

ORIGINAL ARTICLE

Medicine Science 2021;10(2):450-4

Effects of storage conditions on thiol disulfide homeostasis

ID Ahmet Rifat Balik, ID Betul Ozbek Iptec, ID Gulsen Yilmaz, ID Leyla Didem Kozaci, ID Ozcan Erel, ID Cemile Bicer

Ankara Yıldırım Beyazıt University, Faculty of Medicine, Department of Medical Biochemistry, Ankara, Turkey

Received 08 November 2020; Accepted 28 January 2021

Available online 24.04.2021 with doi: 10.5455/medscience.2020.11.236

Copyright © Author(s) - Available online at www.medicinescience.org

Content of this journal is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License.



Abstract

We planned this study to research the effect of freeze-thaw number and storage temperatures on the tests of thiol-disulfide homeostasis. Dynamic thiol-disulfide homeostasis has a crucial role in antioxidant defense reactions. Thiol disulfide homeostasis is detected in serum samples. To gauge the steadiness at different storage temperatures, sera stored at -20°C and -80°C for 1, 2, 3, 6, and 12 months. To work out the effect of the freeze-thaw cycle, sera were frozen at -80°C and subjected to ten freeze-thaw cycles at 24-hour intervals. According to our results, native thiol, total thiol, and disulfide levels in human serum samples were stable at -20°C for three months and at -80°C for 12 months. After the third month at -20°C, native thiol and total thiol levels decreased while disulfide levels increased. Additionally, as the number of freeze-thaw cycles increased, native thiol and total thiol levels decreased, and disulfide levels increased. Our results show that storage at -20°C and an increasing number of freeze-thaw processes affect thiols levels. To get the most accurate results, repeated freeze-thaw cycles should be avoided the maximum amount possible. Also, samples should be frozen at -80°C if long storage times are required.

Keywords: Thiols, freezing-thawing, stability

Introduction

Thiols contain sulfide and a hydrogen (-SH) atom bonded to a carbon atom. Dynamic thiol-disulfide homeostasis plays a critical role in signal transduction, detoxification, regulation of enzymatic activity, apoptosis, cellular signal transduction mechanisms, transcription factors, and also antioxidant protection. Therefore, the determination of thiol-disulfide homeostasis can provide valuable information on various normal or abnormal biochemical changes. In a way developed by Erel & Neşelioğlu, managed to gauge thiol-disulfide homeostasis by measuring the amount of native thiol (SH), total thiol (SH+SS), and disulfide (SS) level in serum samples [1]. In the method, native thiol and total thiol are measured with separate kits. The disulfide is found by subtracting native thiols from total thiols and then dividing the result into two. Quantification of the reduced thiol groups is performed to work out the redox status of proteins containing -SH groups which will undergo reduction or oxidation and form disulfide bonds [2].

Most scientific studies are too long to be administered during a single day or maybe a couple of days. During the gathering of working groups, samples should be stored and frozen, and thawed during the evaluation of samples in terms of various parameters or in cases that need re-analysis. Thiol disulfide homeostasis has been the topic of many studies in recent years. It's not known to what extent storage conditions or freeze-thaw affect the SH, SH+SS, and SS levels analyzed. Our aim during this study is to research the effect of freeze-thaw number and storage temperatures on the tests of thiol-disulfide homeostasis.

Material and Methods

Subjects

Patients who applied to Ankara Yıldırım Beyazıt University, Faculty of Medicine, Ankara City Hospital for complete blood count (CBC), clinical chemistry and hormone test requests, and health examination were included during this prospective study. Serum samples remaining from the routine examinations of the patients were used. Serum samples of fifty patients were analyzed to gauge the steadiness of frozen sera, and 59 patients were analyzed to gauge the freeze-thaw cycle. For the stability of the frozen sera, 23 male and 27 female sera were used in the 18-50 age range (mean age; 34.64±8.94). To evaluate the effects of the freeze-

*Corresponding Author: Ahmet Rifat Balık, Ankara Yıldırım Beyazıt University, Faculty of Medicine, Department of Medical Biochemistry, Ankara, Turkey. E-mail: rifatbalik@hotmail.com

thaw cycle, 26 male and 33 female sera were used in the 18-50 age range (mean age; 34.53±8.86). All samples were taken into yellow capped blood tubes without anticoagulants (BD Vacutainer®, Becton, Dickinson, and Company, New Jersey, USA). Clinical chemistry, hormone, and CBC test results of the patients included within the study were scanned and evaluated from the laboratory data system (LIS). Because the exclusion criteria; liver diseases (aspartate aminotransferase ≥ 50 U/l, alanine aminotransferase ≥ 50 U/l, gamma-glutamyl transferase > 42 U/l, total bilirubin > 1.1 mg/dl, direct bilirubin > 0.3 mg/dl), insufficient kidney function (urea > 48.5 mg/dl, creatinine > 0.9 mg/dl) and untreated hypo or hyperthyroidism (free triiodothyronine < 1.8 or > 4.6 pg/ml, free thyroxine < 0.9 or > 1.7 ng/dl, thyroid-stimulating hormone < 0.27 or > 4.2 uIU/ml), diabetes (HbA1c $\geq 6.5\%$ (48 mmol/mol) and glucose ≥ 126 mg/dl (7 mmol/l)) were used [3, 4]. Yıldırım Beyazıt University Faculty of Medicine Clinical Research Ethics Committee approved the study. Informed consent was obtained from the subjects. The procedures followed were by the current version of the Helsinki Declaration.

Methods

Serum samples collected on the same day were divided into 11 portions to assess the steadiness of frozen sera. One aliquot of every sample was measured on the same day of collection as SH, SH+SS, and SS levels and therefore the results were recorded as fresh serum samples (day 0) data. Five aliquots of every patient were frozen at -20°C and therefore the other five at -80°C . The temperatures of the freezers were checked regularly. At the top of the 1st, 2nd, 3rd, 6th, and 12th month storage periods, samples of both freezing temperatures were thawed and analyzed. All analyzed samples were run twice in each study and therefore the mean of the measurements was utilized in statistical analysis. To research the freeze-thaw effect, SH, SH+SS, and SS values of the serum samples collected on the same day were measured and recorded as fresh serum samples (cycle 0) results.

Then serum samples were frozen at -80°C . After 24 hours, serum samples were removed from the freezer and measured within the same way and recorded because of the first freeze-thaw cycle results. At end of the analysis, serum samples were frozen again at -80°C . The equivalent procedure was repeated at 24-hour intervals until 10 freeze-thaw cycles were obtained. After the serum samples were thawed, two measurements were taken in each study, and therefore the mean of the measurements was included within the statistics.

Serum samples were kept at room temperature for a minimum of 1 hour on the day of study during all thawing procedures. After the samples were inverted 10 times, they were analyzed within 3 hours from the beginning of thawing.

SH, SH+SS, and SS measurements altogether serum samples were studied consistently with Erel&Neşelioğlu method on Siemens Advia 1800 automatic analyzer [1]. All the measurements and cryopreservation procedures were made in the Medical Biochemistry Laboratory of the Ankara City Hospital in Turkey.

Statistical analysis

The results are given as mean and SD (standard deviation) using SPSS 22.0 version. Whether the groups were normally distributed was evaluated using the Shapiro-Wilks test. The variation of the measurements consistent with fresh serum sample results was evaluated using the One-Way ANOVA test. Values with $p < 0.05$ in the Tukey results were considered statistically significant.

Results

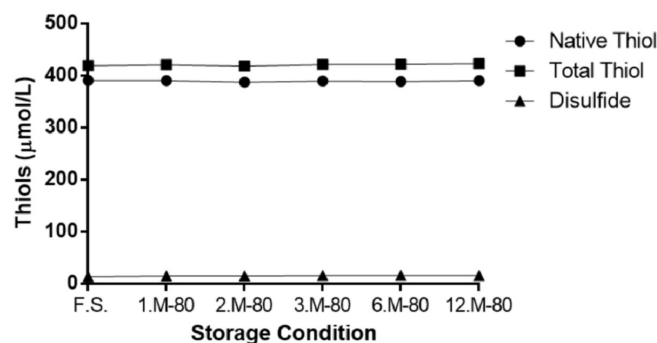
According to our results, SH, SH+SS, and SS levels didn't change significantly in human serum samples at -20°C until the 3rd month. SH, SH+SS, and SS levels of serum samples belonging to the same patients were stable until the end of the 12th month at -80°C (Figure 1). The results obtained consistent with the storage temperature and duration are shown in Table 1.

Table1. Change of native thiol, total thiol, and disulfide levels at -20 and -80°C according to fresh serum results

	Native Thiol ($\mu\text{mol/l}$)		Total Thiol ($\mu\text{mol/l}$)		Disulfide ($\mu\text{mol/l}$)	
	Mean $\pm$ SD	p-Value	Mean $\pm$ SD	p-Value	Mean $\pm$ SD	p-Value
Fresh Serum (Day 0)	390.99 $\pm$ 51.78	1.000	419.24 $\pm$ 55.10	1.000	14.13 $\pm$ 4.00	1.000
1st month -20°C	380.55 $\pm$ 50.40	0.992	411.56 $\pm$ 54.10	1.000	15.51 $\pm$ 4.00	0.857
2nd month -20°C	367.76 $\pm$ 48.71	0.372	399.88 $\pm$ 52.55	0.761	16.06 $\pm$ 3.95	0.425
3rd month -20°C	345.76 $\pm$ 45.79	$< 0.001^*$	383.89 $\pm$ 50.45	0.035*	19.07 $\pm$ 4.02	$< 0.001^*$
6th month -20°C	303.77 $\pm$ 40.23	$< 0.001^*$	356.88 $\pm$ 46.90	$< 0.001^*$	26.56 $\pm$ 4.46	$< 0.001^*$
12th month -20°C	269.94 $\pm$ 35.75	$< 0.001^*$	335.21 $\pm$ 44.05	$< 0.001^*$	32.64 $\pm$ 4.95	$< 0.001^*$
1st month -80°C	390.80 $\pm$ 51.76	1.000	421.00 $\pm$ 55.33	1.000	15.10 $\pm$ 4.06	0.986
2nd month -80°C	387.77 $\pm$ 51.36	1.000	418.56 $\pm$ 55.01	1.000	15.40 $\pm$ 4.05	0.911
3rd month -80°C	389.65 $\pm$ 51.60	1.000	421.54 $\pm$ 55.40	1.000	15.94 $\pm$ 4.11	0.522
6th month -80°C	388.99 $\pm$ 51.52	1.000	421.77 $\pm$ 55.43	1.000	16.39 $\pm$ 4.13	0.199
12th month -80°C	390.65 $\pm$ 51.74	1.000	423.02 $\pm$ 55.59	1.000	16.18 $\pm$ 4.13	0.331

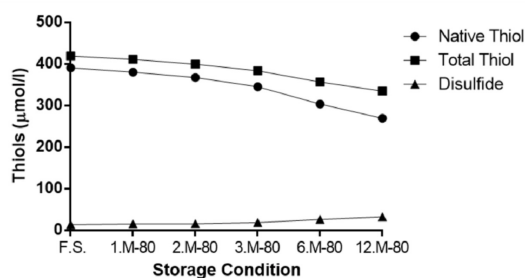
Results were expressed as mean $\pm$ SD

*Values with $p < 0.05$ in the Tukey results were considered statistically significant



F.S.: Fresh Serum, 1.M-80: 1st month -80 °C, 2.M-80: 2nd month -80 °C, 3.M-80: 3rd month -80 °C, 6.M-80: 6th month -80 °C, 12.M-80: 12th month -80 °C.

Figure 1. Changes of native thiol, total thiol, and disulfide levels according to -80 °C



F.S.: Fresh Serum, 1.M-20: 1st month -20 °C, 2.M-20: 2nd month -20 °C, 3.M-20: 3rd month -20 °C, 6.M-20: 6th month -20 °C, 12.M-20: 12th month -20 °C.

Figure 2. Changes of native thiol, total thiol, and disulfide levels according to -20 °C

We observed that because the storage time of the samples was prolonged at -20°C, SH, and SH+SS decreased and SS level increased (Figure 2).

According to the results of freeze-thaw cycles applied to human serum samples, it had been determined that SH didn't change significantly until the 5th cycle, and SH+SS and SS levels did not change until the 6th cycle. Results obtained consistent with freeze-thaw analysis are shown in Table 2. It was determined that SH and SH+SS levels decreased, and SS levels increased as a result of increasing the number of freeze-thaw cycles (Figure 3).

cycles

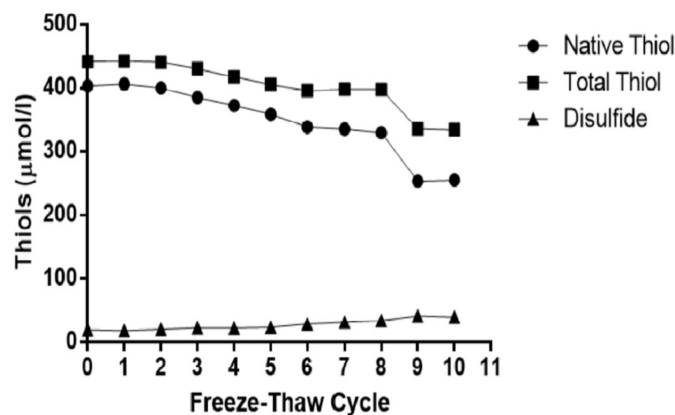


Figure 3. Changes of native thiol, total thiol, and disulfide values according to freeze-thaw cycles

Table 2. Variation of native thiol, total thiol, and disulfide with freeze-thaw cycles according to fresh serum sample

	Native Thiol (μmol/l)		Total Thiol (μmol/l)		Disulfide (μmol/l)	
	Mean ± SD	p-Value	Mean ± SD	p-Value	Mean ± SD	p-Value
Fresh Serum (Cycle 0)	403.57±46.25	1.000	442.08±49.73	1.000	19.25±4.26	1.000
Cycle 1	406.29±51.48	1.000	442.85±53.22	1.000	18.28±3.83	1.000
Cycle 2	400.11±49.51	1.000	440.90±54.31	1.000	20.40±5.82	0.999
Cycle 3	385.05±38.65	0.887	430.54±43.59	0.999	22.75±4.37	0.340
Cycle 4	372.87±66.96	0.234	417.56±72.92	0.749	22.35±6.10	0.531
Cycle 5	358.75±45.88	0.006*	405.76±49.85	0.185	23.50±3.97	0.103
Cycle 6	338.99±63.52	<0.001*	395.87±65.36	0.022*	28.44±6.12	<0.001*
Cycle 7	335.66±48.85	<0.001*	398.25±55.57	0.040*	31.30±4.78	<0.001*
Cycle 8	329.86±51.64	<0.001*	397.91±59.84	0.037*	34.03±5.63	<0.001*
Cycle 9	253.66±100.02	<0.001*	336.10±119.76	<0.001*	41.22±14.70	<0.001*
Cycle 10	255.12±98.45	<0.001*	334.29±117.30	<0.001*	39.58±14.66	<0.001*

Results were expressed as mean ± SD.

*Values with p<0.05 in the Tukey results were considered statistically significant.

Discussion

According to our study, we determined that the amount of SH, SH+SS, and SS in human serum samples were stable for up to three months at -20°C and 12 months at -80°C. On the opposite hand, we determined that the SH levels were 5th, and therefore

the SH+SS, and SS levels remained stable until the 6th freeze-thaw cycle. Additionally, we determined that SH and SH+SS concentrations of the serum samples decreased at -20°C because the storage time was prolonged, and SS concentrations increased.

Samples got to be stored for many scientific studies. Biological

materials collected for research purposes usually have one sample tube and are sometimes exposed to repeated freeze-thaw cycles thanks to multiple measurements or analysis types to be applied within the research protocol. There's limited information about specific sample storage protocols that minimize analytical error and variability in evaluating redox parameters.

Bortolin RC. et al. showed that SH levels decreased significantly in -20°C at 30th and 60th days in homogenized erythrocytes, which storage at -20°C affects quite storing SH at -80°C [5]. In another study conducted by Rubio CP. et al. with dog serum, it had been observed that there was a big decrease in SH+SS concentrations after the 3rd freeze-thaw cycle and at day 60 at -20°C [6]. Our results were compatible with the studies of Bortolin RC et al. and Rubio CP et al. and therefore the prolongation of the storage time of human serum at -20°C caused a decrease in SH and SH+SS levels. Additionally, thiol-disulfide homeostasis tests were observed to be more stable in samples stored at -80°C than at -20°C. The variability of thiol levels measured in rat, dog, and human blood and therefore the stability times at -20°C are thought to result from differences within the population of studies, the proteins that make up the thiols, the levels of albumin, and other amino acids, and their response to oxidation.

The majority of thiols in serum originate mainly from albumin and other proteins, while a little portion consists of low relative molecular mass thiols like glutathione, cysteine, homocysteine, cysteinyl glycine, and γ -glutamyl cysteine.

In a study by Culp-hill R. et al, they stated that glutathione (redox-sensitive tripeptide) tends to deteriorate when stored at -20°C for two weeks, and caution should be exercised for extended storage time than 14 days [7]. Takehana S. et al. Showed that the concentration of some amino acids (arginine, glutaminic acid, and cystine) is more susceptible to change in extended storage times at -20°C than -80°C [8]. In these studies, it had been emphasized that the amino acids that structure a little portion of the thiol sources are more stable at -80°C, and at -20°C, they degrade more easily over time and should cause erroneous results.

Cuhadar S. et al. found that total protein concentrations decreased significantly at the primary freeze-thaw cycle, however, albumin concentration didn't change statistically until the 7th freeze-thaw cycle. They also found that total protein and albumin levels began to decrease significantly at -20°C after the 3rd month [9]. Kale VP. et al. in their study with 30 rats reported that the concentrations of 9 parameters including albumin weren't statistically significant after the primary thawing compared to their levels measured in fresh samples [10]. Albumin and other proteins structure the bulk of the thiol pool. The three-dimensional structure of proteins is critical for the properties and performance of a molecule, and repeated freeze-thaw can cause protein degradation [11]. The freezing-thawing process causes the denaturation and instability of the proteins, causing stress within the protein structure and results in oxidative damage. Freezing can denature proteins and alter their dissolving concentrations thanks to pH changes resulting from the crystallization of water and buffer solutes [12, 13].

Considering all the results together, we proceed with a uniform result: the increasing number of freeze-thaw leads to differences within the parameters of thiol-disulfide homeostasis of frozen

samples for various periods. Samples that are frozen at -20°C for an extended time are more susceptible to oxidative stress and may be considered unstable compared to fresh samples. Our results show that storing serum samples at -20°C affects thiol-disulfide homeostasis parameters quite -80°C. This might flow from the suppression of enzymatic activity and inhibition of self-degradation of amino acids and proteins in plasma at -80°C [8]. additionally, the decrease within the concentration of the -SH groups that form native thiols during storage time at -20°C is perhaps caused by the formation of oxidative S-S bridges of -SH-containing cysteine and protein groups [14]. This explains the oxidative stress that increases at -20°C and with increasing freeze-thaw cycles, and consequently increased disulfide (S-S) levels. These data support other studies showing that thiol groups are vulnerable to oxidation and degradation during sampling and storage [15, 16]. It's recommended to stay the samples of physical body fluids within the freezer for an extended time rather than thawing them repeatedly [11].

As a result, the long-term storage of serum samples at -20°C and therefore the increase in freeze-thaw number may affect the accuracy of the test results obtained from the samples and cause the parameters to vary thanks to oxidative stress. Therefore, samples got to be analyzed as soon as possible. However, if this is often impossible, serum samples should be preserved in portions to stop the effect of freeze-thaw on the samples. A frozen serum sample should be thawed once to get the foremost accurate test results. To scale back interaction within the results, sample freezing time should be checked and repeated freeze-thaw cycles should be avoided. Additionally, if long storage times are required, samples are recommended to be stored at low temperatures, like -80°C.

Acknowledgment

The authors thank the technical service of the biochemistry laboratory of the Ankara City Hospital.

Conflict of interests

The authors declare that they have no competing interests.

Financial Disclosure

All authors declare no financial support.

Ethical approval

Yıldırım Beyazıt University Faculty of Medicine Clinical Research Ethics Committee approved the study.

References

1. Erel O, Neselioglu S. A novel and automated assay for thiol/disulphide homeostasis. *Clin Biochem.* 2014;47:326-32.
2. Gasparotto J, Senger MR, Kunzler A, et al. Increased tau phosphorylation and receptor for advanced glycation endproducts (RAGE) in the brain of mice infected with *Leishmania amazonensis*. *Brain Behav Immun.* 2015;43:37-45.
3. Tanno K, Okamura T, Ohsawa M, et al. Comparison of low-density lipoprotein cholesterol concentrations measured by a direct homogeneous assay and by the Friedewald formula in a large community population. *Clinica Chimica Acta.* 2010;411:1774-80.
4. Cowie CC, Rust KF, Byrd-Holt DD, et al. Prevalence of diabetes and high risk for diabetes using A1C criteria in the U.S. population in 1988–2006. *Diabetes Care.* 2010;33:562-8.
5. Bortolin RC, Gasparotto J, Vargas AR, et al. Effects of freeze-thaw and storage on enzymatic activities, protein oxidative damage, and immunocontent of the blood, liver, and brain of rats. *Biopreserv Biobank.* 2017;15:182-90.

6. Rubio CP, Tvarijonaviute A, Caldin M, et al. Stability of biomarkers of oxidative stress in canine serum. *Res Vet Sci.* 2018;121:85-93.
7. Culp-Hill R, Reisz JA, Hansen KC, et al. Investigation of the effects of storage and freezing on mixes of heavy-labeled metabolite and amino acid standards. *Rapid Commun Mass Spectrom.* 2017;31:2030-4.
8. Takehana S, Yoshida H, Ozawa S, et al. The effects of pre-analysis sample handling on human plasma amino acid concentrations. *Clin Chim Acta.* 2016;455:68-74.
9. Cuhadar S, Koseoglu M, Atay A, et al. The effect of storage time and freeze-thaw cycles on the stability of serum samples. *Biochem Med (Zagreb).* 2013;23:70-7.
10. Kale VP, Patel SG, Gunjal PS, et al. Effect of repeated freezing and thawing on 18 clinical chemistry analytes in rat serum. *J Am Assoc Lab Anim Sci.* 2012;51:475-8.
11. Mitchell BL, Yasui Y, Li CI, et al. Impact of freeze-thaw cycles and storage time on plasma samples used in mass spectrometry based biomarker discovery projects. *Cancer Inform.* 2005;1:98-104.
12. Franks F. Protein destabilization at low temperatures. *Adv Protein Chem.* 1995;46:105-39.
13. Cao E, Chen Y, Cui Z, et al. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. *Biotechnol Bioeng.* 2003;82:684-90.
14. Stein WH, Moore S. The free amino acids of human blood plasma. *J.Biol. Chem.* 1954;211:915-26.
15. Rahman I, Kode A, Biswas SK. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat Protoc.* 2006;1:3159-65.
16. Tipple TE, Rogers LK. Methods for the determination of plasma or tissue glutathione levels. *Methods Mol Biol.* 2012;889:315-24.