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Determination of Storage Time of Saliva Samples Obtained From Patients With and Without Chronic Periodontitis for the Comparison of Some Biochemical and Cytological Parameters

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Background: Salivary glutathione (GSH), malondialdehyde (MDA), protein, sialic acid (SA) levels, cytological parameters, and tissue factor activities (TFa) were investigated when fresh and after 3, 7, 11, 15, 21, and 30 days (d) of storage at −20°C both in the control and the periodontitis group. Moreover, the control and the periodontits groups were compared and continuity of the significances detected between the two groups were evaluated. *Methods:* GSH, MDA, SA, protein, and TFa were determined using the methods of Beutler, Yagi, Warren, Lowry, and Quick, respectively. Saliva imprint samples were stained with Giemsa and microscopically examined. *Results:* When the continuity of the significances of differences between the two groups was investigated, differences continued to be significant for GSH and TFa on days 3, 7, 11, 15, 21, and 30. Cytologically, only the significance detected between leucocyte numbers continued to be significant for 30 d. However significance of differences in total protein, MDA, and SA levels on day 0, were interrupted on days 3, 7, and 11, respectively. *Conclusion:* Saliva samples may be stored for 30 d for GSH and TFa analyses in patients with and without periodontitis. However, to compare salivary MDA, SA, and total protein levels in these groups we suggest fresh samples to be studied. J. Clin. Lab. Anal. 27:261–266, 2013.

Key words: saliva; storage; periodontitis

INTRODUCTION

Diagnosis of disease via the analysis of saliva is potentially valuable for children and adults, since 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 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 (TFa) (6–9). Coagulative function of the saliva derives from the TF found in saliva (10). Sialic acid (SA), a family of acetylated derivatives of neuraminic acid, is widely distributed in mammals. It usually occurs as a terminal component at the nonreducing end of carbohydrate chains of glycoproteins and glycolipids (11). SA is an important component of salivary glycoprotein and enhances bacterial aggregation and participates in the formation of acquired pellicle, and dental plaque (12).

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Periodontal disease is a common chronic adult condition. The bacterium *Porphyromonas gingivalis* has been implicated in the aetiology of this disease, which causes destruction of the connective tissue and bone around the root area of the tooth. Reactive oxygen species (ROS) are implicated in the destruction of the periodontium during inflammatory periodontal diseases. Previous research has found increased LPO and reduced salivary antioxidant activity in patients suffering from periodontal disease (13, 14). On the other hand, research considering salivary antioxidant status and periodontal disease is sparse and has yielded conflicting data due to the different methodology employed by the authors.

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 before analysis. We have previously reported the effects of 6 months storage on the stability of salivary GSH, LPO, and 90 days (d) of storage on the stability of salivary TFa and the stability of saliva imprint samples at −20°C (15). Based on the results, we have suggested that the saliva samples have to be stored under the same circumstances and in the same time period. On the other hand, when working with different patient groups, the storage period of saliva may also affect the significance of the difference in the salivary parameters between the groups. Accordingly in this study, determination of storage time for the comparison of saliva samples of patients with and without chronic periodontitis was aimed. Salivary GSH, malondialdehyde (MDA), the end product of LPO, protein, SA levels, cytological parameters, and TFa were investigated when fresh and after 3, 7, 11, 15, 21, 30 d of storage at −20°C both in the control and the periodontitis group. Moreover hypothesizing that storage period may affect the saliva of healthy individuals and periontitis patients differently, which may in turn affect the significances between the two groups, the continuity of the significances detected between the two groups were evaluated.

METHODS

Saliva Collection and Storage

Saliva was collected from 7 subjects with chronic periodontitis and 12 healthy subjects, having no systemic disease with an age range of 26–59 years. The study was approved by the local Ethical Committee and 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 hours fasting, at 08:00–10:00 a.m. To avoid individual differences the saliva was pooled and two groups were formed as the control and the periodontitis group. Then they were stirred continually in course of dividing aliquots into 70 identical portions. Seven identical series (one for each day) of 10 aliquots were prepared. For each day 10 aliquots were thawed then centrifuged (1715 × *g* for 5 min) for GSH, MDA, total protein, and SA assays as they were determined in the supernatant. However, saliva samples were not cenrifuged for TFa and cytological examinations, aliquots were vortexed at least 10 sec for easy sampling. One aliquot was used to assess all parameters, therefore each parameter was repeated 10 times. Consequently, the effects of freezing at –20°C on these parameters were evaluated in 10 pooled saliva samples when fresh and at 3, 7, 11, 15, 21, and 30 d after storage.

Saliva Analysis

Assay of GSH

Saliva GSH concentration was determined according to Beutler et al. (16) 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 (17).

Assay of SA

Total saliva SA levels were determined by the thiobarbituric acid method described by Warren (18).

Assay of total protein

Total protein content of saliva samples were evaluated by the method of Lowry (19).

Assay of tissue factor activity

TFa of saliva samples were determined according to Quick's one-stage method (20) using normal plasma. This was performed by mixing 0.1 ml of saliva with 0.1 ml of 0.02 M $CaCl_2$, with the clotting reaction being started on addition of 0.1 ml of plasma. Same plasma was used for all TF activity assays in both the control and patients groups. All reagents were brought to the reaction temperature (37°C) before admixture. Since the clotting time is inversely proportional to the TFa, the lengthening of the clotting time is a manifestation of decreased TFa.

Cytological Examinations

Saliva samples were smeared over a glass microscope slide and fixed with air. Then they were stained with Giemsa stain (21) and microscopically examined ($\times 100$) for the presence of ephitelium, leucocyte, and bacterium cell count.

Statistical Analysis

One-way Anova test followed by Fisher's least-significant difference test was used to compare the means of salivary parameters obtained at a storage period with the means obtained at other periods within the control and periodontitis group; whereas, Kruskal–Wallis test followed by Dunn's multiple comparison test was used for the comparison of cytological parameters within each group. Differences between the control and the periodontitis groups were evaluated using one-way Anova test followed by Student *t*-test. For the comparison of cytological parameters of the control and the periodontitis groups Kruskal–Wallis test followed by Mann–Whitney *U* test was used. All statistical analyses were performed with the SPSS software for Windows, Version 11.

RESULTS

Salivary GSH, LPO, SA, total protein levels, and TFa of the control and periodontitis groups are given in Tables 1 and 2, respectively. Only salivary GSH levels were stable up to 11 d in the control group and salivary protein levels were stable up to 11 d in the periodontitis group. Results of the cytological examinations are given for the control and the periodontitis groups in Tables 3 and 4, respectively.

When GSH, LPO, SA, total protein levels, and TFa of the control group were compared with the periodontitis group, significant differences were obtained at the begining of the study (day 0). These differences continued to be significant for GSH and TFa on days 3, 7, 11, 15, 21, and 30. On the other hand the differences detected in LPO, SA, and total protein levels between the control and periodontitis group on day 0, were interrupted on days 7, 11, and 3, respectively (Table 5). For the cytological examinations only the significance detected between leucocyte numbers in the two groups continued to be significant for 30 d (Table 6).

DISCUSSION

Many analytes are not stable at room temperature, and keeping samples cold after collection is important. On the other hand in many research settings, storage of samples prior to analysis is unavoidable. Therefore in our present study we aimed to determine whether different storage periods at -20°C affect the stability of salivary GSH, LPO, total protein, SA levels, and TFa in the saliva samples of subjects with and without periodontitis.

We have previously reported that saliva samples may be stored up to 1 month for LPO assay and up to 90 d at -20°C for cytological examinations (15). On the other hand, we have suggested the need of further studies to determine the stability of salivary GSH and saliva TFa stored for less than 30 d at -20°C. Therefore, in our present study we aimed to explore and compare the effects of short-term storage at -20°C on the stability of salivary GSH, LPO, total protein, SA levels, and TFa. According to our results storage periods led to some changes in the parameters investigated, both in the control group and the periodontitis group. Significant decreases were observed in GSH, SA, and protein levels both in the control and the periodontitis groups, whereas TF activities increased in all periods both in the control and the periodontitis group. Although significant decreases were observed in MDA levels in the control group, they increased significantly in the periodontitis group. Only salivary GSH levels were stable up to 11 d in the control group and salivary protein levels were stable up to 11 d in the periodontitis group.

The data about the stability of TFa are limited. Lwaleed et al. (22) investigated the activity of urinary TFa stored at room temperature, 4°C, -20°C, or -72°C after 0, 2, 4, 6, 8, and 12 hours of storage and the effects of storage at -20°C and -72°C daily for 7 d after collection. In contrast to our study they reported reduced urinary TFa in stored samples. This may be due to the source of TF since salivary TFa was measured in our study instead of urinary TFa. Moreover Bach et al. (23) showed that freezing and thawing either cause internal reorientation of the vesicles toward the TF or trap the TF within the associated vesicles.

Periodontal diseases (gingivitis and periodontitis) are among the most widespread chronic conditions affecting populations worldwide. Tissue injury due to free radical production has been suggested to be enhanced in individuals with periodontal disease due to a lack of adequate antioxidant defence (24–26). Exaggerated neutrophil activity may be attributable to defects of the inflammatory response in some individuals (27), but low antioxidant capacity may be caused by a number of factors including smoking and poor nutritional status. Chapple et al. have reported that patients with periodontal disease have reduced total antioxidant capacity in whole saliva (24) and lower concentrations of glutathione in serum and in gingival crevicular fluid (25). In contrast, Moore et al. (28) reported similar salivary antioxidant profiles in periodontal patients and apparently healthy controls. Glutathione can be synthesized in the body from the amino acids L-cysteine, L-glutamic acid, and glycine. The sulfhydryl

TABLE 1. GSH, LPO, SA, Total PR Levels, and TFa of the Control Group

Parameters → Days↓	GSH (mg/dl)	LPO (nmol MDA/ml)	SA(mg/dl)	PR (mg/dl)	TFa (s)
0	0.38 ± 0.04	0.55 ± 0.1	2.89 ± 0.3	190.2 ± 8.63	52.6 ± 4.38
3	0.35 ± 0.05	0.33 ± 0.04[a,b]	3.32 ± 0.4[a,b]	167.9 ± 4.48[a,b]	49.5 ± 2.37[a,b]
7	0.39 ± 0.03	0.49 ± 0.01[a]	3.33 ± 0.45[b]	152.2 ± 12.49[a,b]	37.9 ± 2.0[a,b]
11	0.27 ± 0.03[a,b]	0.71 ± 0.02[a,b]	2.92 ± 0.5[a]	142.1 ± 10.23[a,b]	48 ± 1.56[a,b]
15	0.3 ± 0.03[b]	0.4 ± 0.1[a,b]	3.35 ± 0.3[a,b]	161.3 ± 14.3[a,b]	38.1 ± 1.29[a,b]
21	0.31 ± 0.05[b]	0.42 ± 0.08[b]	3.43 ± 0.32[b]	164.5 ± 13.7[b]	36.1 ± 1.37[b]
30	0.33 ± 0.03[b]	0.32 ± 0.06[a,b]	2.79 ± 0.38[a]	148.4 ± 10.24[a,b]	43 ± 1.89[a,b]

P Anova: 0.001.
[a] $P < 0.05$, significantly different from the previous period, analyzed by Fisher's least-significant difference test.
[b] $P < 0.05$, significantly different from day 0, analyzed by Fisher's least-significant difference test.
GSH, glutathione; LPO, lipid peroxidation; SA, sialic acid; PR, protein; TFa, tissue factor activity.

group of cysteine serves as a proton donor and is responsible for the biological activity of glutathione. Cysteine is the rate-limiting factor in cellular glutathione synthesis, since this amino acid is relatively rare in foodstuffs (29). Accordingly, Zappacosta et al. (30) reported a statistically significant increase of the salivary parameters level of cysteine, cysteinylglycine, glutathione, aspartate aminotransferase, and lactate dehydrogenase in the patient subgroup with periodontal probing pocket depth >5 mm compared with the control group. Salivary cysteine concentrations

TABLE 2. GSH, LPO, SA, Total PR Levels, and TFa of the Periodontitis Group

Parameters → Days↓	GSH (mg/dl)	LPO (nmol MDA/ml)	SA(mg/dl)	PR (mg/dl)	TFa (s)
0	0.69 ± 0.04	0.38 ± 0.11	6.95 ± 0.28	171.9 ± 8.06	46.9 ± 2.02
3	0.57 ± 0.07[a,b]	0.55 ± 0.1[a,b]	4.7 ± 0.43[a,b]	163.9 ± 10.56	40.3 ± 3.37[a,b]
7	0.66 ± 0.03[a]	0.56 ± 0.11[b]	3.83 ± 0.39[a,b]	170 ± 13.8	41.7 ± 2.06[b]
11	0.53 ± 0.07[a,b]	0.55 ± 0.17[b]	2.99 ± 0.37[a,b]	147.5 ± 7.85[a,b]	35.4 ± 1.71[a,b]
15	0.64 ± 0.07[a,b]	0.53 ± 0.07[b]	2.02 ± 0.18[a,b]	155.2 ± 9.72[b]	35 ± 2.79[b]
21	0.64 ± 0.04	0.51 ± 0.12[b]	2.39 ± 0.35[a,b]	149 ± 9.04[b]	39.2 ± 1.48[a,b]
30	0.40 ± 0.04[a,b]	0.53 ± 0.08[b]	2.52 ± 0.38[b]	145.6 ± 10.41[b]	36.2 ± 4.42[a,b]

P Anova: 0.001.
[a] $P < 0.05$, significantly different from the previous period, analyzed by Fisher's least-significant difference test.
[b] $P < 0.05$, significantly different from day 0, analyzed by Fisher's least-significant difference test.
GSH, glutathione; LPO, lipid peroxidation; SA, sialic acid; PR, protein; TFa, tissue factor activity.

TABLE 3. Epithelial Cells, Leucocytes, and Bacteria in the Saliva Imprint Samples of the Control Group

Epithelial cells[a]	Day 0	Day 3	Day 7	Day 11[b,c]	Day 15	Day 21[b,c]	Day 30[b]
1	0%	0%	0%	0%	0%	30%	0%
2	0%	0%	30%	90%	50%	70%	0%
3	100%	100%	70%	10%	50%	0%	100%
Leucocytes	Day 0	Day 3	Day 7	Day 11	Day 15	Day 21	Day 30
0	90%	90%	90%	100%	100%	100%	100%
1	10%	10%	10%	0%	0%	0%	0%
Bacteria[a]	Day 0	Day 3	Day 7[b,c]	Day 11[c]	Day 15[b,c]	Day 21[c]	Day 30[c]
1	0%	50%	0%	0%	0%	0%	0%
2	60%	50%	40%	40%	0%	0%	0%
3	0%	0%	60%	60%	30%	40%	40%
4	40%	0%	0%	0%	70%	60%	60%

[a] *P* Kruskal–Wallis: 0.001.
[b] $P < 0.05$, significantly different from the previous period, analyzed by Dunn.
[c] $P < 0.05$, significantly different from day 0, analyzed by Dunn test.
0 = none, 1 = rare, 2 = few, 3 = moderate, 4 = many.

TABLE 4. Epithelial Cells, Leucocytes, and Bacteria in the Saliva Imprint Samples of the Periodontitis Group

Epithelial cells	Day 0	Day 3	Day 7	Day 11	Day 15	Day 21	Day 30
1	0%	0%	0%	0%	0%	0%	10%
2	60%	100%	50%	90%	80%	60%	80%
3	40%	0%	50%	10%	20%	40%	10%
Leucocytes	Day 0	Day 3	Day 7	Day 11	Day 15	Day 21	Day 30
0	40%	40%	30%	20%	0%	0%	0%
1	30%	20%	70%	80%	100%	100%	80%
2	30%	40%	0%	0%	0%	0%	20%
Bacteria[a]	Day 0	Day 3	Day 7	Day 11	Day 15	Day 21[b]	Day 30[b]
1	0%	0%	0%	0%	0%	0%	0%
2	30%	0%	10%	10%	0%	0%	0%
3	70%	100%	90%	50%	70%	40%	50%
4	0%	0%	0%	40%	30%	60%	50%

[a] P Kruskal–Wallis: 0.001.
[b] $P < 0.05$, significantly different from day 0, analyzed by Dunn test.
0 = none, 1 = rare, 2 = few, 3 = moderate, 4 = many.

showed the most significant correlation and the authors suggested that the increase of cysteine in these patients could be related to some proteolytic activity of bacteria in the oral cavity. Similarly in our study salivary GSH level was significantly higher in the periodontitis group compared with the control group. Due to high GSH levels in the periodontitis group LPO was significantly lower in this group. On the other hand as an index of increased inflammation salivary SA was significantly higher in the periodontitis group compared with the control group. TFa was also significantly higher in the patient group as inflammation has been known to induce TFa (31).

When we investigated the continuity of the significances of the differences detected between the two groups, we found out that these differences continued to be significant for GSH and TFa on days 3, 7, 11, 15, 21, and 30. For the cytological examinations only the significance detected between leucocyte numbers in the two groups continued to be significant for 30 d. On the other hand the differences detected in MDA, SA, and total protein levels between the control and periodontitis group on day 0, were interrupted on days 7, 11, and 3, respectively. This finding is important to set up saliva storage conditions of different groups. For instance, when compared with

TABLE 5. Significance Values (P) Obtained From the Comparision of GSH, LPO, SA, Total PR Levels, and TFa of the Saliva Samples of Healthy and Chronic Periodontitis Patients on Days 0, 3, 7, 11, 15, 21, and 30

Days→ Parameters↓	0	3	7	11	15	21	30
GSH (mg/dl)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
LPO (nmol MDA/ml)	0.001	0.0001	NS	NS	0.002	NS	0.0001
SA(mg/dl)	0.0001	0.0001	0.015	NS	0.0001	0.0001	NS
PR (mg/dl)	0.0001	NS	0.007	NS	NS	0.008	NS
TFa (s)	0.002	0.0001	0.001	0.0001	0.007	0.0001	0.001

Note: Student t-test was used to obtain the significance values.
NS, non significant; GSH, glutathione; LPO, lipid peroxidation; SA, sialic acid; PR, protein; TFa, tissue factor activity.

TABLE 6. Significance Values (P) Obtained From the Comparision of Epithelium Cells, Leucocytes, Bacterium, and Yeast Cells of the Saliva Samples of Healthy and Chronic Periodontitis Patients on Days 0, 3, 7, 11, 15, 21, and 30

Days→ Parameters↓	0	3	7	11	15	21	30
Epithelium cell	0.004	0.0001	NS	NS	NS	0.010	0.0001
Leucocyte	0.018	0.016	0.008	0.0001	0.0001	0.0001	0.001
Bacterium	0.001	0.0001	NS	0.024	NS	NS	NS
Yeast cell	0.005	0.008	NS	NS	NS	0.001	NS

Note: Mann–Whitney test was used to obtain the significance values.
NS, non significant.

the control group although salivary LPO significantly decreased in the patient group, when the same experimental protocol was applied on saliva samples that were stored for 7 d no significant difference was detected between the groups. On the other hand, researchers should also take into account the methods used for the analysis. Therefore based on the results of our study we may suggest that saliva samples may be stored for 30 d for the studies comparing salivary GSH and TFa in patients with and without periodontitis. However in order to compare salivary MDA, SA, and total protein levels in these groups we suggest fresh samples to be studied.

CONFLICTS OF INTEREST

The authors state no conflicts of interest.

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