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Effect of Sample Storage on Stability of Salivary Glutathione, Lipid Peroxidation Levels, and Tissue Factor Activity

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Saliva samples are often required to be stored for longer periods of time either because of the project protocol or because of lack of funding for analysis. The effects of 6 months storage (fresh, 30, 60, 90 120, 150, and 180 d) on the stability of salivary reduced glutathione (GSH), lipid peroxidation (LPO) and 90 days of storage (fresh, 15, 30, 60, and 90 d) on the stability of salivary tissue factor (TF) activity and the stability of saliva imprint samples at −20°C were evaluated in this study. Salivary GSH, malondialdehyde (MDA) levels as an index of LPO, and TF activities were determined using the methods of Beutler, Yagi, and Quick, respectively. Saliva imprint samples were stained with Giemsa and microscopically examined. Salivary GSH levels and TF activities decreased, whereas MDA levels increased significantly after 6 months of storage at −20°C. Leucocyte, epithelium and bacterium cell counts did not significantly change at the end of 90 d of storage. Saliva samples may be stored up to 1 month at −20°C for LPO assay. For cytological examinations, saliva samples may be stored for 90 d at −20°C. Further studies are needed to determine the stability of salivary GSH, and salivary TF activity stored less than 30 days at −20°C. On the other hand, if saliva samples are required to be stored, to avoid the changes because of different storage periods, we recommend that they must be stored under the same circumstances and in the same time period. J. Clin. Lab. Anal. 23 : 93–98, 2009.

Key words: saliva; storage; tissue factor; glutathione; lipid peroxidation

INTRODUCTION

Diagnosis of disease via the analysis of saliva is potentially valuable for children and adults, because collection of the fluid is associated with fewer compliance problems and may provide a cost-effective approach for the screening of large populations (1). Therefore, saliva is especially suitable for study because of its ready availability.

Oxidative stress constitutes the basis for many diseases and it may account for the severity of systemic and oral disease complications. Salivary-reduced glutathione (GSH) and lipid peroxidation (LPO) have been suggested to be used to detect the oral oxidant–antioxidant status (2–4).

Tissue factor (Thromboplastin, Factor III, TF) is an essential initiator of the coagulation cascade and it is considered to be a major regulator of normal hemostasis and thrombosis (5). Saliva, other body fluids (amniotic fluid, bile, semen, sweat, or tears) and various tissues have been known to have TF activity (6–9). Coagulative function of the saliva derives from the TF found in saliva (9).

In research projects, samples are often required to be stored for longer periods of time either because of the protocol of the project or because of lack of funding for analysis. Currently, little consistency exists in the literature regarding the feasibility of saliva storage

Abbreviations: MDA, malondialdehyde; LPO, lipid peroxidation; GSH, reduced glutathione; TF, tissue factor; TBARS, thiobarbituric acid reactive substances

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before analysis. Therefore, the aim of this study was to explore the effects of long-term storage on stability of salivary GSH, malondialdehyde (MDA) levels as an index of LPO and TF activity. In addition, the effect of storage on the stability of saliva imprint samples was evaluated cytologically.

MATERIALS AND METHODS

Saliva Collection and Storage

Saliva was collected from employees of a research laboratory, having no systemic disease with an age range of 26–59 years. All subjects gave informed consent to participate in the study. Following flushing of mouth with distilled water, unstimulated saliva samples were collected into plastic tubes after 2 hr-fasting at 08:00–10:00 a.m. The saliva was pooled, stirred continually in course of dividing aliquots into 70 as identical portions. Seven series of identical 10 aliquots were prepared for GSH and LPO assays. Consequently, the effects of freezing at −20°C on the level of GSH and MDA were evaluated in 10 pooled saliva samples when fresh, and at 30, 60, 90, 120, 150, and 180 d after storage. In addition, 5 series of identical 10 aliquots were prepared for TF activity determination and cytological examinations. Accordingly, 10 saliva samples were evaluated for the effect of freezing at −20°C on the TF activities and cytological examinations when fresh, and 15, 30, 60, and 90 d after storage. After thawing, saliva samples were centrifuged (1,715 × *g* for 5 min), and GSH and MDA concentrations were determined in the supernatant. However, saliva samples were not centrifuged for TF activity and cytological examinations, they were vortexed at least 10 sec for easy sampling.

Saliva Analysis

Assay of GSH

Saliva GSH concentration was determined according to Beutler et al. (10) using metaphosphoric acid for protein precipitation and 5′5′-dithiobis-2-nitro-benzoic acid for color development.

Assay of LPO

LPO was assayed by measuring MDA levels in saliva. MDA levels in saliva were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Yagi (11).

Assay of TF activity

TF activities of saliva samples were determined according to Quick's one-stage method (12) using normal plasma. This was performed by mixing 0.1 ml saliva with 0.1 ml of 0.02 M $CaCl_2$, with the clotting reaction being started on an addition of 0.1 ml of plasma. All reagents were brought to the reaction temperature (37°C) before admixture. As the clotting time is inversely proportional to the TF activity, the lengthening of the clotting time is a manifestation of decreased TF activity.

Cytological examinations

Saliva samples were smeared over a glass microscope slide and fixed with air. Then they were stained with Giemsa stain (13) and microscopically examined (× 100) for the presence of epithelium, leucocyte, and bacterium cell counts.

Statistical analysis

Fisher's least-significant difference test and Spearman correlation analysis were used to analyze the data. All statistical analyses were performed with the SPSS software for Windows, Version 11.

RESULTS

Salivary GSH, MDA levels, TF activities, and 95% confidence intervals are given in Tables 1 and 2. The coefficients of variation of fresh, 30, 60, 90, 120, 150,

TABLE 1. GSH and MDA Levels With 95% CI of Saliva Samples Stored at −20°C for 180 Days

	GSH (mg/dl)	SE	95% CI	MDA (nmol MDA/ml)	SE	95% CI
Day 0	0.266	0.009	0.245–0.287	0.332	0.044	0.233–0.431
1 mo	0.835*	0.011	0.810–0.860	0.339	0.021	0.292–0.386
2 mo	0.876♠	0.014	0.843–0.909	0.539*	0.015	0.506–0.572
3 mo	0.377*	0.017	0.339–0.415	0.380*	0.018	0.339–0.421
4 mo	0.293♣	0.005	0.281–0.305	0.206*	0.016	0.169–0.243
5 mo	0.231*	0.007	0.215–0.247	0.638*	0.014	0.607–0.669
6 mo	0.202*♦	0.005	0.191–0.213	0.846*	0.011	0.820–0.872

Values are expressed as mean and SE (standard error); mo: month; *$P<0.0001$ significantly different from the previous month; ♠$P<0.05$ significantly different from the previous month; ♣$P<0.01$ significantly different from the previous month; ♦$P<0.0001$ significantly different from day 0. GSH, reduced glutathione; MDA, malondialdehyde; CI, confidence interval.

and 180 d-stored saliva samples' GSH and LPO assays were 3.38, 1.32, 1.60, 4.5, 1.71, 3.03, and 2.48%, and 13.25, 6.19, 2.78, 4.73, 7.77, 2.19, and 1.30%, respectively. The coefficients of variation of fresh, 15, 30, 60, and 90 d-stored saliva samples' TF activities were 0.56, 0.42, 0.92, 1.14, and 1.83%, respectively. The coefficients of variations for GSH, LPO, and TF activity assays indicate relatively good reproducibility of the assays. Salivary GSH levels significantly decreased after 6 months of storage at −20°C compared with fresh samples ($P<0.0001$). On the other hand, a significant increase was observed between fresh GSH levels and 1 month-stored GSH samples ($P<0.0001$). GSH levels at the end of 2 months also increased significantly when compared with the first month ($P<0.05$). After 2 months, GSH levels of the saliva samples decreased significantly per month (Fig. 1).

Compared with fresh saliva samples, salivary MDA levels increased significantly after 6 months ($P<0.0001$). However, the increase was not significant at the end of the first month compared with fresh saliva samples, but significant at the end of 2 months compared with first month ($P<0.0001$). On the other hand, MDA levels decreased significantly at the end of third and fourth months of storage ($P<0.0001$). At the end of fifth and sixth months, MDA levels increased significantly per month ($P<0.0001$) (Fig. 2).

Salivary TF activity decreased significantly after 15, 30, 60, and 90 d when compared with the previous activities, and there was a significant decrease after 90 d of storage as compared with fresh samples ($P<0.05$) (Fig. 3).

GSH levels of the fresh saliva samples showed an inverse correlation with the MDA levels (day 0) ($r=-0.488$; $P<0.01$). In general, salivary TF activity was positively correlated with GSH levels ($r=0.341$; $P<0.01$) and negatively correlated with MDA levels ($r=-0.391$; $P<0.01$).

Leucocyte, epithelium and bacterium cell counts did not significantly change at the end of 15, 30, 60, and 90 d of storage. In general, salivary TF activity was strongly and positively correlated with epithelium ($r=0.729$; $P<0.01$) and leucocyte cell counts ($r=0.849$; $P<0.01$) and negatively correlated with bacterium cell count ($r=-0.822$; $P<0.01$).

TABLE 2. TF Activities With 95% CI of Saliva Samples Stored at −200°C for 90 Days

	TF activity (sec)	SE	95% CI
Day 0	54.60	0.306	53.90–55.29
Day 15	69.80*	0.291	69.14–70.46
Day 30	93.60*	0.859	91.66–95.54
Day 60	94.60*	1.077	92.16–97.04
Day 90	114.00*	2.082	109.29–118.71

Values are expressed as mean and SE (Standart Error). *$P<0.0001$ significantly different from the previous period. TF, tissue factor; CI, confidence interval.

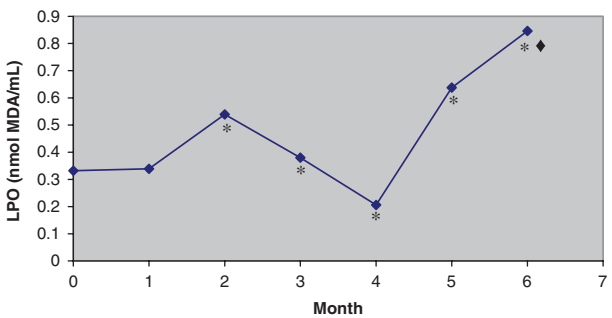

Fig. 2. The effect of storage at −20°C for 6 months on salivary MDA levels. *$P<0.0001$ significantly different from the previous month; ♦ $P<0.0001$ significantly different from day 0. MDA, malondialdehyde.

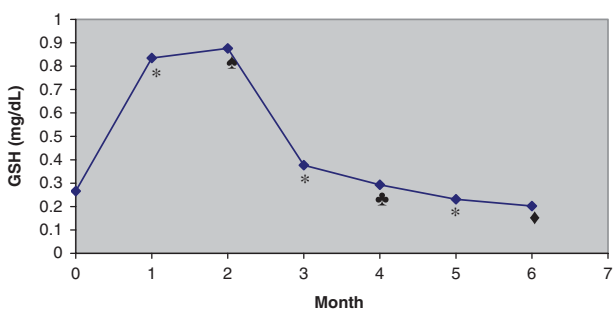

Fig. 1. The effect of storage at −20°C for 6 months on salivary reduced GSH levels. *$P<0.0001$ significantly different from the previous month; ♦ $P<0.0001$ significantly different from day 0; ♠ $P<0.05$ significantly different from the previous month; ♣ $P<0.01$ significantly different from the previous month; GSH, reduced glutathione.

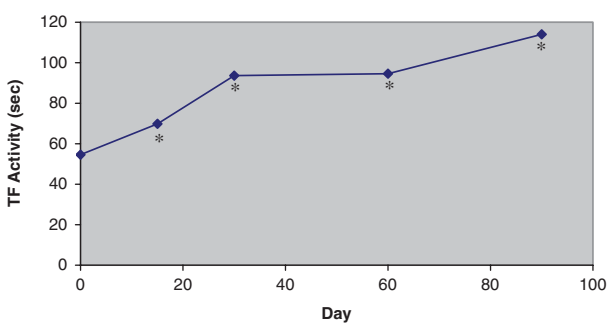

Fig. 3. The effect of storage at −20°C for 90 days on salivary TF activity (lengthening in seconds is a manifestation of decreased TF activity). *$P<0.0001$ significantly different from the previous period. TF, tissue factor.

DISCUSSION

Saliva is increasingly used and well validated in diagnosing. Moreover, saliva is reported to be suitable to detect the body's oxidative stress level (1–4). GSH is present in saliva, cerebrospinal fluid, sweat, seminal fluid, and milk. Thus, release of GSH appears to be a general property of cells, which suggests that extracellular GSH has specific biological functions. One of these functions is control of the extracellular redox state, but other functions of extracellular GSH include: acting as a source of cysteine, acting as a substrate for extracellular GSH-dependent enzymes, direct protection of critical thiol groups of transporters and receptors present on the extracellular surface of cells, the reduction of disulfides to control the fluidity of mucus and the maintenance of other antioxidant systems (e.g., ascorbate) (14,15).

Stability of GSH in stored blood samples has been studied before in several studies (16,17). Whole blood stored at $-70^{\circ}C$ for up to 10 days was shown to be the best storage condition for erythrocyte GSH determination (16). Jodwik et al. (17) preserved blood with CPDA-1 anticoagulant (citrate, phosphate, dextrose, and adenine) and determined the concentrations of GSH in plasma on days 1, 3, 7, 12, 16, 20, and 25 of storage. They concluded that a 12-day period could be considered as a safe storage limit.

To our knowledge, the stability of reduced GSH in stored saliva samples was investigated for the first time in this study. Salivary GSH levels significantly decreased after 6 months of storage at $-20^{\circ}C$. On the other hand, a significant increase was observed at the end of 1 month of storage compared with fresh samples and at the end of 2 months compared with the first month. After 3 months, GSH levels of the saliva samples decreased significantly per month. Increased GSH levels at the end of the first and the second months are unexpected findings. However, a similar situation has been reported for serum alkaline phosphatase (ALP) activity. Massion et al. (18) reported that ALP activities of 10 fresh sera increased by an average of 0.9, 2.7 and 6.1% in 6, 24 and 96 hr; that of pooled serum, frozen, and thawed, increased about 1% per hour. These increases may be the result of the release of ALP from complexes it forms with lipoproteins in vitro, as the noncomplexed enzyme has greater activity (19). Saliva is mainly composed of water (99.5%), organic (0.3%), inorganic, and trace elements (0.2%) (20). Copper is one of the trace elements present in saliva (21). Several studies have pointed out a role for GSH in the intracellular traffic of copper (22,23). It has been proposed that GSH is capable of chelating and detoxifying metals soon after they enter the cell, and copper is rapidly complexed by GSH after entering the cell and is then transferred to metallothioneins (24). Increased GSH levels after storage may be the result of the release of GSH from the complexes it forms, in particular with copper. On the other hand, further studies are needed to measure the stability of saliva samples stored for periods <30 d.

Saral et al. (25) showed that the differences in the level of MDA in serum were reflected in saliva, suggesting that the saliva can be used as a biological sample in monitoring LPO in patients with recurrent aphthous ulceration. Therefore, storage of saliva for LPO determination is an important issue. In this study, compared with fresh saliva samples, salivary MDA levels increased significantly after 6 months of storage at $-20^{\circ}C$ ($P<0.0001$). MDA levels did not change significantly after 1 month of storage at $-20^{\circ}C$ but significantly increased after 2 months of storage both when compared with fresh saliva samples and 1 month-stored samples. On the other hand, at the end of third and fourth months of storage MDA levels were found to be decreased significantly when compared with their previous month. Beginning from the fifth month, MDA levels increased significantly per month. Decreased MDA levels at the end of the third and fourth months might be because of increased GSH levels at the end of 2 months.

Stressful environments, such as prolonged freezing or repeated freeze–thaw cycles, impose several interconnected stresses including dehydration, hyperosmotic stress, ice formation, oxidative stress, and low temperature (26,27). Disruption of the plasma membrane is a primary cause of freezing injury (28). Moreover, Alvarez and Storey (29) suggested that cryopreservation enhances LPO in human sperm and that this enhancement is mediated at least in part by the loss of superoxide dismutase activity occurring during the process. Therefore, in this study, increased GSH levels at the end of 2 months might have prevented the increase in LPO at the end of the third and fourth months. Then, owing to the loss of GSH levels, MDA levels were found to be increased significantly at the end of fifth and sixth months. As effect of storage time on the stability of salivary LPO has not been studied before, we cannot compare other researchers' findings on this issue. However, on the strength of unchanged MDA levels after 1 month of storage, we may suggest that saliva samples may be stored up to 1 month for LPO assay.

As the principal biological initiator of blood coagulation, TF is believed to play a critical role in thrombosis and thrombogenesis (5), and concentrations in several biological fluids correlate with different pathological conditions. Although saliva has long been recognized as having TF activity (3,8,9), there is only one study in the

literature that examined the effect of storage time and temperature on salivary TF activity. Zacharski and Rosenstein (9) reported that salivary coagulant activity was not increased by freezing and thawing and was stable at −20°C for at least 2 weeks. When we compared TF activity of the stored saliva samples, we found that TF activity decreased significantly per month and there was a significant decrease after 6 months of storage as compared with the first month. Similar to our results, urinary TF (uTF) is shown to be relatively unstable on storage (30,31). Carty et al. (30) showed a fall in uTF activity after 13 days at 4°C, after 10 days at room temperature and after only four days at −20°C. Matsumura and Von Kaulla (31) found that urine began to lose procoagulant activity after 1 day of storage at room temperature and after 3 days at −20°C. Freezing urine could be a major cause of instability. Bach et al. (32) showed that freezing and thawing either cause internal reorientation of the vesicles toward TF or trap TF within the associated vesicles. In either case, not all TF would be available to factor VII and therefore not all the coagulant activity will be measured. These workers have also shown that digestion of TF, using subtilisin protease proteolytic enzyme, causes a 50% loss in activity on frozen storage.

Leucocyte, epithelium, and bacterium cell counts were not significantly changed at the end of 15, 30, 60, and 90 d of storage. On the basis of this result, we may suppose that, for cytological examinations, saliva samples can be stored at −20°C for 90 d. On the other hand, we believe that further work is necessary for the determination of saliva stability for longer periods than 90 days.

Approximately 78% of salivary TF activity is attributed to cells found in saliva (9). On the other hand, in our study a negative correlation was observed between bacterium cell count and salivary TF activity. This negative correlation may be the result of significantly decreased TF activity because of storage period.

The acute responses of bacterial cells to freezing and thawing, including the effects of earlier exposure to cold and other stresses on survival are different. Many other variables also contribute to whether bacteria survive freezing and thawing, including their nutritional status and growth phase as well as the cooling rate employed (26). Moreover, loss of viability is proportional to the number of freeze–thaw cycles that cells experience (27). Therefore, the elapsed time that cells are frozen generally influences viability less than the processes of freezing and thawing (26). Freezing and thawing *E. coli* cells without an exogenously supplied cryoprotective agent, such as glycerol, severely decreases their viability (32). On the other hand, in our study, as bacterial cells in saliva samples experienced the freeze–thaw cycle one time, their viability could be less affected. This may explain why bacterium cell count did not significantly change. By contrast, much less is known about how and why different bacterial strains and species vary in their capacity to survive these stresses (33).

In this study, there was a positive correlation between salivary TF activity and GSH levels, and a negative correlation between salivary TF activity and MDA levels. As TF contains phospholipids in its structure (9), protection of the phospholipids from oxidation by GSH might be the reason for this positive correlation. On the other hand, the decrease in GSH levels owing to storage period may also lead to a decrease in TF activity. Similarly as MDA increases, phospholipids in the structure of TF might be prone to oxidation and this could decrease its activity.

In many research settings, storage of samples before analysis is unavoidable. Therefore, to validate the potential use of salivary GSH and LPO as biological indicators, we performed a longitudinal study to examine the long-term stability of GSH and LPO in stored saliva samples. Furthermore, saliva samples stored at −20°C were also cytologically investigated and for the stability of TF activity. On the basis of the results of this study, we suggest that, saliva samples may be stored up to 1 month for LPO assay. For cytological examinations, saliva samples may be stored for 90 d at −20°C. On the other hand, further studies are needed to determine the stability of salivary GSH and saliva TF activity stored less than 30 days at −20°C.

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