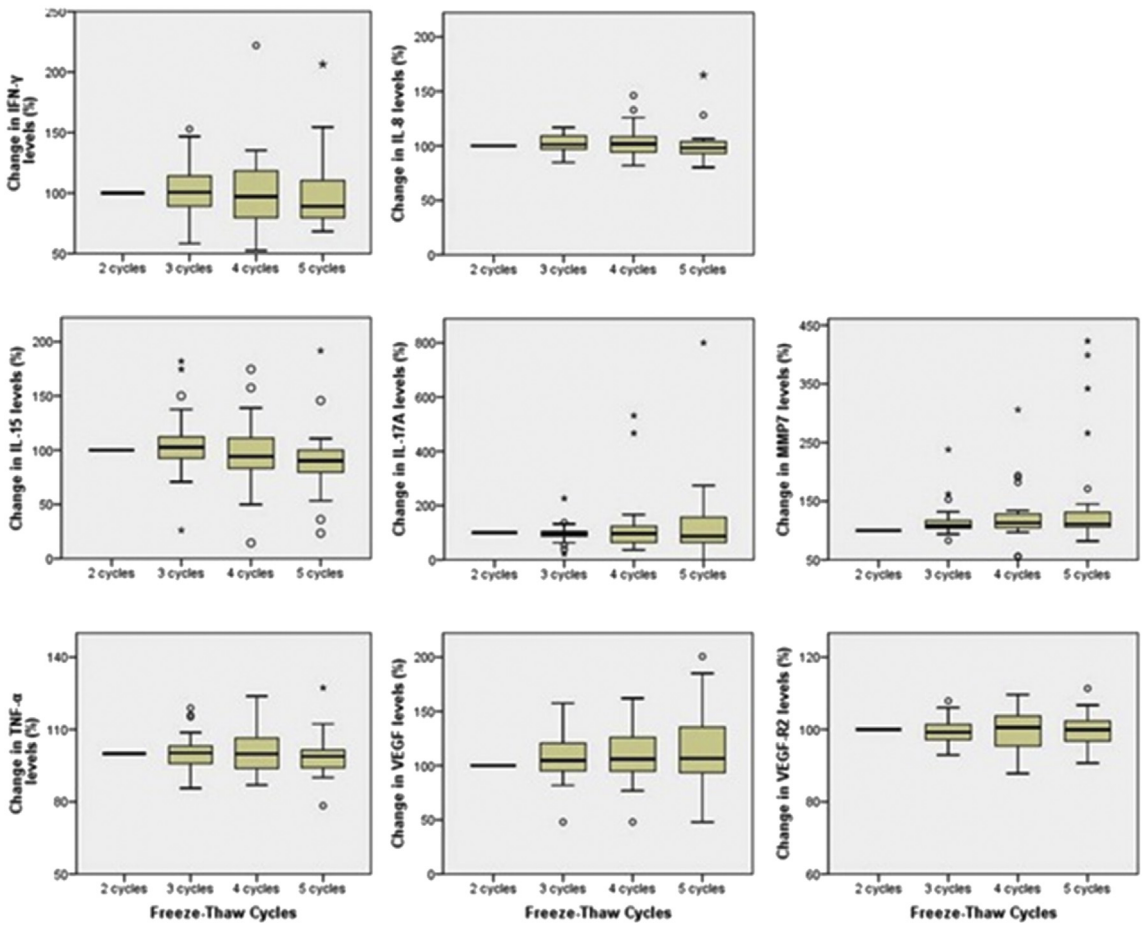

Figure 1. Box plot showing the effect of repeated freezing and thawing on plasma concentrations of analytes. Lines on the box plot represent the median, 1st, and 3rd quartiles. Vertical lines represent the 10th and 90th centiles. IFN = interferon; IL = interleukin; MMP = matrix metalloproteinase; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor.

Table 2. Concentration changes of analytes induced by repeated freezing and thawing of serum samples.

Analyte (pg/mL)	Freeze−thaw cycles				
	2 cycles (baseline)	3 cycles*	4 cycles*	5 cycles*	*p*
IFN-γ	8.11 ± 12.98	8.66 ± 14.13 (+6.8)	8.82 ± 14.71 (+8.8)	8.36 ± 13.76 (+3.2)	0.327
IL-8	35.54 ± 71.31	36.25 ± 71.46 (+2.0)	36.40 ± 71.28 (+2.4)	34.22 ± 67.01 (−3.7)	0.208
IL-15	1.54 ± 0.74	1.67 ± 0.73† (+8.7)	1.73 ± 0.71† (+12.4)	1.46 ± 0.66 (−4.7)	0.001
IL-17A	3.07 ± 3.45	3.59 ± 4.06† (+17.2)	3.29 ± 3.61 (+7.4)	3.02 ± 3.39 (−1.6)	0.110
MMP7	2.48 ± 1.50	2.59 ± 1.57† (+4.1)	2.65 ± 1.43† (+6.9)	2.64 ± 1.41† (+6.5)	0.004
TNF-α	8.74 ± 5.09	9.04 ± 5.63† (+3.4)	9.17 ± 5.80† (+4.9)	8.82 ± 5.57 (+0.9)	0.126
VEGF	195.13 ± 99.52	197.53 ± 97.88 (+1.2)	197.21 ± 97.99 (+1.1)	208.84 ± 108.75† (+7.0)	0.107
VEGFR2	9,230.32 ± 1,877.97	9,264.60 ± 1,874.35 (+0.4)	9,237.86 ± 1,806.16 (+0.1)	9,267.74 ± 1,845.64 (+0.4)	0.877

*Values within"( )" show the percentage change with a "+" for an increase and a "−" for a decrease compared with baseline; †Indicates $p < 0.05$ calculated with paired 2-tailed *t* test. The *p* value was measured with repeated-measures analysis of variance. IFN = interferon; IL = interleukin; MMP = matrix metalloproteinase; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor.

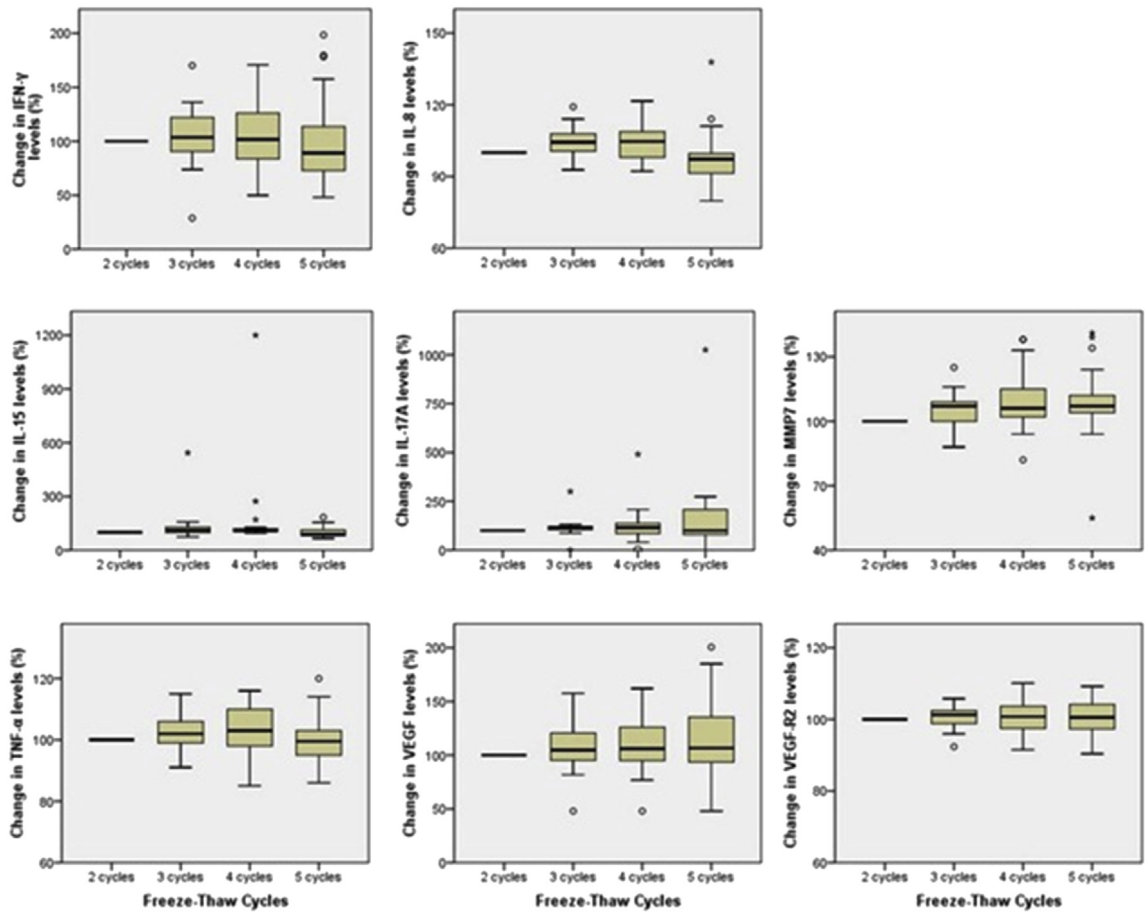

Figure 2. Box plot showing the effect of repeated freezing and thawing on serum concentrations of analytes. Lines on the box plot represent the median, 1st, and 3rd quartiles. Vertical lines represent the 10th and 90th centiles. IFN = interferon; IL = interleukin; MMP = matrix metalloproteinase; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor.

circulating proteins. IFN-γ, IL-8, and VEGF-R2 are not susceptible to freeze−thawing-induced protein concentration changes, while MMP7, TNF-α, and VEGF are slightly susceptible. Furthermore, we identified that the tube type used for collection of whole blood and the thawing temperature of samples may influence the stability of the circulating proteins. We believe that these findings will aid in sample selection according to the type of analyte and in the further development of standard operating procedures for biobanking.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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