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ORIGINAL ARTICLE

Effects of Freeze-Thaw Cycles and Assessment of Short-Term Storage Stability on Serum Iron, Ferritin, and Transferrin

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SUMMARY

Background: **Most errors in the laboratory occur due to processes related to the pre-analytical phase. The purpose of this study was to investigate the effects of multiple freeze-thaw cycles on serum iron, transferrin, and ferritin, as well as the impact of short-term storage on the stability of these analytes.**
Methods: **Serum samples from ten volunteers were submitted to three consecutive freeze-thaw cycles at -20°C and -80°C. Serum aliquots were also kept at 4°C, -20°C, and -80°C for 28 days. Iron, ferritin, and transferrin were measured after 1, 7, 14, 21, or 28 days of storage.**
Results: **Serum ferritin and transferrin showed variations from -2.6 to 2.6% and -1.7 to 2.4%, respectively, in their concentrations during the three freeze-thaw cycles of -20°C and -80°C. However, the variations were statistically significant only at -20°C. No significant changes were found for iron at both temperatures. The storage at temperatures of 4°C, -20°C, and -80°C for up to 4 weeks significantly affected the serum concentrations of iron, ferritin, and transferrin ($p < 0.01$ for all).**
Conclusions: **Serum iron, ferritin, and transferrin were affected by the storage of samples at temperatures of 4°C, -20°C and -80°C for up to 4 weeks, and the freeze-thaw cycles at -20°C influenced the measurement of serum ferritin and transferrin.**
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KEY WORDS

iron, ferritin, freeze-thaw cycle, stability, transferrin

INTRODUCTION

The assessment of pre-analytical interference on laboratory tests is of great importance, since this step is considered the most sensitive phase of the entire analytical process, and must guarantee quality and reliability [1-3]. Errors in the pre-analytical phase are responsible for up to 75% of laboratory exam errors [1,4]. Therefore, it is of interest to investigate some aspects of the influence of freeze-thaw cycles and short-term storage on the stability of laboratory parameters such as iron, ferritin, and transferrin.

More than two-thirds of the iron present in the body is located in the reticuloendothelial system, encompassing hemoglobin and bone marrow [5]. Iron is essential for the transport of oxygen, DNA synthesis, and homeosta-

sis maintenance [6,7]. Transferrin is synthesized mainly in liver hepatocytes [8-10], and it is essential to transport iron ions in the ferric form [9-11]. Ferritin is another protein that acts on homeostasis and in iron storage [7,12]. Besides, this protein is useful to differentiate anemia caused by iron deficiency from other types of anemia, as well as to quantify the iron content stored in the body [12].

Evidence related to the assessment of the stability of iron, ferritin, and transferrin is mainly focused on long-term storage [13-15]. For this reason, it is important to investigate the stability of these analytes under other conditions. Therefore, the present study aimed to investigate the effects of multiple freeze-thaw cycles on serum iron, transferrin, and ferritin, as well as the impact of short-term storage on the stability of these analytes.

MATERIALS AND METHODS

Study population

Ten supposedly healthy subjects were enrolled at the University Hospital of Santa Maria, located in Santa Maria, Rio Grande do Sul, Brazil. None of the individuals recruited had urinary tract diseases, previous kidney disease, inflammatory or infectious diseases, neoplasms, liver disease, thyroid disorders or were undergoing kidney transplantation. Blood samples were collected via the venous puncture technique into Vacutainer® (BD Diagnostics, Plymouth, UK) tubes without anticoagulant, after at least an 8-hour fasting period. The samples were then centrifuged at 2,500 x g for 15 minutes. The serum was used to measure the analytes. The samples were aliquoted and stored under the necessary conditions for the assays. The local Research Ethics Committee approved this study (number 11559119.4. 0000.5346), and all participants signed a consent form.

Laboratory measurements

Dosages were performed via the BS 380® automated biochemical analyzer (Mindray, Shenzhen, China) using commercial kits (Bioclin®, Belo Horizonte, Brazil). Baseline fasting glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, HDL-cholesterol, and triglycerides were quantified using commercial assays. The body mass index (BMI) was calculated by dividing the weight in kilograms by the square of the height in meters. Iron was determined by a colorimetric technique using the modified Goodwin/Ferrozine methodology, in which the iron is released from transferrin in acidic medium and reduced to the ferrous state through hydroxylamine and then reacts with ferrozine leading to the formation of a violet complex. Transferrin and ferritin were determined by immunoturbidimetric assays, where the analytes react with specific antibodies forming an antigen-antibody complex. The turbidity was proportional to the concentration of the analyte in the sample.

Effects of freeze-thaw cycles on serum analytes

Serum samples were kept at -20°C and -80°C. Every 75 minutes, the samples were thawed for 15 minutes at 37°C, and the levels of iron, ferritin, and transferrin were measured. Then, the samples were refrozen, and a new cycle began. Three consecutive cycles were performed on the same day as the blood collection.

Stability of iron, transferrin, and ferritin at 4°C, -20°C, and -80°C

For this investigation, 15 aliquots were taken from each sample, of which five tubes were kept at each temperature: 4°C, -20°C, and -80°C. After 1, 7, 14, 21, or 28 days of storage, one tube from each individual was thawed at 37°C for 15 minutes, and the analytes were measured.

Statistical Analysis

The distribution of the variables was investigated by the use of the D'Agostino-Pearson omnibus normality test. The parametric variables were expressed as mean ± standard deviation (SD) and the non-parametric as median and interquartile range. The one-way analysis of variance (ANOVA) for repeated measures was used to verify the influence of storage conditions on analyte stability. A p-value of less than 0.05 was considered statistically significant. Data were analyzed using GraphPad Prism® software version 6.01 (GraphPad Software, La Jolla, CA, USA).

RESULTS

The baseline characteristics of the study population are shown in Table 1. The effects of the freeze-thaw cycles on serum iron, ferritin, and transferrin were reported in Table 2. Serum iron presented a reduction between 3.4% and 11.2% along with the three freeze-thaw cycles compared to the baseline values at the temperatures of -20°C and -80°C. The highest variations were observed in the samples kept at -80°C; however, the differences were not statistically significant for both temperatures ($p = 0.239$ and $p = 0.088$, respectively). Serum ferritin and transferrin showed variations in their concentrations during the three freeze-thaw cycles at temperature of -20°C and -80°C. The values varied from -2.6 to 2.6% for ferritin and from -1.7 to 2.4% for transferrin over all three cycles. However, the variations were statistically significant for ferritin and transferrin only in samples maintained at -20°C ($p = 0.003$ and $p = 0.002$, respectively).

The storage of serum samples at temperatures of 4°C, -20°C, and -80°C for up to 4 weeks significantly affected the serum concentrations of iron, ferritin, and transferrin (Table 3). Iron showed a variation of -12.3 to 29.2% concerning its basal value found in fresh samples, and the most prominent variations were found in samples stored at 4°C ($p < 0.001$). In addition, the storage at all temperatures significantly affected ($p < 0.001$)

Table 1. Baseline characteristics of the study population.

Variables	Values
Age (years)	43.4 ± 12.4
Male (%)	60
BMI (kg/m^2)	27.9 ± 4.2
Fasting glucose (mg/dL)	91 (82 - 103)
AST (U/L)	24 (18 - 40)
ALT (U/L)	24 (15 - 36)
Total cholesterol (mg/dL)	219 ± 45
HDL cholesterol (mg/dL)	59 ± 15
Triglycerides (mg/dL)	160 (96 - 254)

Data are expressed as mean ± SD or median and interquartile range. BMI - body mass index, AST - aspartate aminotransferase, ALT - alanine aminotransferase, HDL - high-density lipoprotein.

Table 2. Effects of the freeze/thaw cycles on serum iron, ferritin, and transferrin.

	Basal	Cycle 1	Cycle 2	Cycle 3	p-value
Iron					
-20°C	89 ± 19	86 ± 22	82 ± 18	85 ± 21	0.239
-80°C	89 ± 19	81 ± 19	80 ± 19	79 ± 13	0.088
Ferritin					
-20°C	117 ± 81	115 ± 80	116 ± 81	118 ± 83	0.003
-80°C	117 ± 81	114 ± 80	116 ± 80	120 ± 86	0.191
Transferrin					
-20°C	287 ± 46	282 ± 45	284 ± 44	294 ± 44	0.002
-80°C	287 ± 46	283 ± 43	284 ± 44	291 ± 40	0.074

Serum concentrations of iron and ferritin were expressed in µg/L, and serum transferrin values were expressed in mg/dL. Values were expressed as mean ± SD.

Table 3. Stability of serum iron, ferritin, and transferrin in different temperatures up to 28 days.

	Fresh	Days					p-value
		1	7	14	21	28	
Iron							
4°C	89 ± 19	89 ± 18	97 ± 21	106 ± 22	104 ± 22	115 ± 20	< 0.001
-20°C	89 ± 19	89 ± 17	90 ± 18	79 ± 18	89 ± 17	97 ± 18	0.003
-80°C	89 ± 19	90 ± 16	90 ± 17	78 ± 20	91 ± 19	95 ± 18	0.004
Ferritin							
4°C	117 ± 8	174 ± 86	110 ± 64	174 ± 87	184 ± 95	185 ± 94	< 0.001
-20°C	117 ± 8	175 ± 87	140 ± 69	168 ± 87	169 ± 87	167 ± 84	< 0.001
-80°C	117 ± 8	173 ± 87	150 ± 73	168 ± 87	175 ± 89	151 ± 81	< 0.001
Transferrin							
4°C	287 ± 46	263 ± 38	264 ± 36	251 ± 34	277 ± 45	259 ± 35	0.001
-20°C	287 ± 46	257 ± 37	248 ± 36	223 ± 29	248 ± 34	226 ± 33	< 0.001
-80°C	287 ± 46	271 ± 39	248 ± 36	235 ± 43	250 ± 37	223 ± 33	< 0.001

Serum concentrations of iron and ferritin were expressed in µg/L, and serum transferrin values were expressed in mg/dL. Values were expressed as mean ± SD.

the concentrations of ferritin and transferrin, with variations ranging from -6.0 to 58.1% and from -22.3 to -3.5%, respectively.

DISCUSSION

This study demonstrated that freeze-thaw cycles at -20°C affected the measurement of serum ferritin and transferrin. Besides, the stability of serum iron, ferritin, and transferrin was affected by the three storage temperatures at times investigated in the present study. Although the stability of these analytes was previously investigated [3,13,14,16], the importance of the present study is to provide a better understanding of short-term stability of serum iron, ferritin, and transferrin, as well as the impact of three consecutive freeze-thaw cycles on these analytes in other conditions.

The successive freeze-thaw cycles at -20°C promoted slight but significant changes in serum concentrations of ferritin and transferrin. These variations were discreet, which does not affect the clinical interpretation of the result. However, it is recommended to quantify these proteins in fresh samples, since the successive freeze-thaw cycles can affect the conformational structure of the proteins.

Serum iron, ferritin, and transferrin were affected by the storage of samples at temperatures of 4°C, -20°C, and -80°C for up to 4 weeks. It is speculated that the storage on these conditions may promote conformational changes and even mild denaturation of proteins, which may affect the results of ferritin and transferrin. Freezing urine samples, for example, can cause conformational changes in other proteins such as albumin [17, 18]. Also, the freezing and thawing of serum samples contribute to the occurrence of protein denaturation [19, 20]. This could also explain the changes found for ferritin and transferrin in the freezing and thawing cycles at -20°C. Since immunological assays are usually employed for measuring ferritin and transferrin, it is plausible to speculate that antibodies may not recognize modified proteins, which may affect the quantification of these proteins. For serum iron, we hypothesized that the conformational changes in transferrin might have influenced the amount of free iron available in the serum. Another hypothesis may be related to the evaporation of the samples during storage, as previously suggested [15]. This evaporation can result in the concentration of the samples and, thereby, may be related to falsely elevated serum iron results.

CONCLUSION

Serum concentrations of iron, ferritin, and transferrin were affected by the storage of samples at temperatures of 4°C, -20°C, and -80°C for up to 4 weeks. In addition, freeze-thaw cycles at -20°C influenced the measurement of serum ferritin and transferrin. Therefore, it is recommended to preferably measure these analytes in fresh samples.

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Declaration of Interest:
There are no conflicts of interest to declare.

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