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Salivary markers of oxidative stress and antioxidant status: Influence of external factors

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Abstract.
BACKGROUND: Salivary markers of oxidative stress and antioxidant status represent promising tool for the research of oral diseases. One of the criteria is the validation of these biomarkers from the perspective of the confounding and modifying factors.
AIM: To examine the effect of circadian rhythm, tooth-brushing and ascorbic acid treatment on selected salivary markers of oxidative and carbonyl stress, and antioxidant status.
SUBJECTS AND METHODS: Whole unstimulated saliva samples were collected from 19 healthy participants three times during a day, before and after tooth-brushing, and before and after the administration of vitamin C (250 mg). Advanced oxidation protein products (AOPP), thiobarbituric acid reactive substances (TBARS), advanced glycation end products (AGEs), ferric reducing antioxidant power (FRAP) and total antioxidant capacity (TAC) were measured.
RESULTS: Salivary AGEs levels varied significantly during the day ($p < 0.05$) with the highest concentrations in the morning. FRAP levels varied during the day ($p < 0.01$) with the highest concentrations in the afternoon. Tooth-brushing decreased AGEs ($p < 0.05$) and TBARS levels ($p < 0.01$) and increased FRAP levels ($p < 0.05$). Single intake of vitamin C significantly decreased AGEs ($p < 0.001$) and increased both FRAP ($p < 0.01$) and TAC ($p < 0.01$) concentrations.
CONCLUSION: Significant daily variations were observed in salivary AGEs and FRAP levels. Tooth-brushing and treatment with vitamin C decreased carbonyl stress and increased the antioxidant status. These results are important from the perspective of using saliva for the research of oral diseases.

Keywords: Oxidative stress, saliva, antioxidant status, circadian rhythm, tooth-brushing, ascorbic acid

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an important role in physiological conditions in cell signaling and metabolic pathways. On the other hand, decreased antioxidant levels and/or increased production of reactive metabolites can lead to the destruction of homeostasis and drive the oxidative damage [3,16]. Oxidative/nitrosative stress can seriously influence the cell viability and induce cellular responses leading to cell death [7]. Many studies have shown connection between oxidative damage of molecules and patophysiological mechanisms of severe diseases like atherosclerosis [14], neurodegenerative diseases [15] and diabetes [41]. There is also a relationship between oxidative stress and aging processes [49].

In association with oral and dental diseases (especially periodontitis), changed oxidative stress markers in saliva were reported [2,46,51]. Being a noninvasive biofluid, saliva allows easy, noninvasive and safe sam-

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ple collection. It is therefore suitable for monitoring of general health and disease in children, elderly people, and non-collaborative subjects. Salivary analysis is very attractive due to the possibility of repeated sampling and longitudinal monitoring [22,25]. To use the potential of salivary oxidative stress markers for diagnostics of oral diseases, various criteria must be optimized.

Three main groups of endproducts are used as biomarkers of oxidative damage: – lipid peroxidation products, oxidized proteins and products of DNA oxidation and fragmentation [40]. One of the main validation criteria of a biomarker is the influence of confounding and modifying factors [7]. As reported previously, the limitations for assessing salivary thiobarbituric acid reacting substances (TBARS) – markers of lipoperoxidation- are the external influences and time-dependent variations [18]. To our knowledge, the influence of external factors on salivary biomarkers of oxidative stress has not been examined besides this study. Thus, the aim of our study was to question the effect of circadian rhythm, tooth-brushing, and treatment with vitamin C on selected salivary biomarkers of oxidative stress and antioxidant status. In the presented work, advanced oxidation protein products (AOPP) – markers of oxidative damage to proteins, thiobarbituric acid reactive substances (TBARS) markers of lipoperoxidation, and advanced glycation end products (AGEs) – markers of carbonyl stress were analyzed. The antioxidant status was assessed using two assays – ferric reducing antioxidant power (FRAP) and total antioxidant capacity (TAC).

2. Subjects and methods

2.1. Subjects and sampling

Whole unstimulated saliva samples were obtained from 19 young healthy probands (9 females and 10 males) aged 20–30 (25.9 $\pm$ 2.5) years during two consecutive days. On the first day, the sampling was performed at 08:00, 14:00 and 21:00 before tooth-brushing. An additional sampling was carried in the morning 10 minutes after tooth-brushing. On the following day, the saliva samples were collected before and 30 minutes after an oral administration of 250 mg of vitamin C. Specifically, powder vitamin C was dissolved in water to final concentration 125 g/L. The probands were asked to drink 2 mL of this vitamin C solution provided in microtube (Eppendorf, Hamburg, Germany). One male proband was excluded from analyses before and after vitamin C, because he did not provide saliva sample after vitamin C consumption. Collected saliva samples were stored at -20°C until analyses were performed. All saliva samples were collected after rinsing the mouth with water and the volunteers were instructed not to eat or drink 30 minutes before any sampling. Smokers and people with systemic disorders were excluded from the study. The study was approved by the Ethics committee of the Institute of Molecular Biomedicine, Comenius University in Bratislava, Slovakia. The volunteers gave informed consent for participation in the study. All reagents or chemicals used in our experiments were purchased from Sigma-Aldrich, Steinheim, Germany.

2.2. AOPP determination

Salivary AOPP levels were determined using a spectrophotometric method by Witko-Sarsat et al. [18]. Briefly, 200 μL of saliva was incubated with 20 μL of glacial acetic acid. The absorbance was read immediately at 340 nm. AOPP concentration was expressed in μmol/L on the basis of the calibration curve of chloramin T with potassium iodide.

2.3. AGEs determination

Salivary AGEs were assessed using spectrofluorometric method (λex. = 370 nm, λem. = 440 nm) according to Münch et al. [34]. Saliva samples were diluted 10-fold with PBS (phosphate buffered saline, pH 7.2) and the specific fluorescence of AGEs was expressed in arbitrary units.

2.4. FRAP determination

Salivary FRAP levels were measured according to Benzie and Strain [20]. Two hundred μL of prewarmed 37°C FRAP reagent (1 volume of 3 mol/L acetate buffer, pH 3.6 + 1 vol of 10 mmol/L 2,4,6-tripyridyl-S-triazine in 40 mmol/L HCl + 1 vol of 20 mmol/L $FeCl_3$) was mixed with 20 μL of saliva. Absorbance was read at 593 nm. Ferrous sulphate was used as standard and the concentration of FRAP was expressed in μmol/L.

2.5. TAC determination

Salivary TAC was determined according to Erel [19]. Saliva was mixed with 0.4 mol/L acetate buffer (pH

5.8), incubated with ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) and oxidized with hydrogen peroxide in 0.03 mol/L acetate buffer (pH 3.6). Absorbance was taken at 660 nm. TAC was expressed in μmol/L on the basis of the calibration curve of trolox.

2.6. TBARS determination

Salivary TBARS levels were determined using the spectrofluorometric method according to Behuliak et al. [4]. Twenty μL of saliva was derivatized with 0.67% thiobartituric acid in acidic medium of acetic acid (95°C, 45 min), extracted with n-butanol, and measured at λex. = 515 nm, λem. = 535 nm. TBARS concentration was expressed in μmol/L on the basis of 1,1,3,3-tetraethoxypropane standard.

2.7. Total proteins determination

Proteins were quantified using BCA protein assay kit (Sigma Aldrich, Steinheim, Germany). Briefly 10 μL of saliva was mixed with 200 μL of BCA working reagent, incubated 30 min at 37°C and measured at 562 nm. Levels of salivary oxidative stress markers were normalized for total proteins.

2.8. Statistical analysis

Repeated measures ANOVA, Tukey's multiple comparison test and paired t-test were used for statistical analysis of obtained data. Analysis was performed with XLStatistics 10.05.30 and GraphPad Prism 5.03. Data were presented as mean $\pm$ SD.

3. Results and discussion

No significant differences in salivary levels of oxidative stress markers were observed between men and women. Based on these results, data from both genders were combined for further analyses.

3.1. Effect of circadian rhythm

The results showed significantly higher levels of salivary AGEs in the morning (by 76%, $p < 0.05$, Tukey's test, Fig. 1A). The levels of salivary AOPP did not differ significantly during the day (Fig. 1B). TBARS concentrations were highest in the morning hours, although the differences between morning and afternoon concentrations were not statistically significant (Fig. 1C). Previously, significantly highest TBARS levels were observed in the morning hours [18]. On the other hand Su et al. observed diurnal variations in salivary protein carbonyl concentrations with highest concentrations at 2:00 p.m. [50]. Significant daily variations were observed in salivary FRAP levels ($F = 3.019$, $p < 0.01$, ANOVA) representing antioxidant status. FRAP concentrations were lowest in the morning and were significantly higher in the afternoon (by 53%, $p < 0.01$, Tukey's test, Fig. 1D). It is interesting that the antioxidant profile expressed using TAC assay did not differ significantly during the day (Fig. 1E). The difference between the FRAP and TAC assays might have occurred due to the fact that the FRAP assay measures primarily the non-protein total antioxidant capacity while TAC assay developed by Erel [11] measures the antioxidative effect of proteins as well, which has an important contribution to antioxidant status of saliva. The increase in FRAP levels could be the consequence of fluctuations in amount of low molecular antioxidants. In contrast to our results, Borisenkov et al. [6] reported circadian variations in salivary TAC concentrations with maximum at 6.00 a.m. and minimum at 3 p.m. and suggested that the influence of melatonin in the morning increases TAC. Melatonin, an important circadian marker which synchronizes the internal hormonal environment according to the light-dark cycle, has been proved to be an effective antioxidant [31,44]. Melatonin is measurable in saliva and significant correlation was reported between melatonin concentrations in blood and saliva [35]. The study of Benot et al. [5] reported the impact of melatonin on total antioxidant status (TAS) in human serum with maximal values of TAS at 1:00 a.m. which was similar to our study and contrasted the study of Borisenkov et al. [6]. The TAS levels were not increased at 7.00 a.m. [5]. The contribution of individual antioxidants to salivary antioxidant status should be assessed in detail in future experiments.

The nocturnal levels of markers were not analyzed in our study. Although the nocturnal levels would be interesting in terms of monitoring circadian rhythm, the perspective of diagnostics are important mainly the diurnal levels. The circadian variations in salivary concentrations of other individual antioxidants were not examined in previous studies. The limitation of our study is that salivary flow rates were not assessed dur-

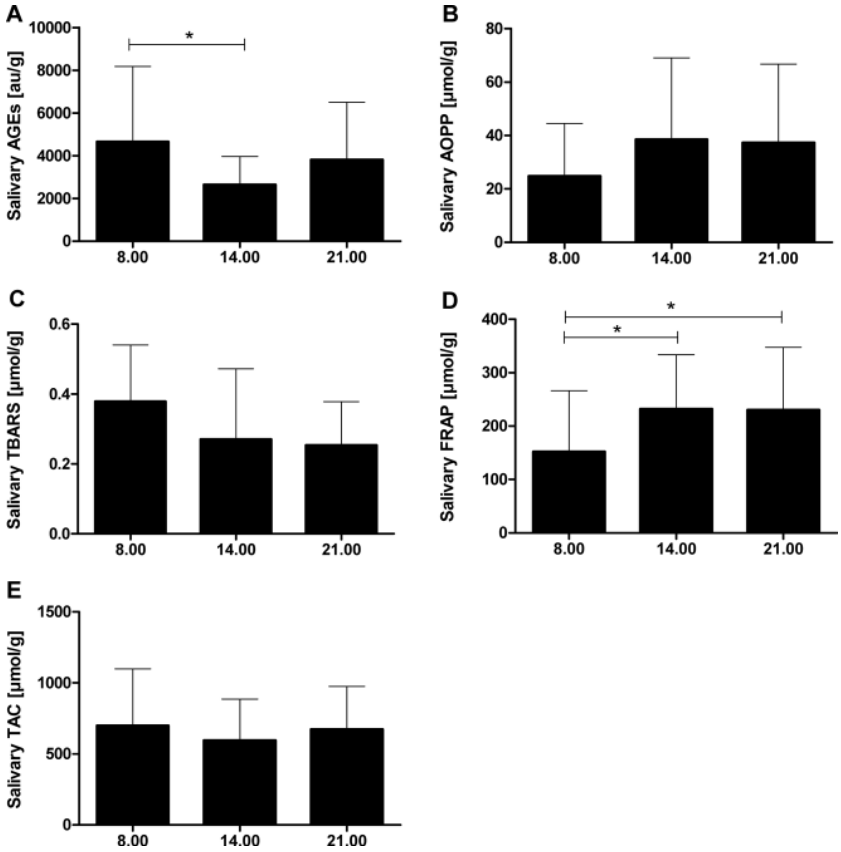

Fig. 1. Effect of daily variations on salivary markers of oxidative stress and antioxidant status. (A) Salivary AGEs levels, biomarker of carbonyl stress. (B) Salivary AOPP levels, biomarker of oxidative damage to proteins. (C) Salivary TBARS levels, biomarker of lipid peroxidation. (D) Salivary FRAP levels, biomarker of antioxidant status. (E) Salivary TAC levels, biomarker of antioxidant status. Data are presented as mean + SD, *denotes $p < 0.05$.

ing saliva collection. Salivary flow rates could vary in relation to circadian cycle, individual hydration, food stimulation, oral hygiene, etc. [8]. Normalization to total proteins is used as alternative to salivary flow to look for differences in the ratio of biochemical analytes present in saliva [12,26]. As in previous studies on salivary oxidative stress markers [9,37], we have decided to normalize levels of salivary oxidative stress markers to total proteins.

The knowledge of circadian variations in salivary biomarkers of oxidative stress is important for designing the collection of samples, especially in studies oriented on oral and other systemic diseases. Assessment of circadian rhythm could be also valuable for optimization of therapeutic interventions according to expected redox homeostasis [50].

3.2. *Effect of tooth-brushing*

Cleaning the teeth decreased the AGEs levels in 12 out of 19 probands on average by 46% ($p < 0.05$, paired t-test, Fig. 2A). Salivary AOPP concentrations were higher in 16 out of 19 probands after tooth-brushing on average by 56% ($p < 0.05$, paired t-test, Fig. 2B). The effect of cleaning the teeth on salivary AOPP levels is rather surprising. On the other hand these data look to be in agreement with recently published review in which AOPP concentrations are suggested as a measure of non-enzymatic antioxidant proteins such as oxidized fibrinogen, at least in plasma [48]. Significant decrease in salivary TBARS after tooth-brushing was reported previously by our group and the local origin of salivary TBARS and oxidative stress were hypothesized [18]. Presented results are in accordance to our previous study [18]. Salivary TBARS concentrations were significantly lower after tooth-brushing in 17 out of 19 probands (by 42%, $p < 0.01$, paired t-test, Fig. 2C). The similar trend in reduction of salivary TBARS and AGEs levels is probably caused by close link between oxidative and carbonyl stress. Reactive carbonyl compounds formed

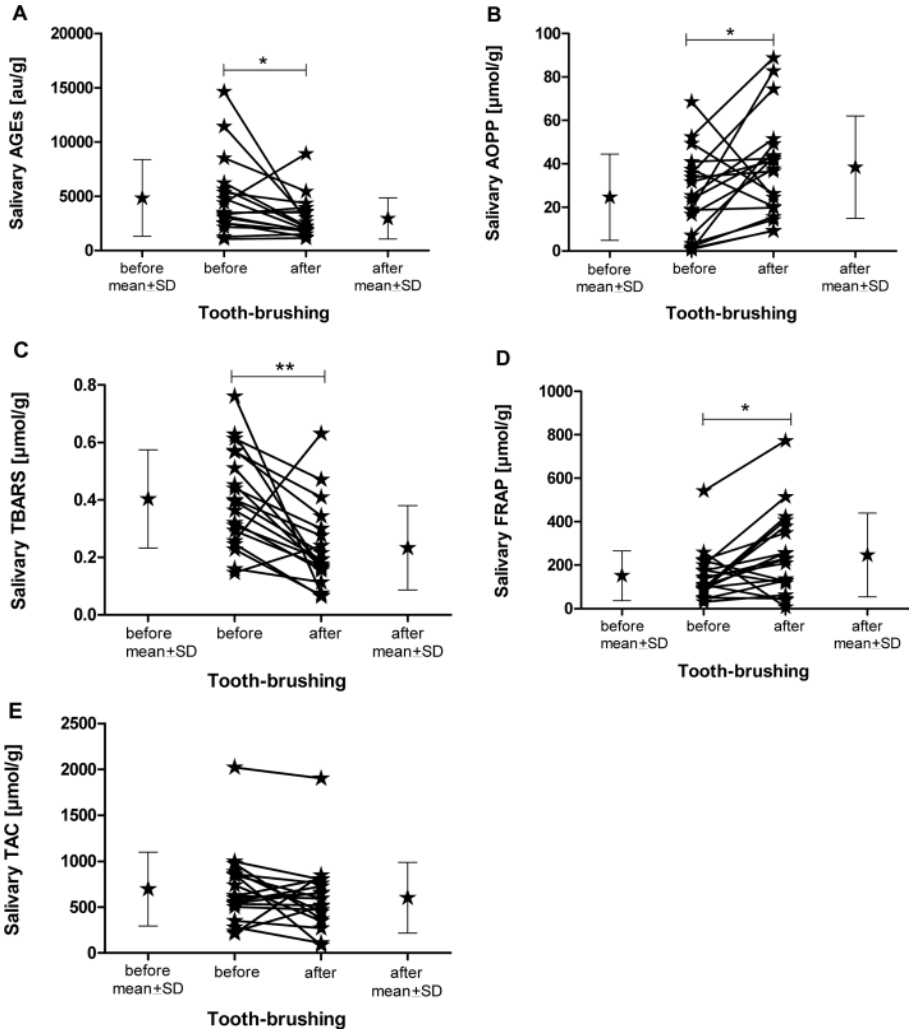

Fig. 2. Effect of tooth-brushing on salivary markers of oxidative stress and antioxidant status. (A) Salivary AGEs levels, biomarker of carbonyl stress. (B) Salivary AOPP levels, biomarker of oxidative damage to proteins. (C) Salivary TBARS levels, biomarker of lipid peroxidation. (D) Salivary FRAP levels, biomarker of antioxidant status. (E) Salivary TAC levels, biomarker of antioxidant status. Data are presented as mean $\pm$ SD, **denotes $p < 0.01$ and $^{*}p < 0.05$.

from carbohydrates react nonenzymaticaly with protein amino groups and yield AGEs during the Maillard reaction. Reactive carbonyl compounds, e.g. glyoxal, malodialdehyde, hydroxynonenal, reacting with amino groups in proteins are also produced during lipid peroxidation [32]. Significant increase was detected in salivary FRAP after tooth-brushing in 14 out of 19 probands on average by 89% ($p < 0.05$, paired t-test, Fig. 2D) but analogously to the influence of daily variations, the salivary TAC levels were not significantly influenced (Fig. 2E). Salivary TAC was decreased in 13 out of 19 probands after tooth-brushing.

Several studies have addressed the role of oxidative stress in the pathology of oral and dental diseases, especially periodontitis [1,2,13,54] and the hypothesis that good oral hygiene may decrease oxidative stress in saliva was proposed [53]. In our study, saliva samples collected before and 10 minutes after tooth-brushing were monitored. Traditionally, tooth-brushing is viewed as procedure for mechanical removing of dental plaque [23]. The removal of bacteria potentially producing free radicals or reduced response of host inflammatory system could lead to decreased oxidative stress in saliva. On the other hand, more recent studies indicate that mechanical stimulation promotes cell proliferation of basal cells and fibroblasts and reduces inflammatory processes, rather than removal of periodontal pathogens [10,20]. The effect

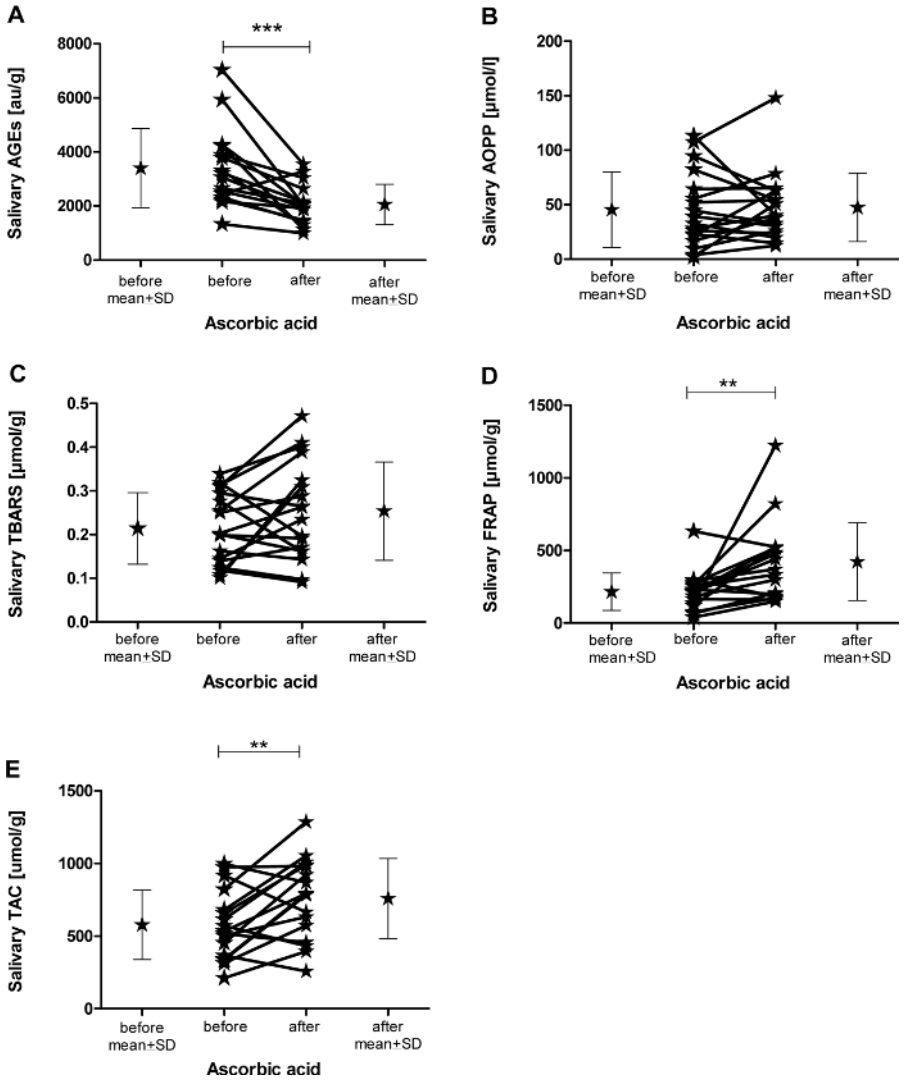

Fig. 3. Effect of ascorbic acid treatment on salivary markers of oxidative stress and antioxidant status. (A) Salivary AGEs levels, biomarker of carbonyl stress. (B) Salivary AOPP levels, biomarker of oxidative damage to proteins. (C) Salivary TBARS levels, biomarker of lipid peroxidation. (D) Salivary FRAP levels, biomarker of antioxidant status. (E) Salivary TAC levels, biomarker of antioxidant status. Data are presented as mean $\pm$ SD, ***denotes $p < 0.001$ and $^{**}p < 0.01$.

of bacterial flora on salivary oxidative stress should be examined in further studies. Mechanical tooth-brushing led to decreased 8-hydroxydeoxyguanosine levels (marker of oxidative DNA damage) in periodontitis rat model. The study demonstrated decreased oxidative damage in gingiva and potential contribution to reduced circulating oxidative stress [10]. Higher antioxidant values may also be caused by expulsion of gingival crevicular fluid following tooth-brushing and artificially increased antioxidant concentration in saliva [21]. Hoek et al. has reported increased albumin levels even more than 45 minutes after tooth-brushing [19]. Albumin is an important salivary antioxidant [33] and may therefore represent another factor contributing to increased total antioxidant capacity after tooth-brushing.

Based on our results it can be concluded that brushing of teeth can significantly influence oxidative stress markers and result in bias in diagnostic tests. To avoid this problem another study should explore the concentrations of salivary oxidative stress markers at different time points after tooth-brushing. Based on these results the optimal time after tooth-brushing should be set for collection of saliva. On the other hand, better parodontal status in periodontitis patients with better oral hygiene [53] could explain decreased oxidative stress as

the result of better oral hygiene. One of the drawbacks of our study is that oral hygiene and health status of the subjects were not determined.

3.3. Effect of vitamin C

Single dose of vitamin C (250 mg) induced significant decrease in AGEs levels in 17 out of 18 probands on average by 64% ($p < 0.001$, paired t-test, Fig. 3A). No significant changes were detected in AOPP levels (Fig. 3B). Salivary TBARS levels were not significantly influenced after vitamin C treatment. It is interesting that tendency to higher levels of TBARS concentrations after vitamin C was detected in 10 out of 18 probands. In our previous study, significantly decreased TBARS concentrations were observed after ascorbic acid treatment [18]. An increase in salivary FRAP was observed in 14 out of 18 probands on average by 107% ($p < 0.01$, paired t-test, Fig. 3D) and TAC in 12 out of 18 probands on average by 1% ($p < 0.01$, paired t-test, Fig. 3E). Vitamin C is free radical scavenging nonenzymatic antioxidant. Vitamin C also acts as cofactor for enzymes involved in collagen hydroxylation, biosynthesis of carnitine and norepinephrine, tyrosine metabolism, and amidation of peptide hormones [28]. Vitamin C works directly as free radical scavenger in watery environment of the cells and by interacting with vitamin E in the lipid rich areas of the cells [45]. It was shown that plasma vitamin C is lowered in chronic or acute oxidant states [24,30,36]. Only few studies have monitored concentrations and vitamin C supplementation in saliva [17,39,42,43,45,47]. Requirement for vitamin C is in humans satisfied by the ordinary diet [29]. Our results have shown that vitamin C supplementation led to significantly decreased carbonyl stress and increased antioxidant status. The conditions of saliva samples collection for oxidative stress monitoring should be standardized in terms of diet and vitamin supplementation. On the other hand, vitamin C supplementation could be potentially used for antioxidative therapy of oral diseases in which increased oxidative stress was reported. Orally administered vitamin C led to decreased 8-hydroxydeoxyguanosine, improved glutathione ratio, and decreased expression of IL1α and IL1β in experimental rat periodontitis model [52]. In depletion-supplementation study in healthy men significantly improved gingival index after supplementation with 600 mg/day of vitamin C and this effect was reversed during the following period of depletion to 5 mg/day. In addition, bleeding scores decreased significantly after the supplementation period and increased during the period of depletion [27].

One of the limitations of our study is that the concentrations of vitamin C before and after vitamin C supplementation were not monitored in saliva. Plasma concentration of vitamin C are highly controlled when the vitamin is taken orally [38]. But no pharmacokinetic studies on vitamin C were conducted in saliva. In the future it would be beneficial to perform dose concentration, dynamics and long-term studies of salivary vitamin C in relation to oxidative stress biomarkers.

3.4. Conclusion

Salivary carbonyl stress and antioxidant status are influenced by daily variations. Tooth-brushing and treatment with vitamin C decrease carbonyl stress and increase antioxidant status. The results are important for further research on the role of oxidative and carbonyl stress in the pathogenesis of oral diseases and for the potential use of salivary markers of oxidative and carbonyl stress in the diagnostics of oral diseases, at least on a population level.

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