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Assessment of Salivary and Serum Antioxidant Vitamins and Lipid Peroxidation in Patients with Recurrent Aphthous Ulceration

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Saral, Y., Coskun, B.K., Ozturk, P., Karatas, F. and Ayar, A. *Assessment of Salivary and Serum Antioxidant Vitamins and Lipid Peroxidation in Patients with Recurrent Aphthous Ulceration*. Tohoku J. Exp. Med., 2005, **206** (4), 305-312 —— Recurrent aphthous ulceration (RAU) is a common oral mucosal disorder characterized by recurrent, painful oral aphthae. Although the exact cause of RAU is not known, local trauma, microorganisms, nutritional deficiencies, hormonal changes, genetics, and immunological factors have been suggested to contribute to its pathogenesis. The aim of this study was to assess the level of lipid peroxidation and status of antioxidant vitamins in patients with RAU. Thirty patients with RAU and 20 healthy controls were recruited. Vitamins A, E, and C and malondialdehyde (MDA) levels were measured in both serum and saliva of patients with RAU and control subjects by high performance liquid chromatography. Levels of vitamins A, E and C in both fluids were significantly lower ($p < 0.05$ for vitamins A and E, and $p < 0.005$ for vitamin C, respectively) in patients with RAU than in healthy control subjects. Conversely, the levels of MDA in serum and saliva were significantly higher ($p < 0.005$) in patients with RAU than in the control group. Furthermore, strong and highly significant correlation was found between serum and salivary levels of vitamins A, E and C, and MDA in patients with RAU ($r \geqq 0.90$, $p < 0.0001$). The present study demonstrates that the serum and saliva levels of selected antioxidant vitamins are lower, while the degree of lipid peroxidation, as judged by the MDA levels, is higher in patients with RAU than in the control subjects. This is the first to measure specific antioxidant levels in both saliva and blood in the same patients, and indicates that the non-enzymatic anti-oxidant ability is impaired in patients with RAU. ———— recurrent aphthous ulceration; antioxidant vitamins; MDA; nonenzymatic defense system; saliva

Recurrent aphthous ulceration (RAU), or Canker sores, is a chronic inflammatory disease of oral mucosa characterized by single or multiple painful ulcers which persist and recur for variable periods of time (Ruah et al. 1988). It is one of the most common oral mucosal disorders, affecting

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up to 20% of the world population. It affects men and women of all ages, races and geographic regions. Patients with RAU experience oral pain causing discomfort, ranging from simple annoyance to an intensely painful period that interferes with normal oral activities, at some times in their lives (Nolan et al. 1991; Ship et al. 2000; Cimen et al. 2003; Zunt 2003).

Although the exact pathophysiology of this clinical entity remains unclear, several factors have been identified to trigger the onset of this disorder. These include genetic backgrounds, immunological and nutritional deficiencies, and infectious agents (both bacterial and viral). Local mechanical trauma, smoking, emotional stress, medications, vitamin and trace element deficiencies, allergies and sensitivities and hormonal changes have also been identified as potential etiological factors, alone or in combination (Ship et al. 2000; Stoopler and Sollectio 2003; Zunt 2003).

The clinical characteristics of RAU are well defined and successful diagnosis can be made through a careful history and clinical examination. It is characterized by small (usually 1-2 mm wide) painful ulcers which typically have red borders and yellow-gray centers. They occur in inner side of the lips, the tongue, the wall of the cheeks and the back and floor of the mouth. Minor, major, and herpetiform are the 3 classic forms of RAU. Minor aphthous ulcers appear as recurrent, round, closely defined, small painful ulcers with shallow necrotic centers, raised margins and erythematous halos in the labial, buccal and floor of the mouth mucosa. Major aphthous ulcers are similar to the minor but are larger, deeper, often scars and can last for weeks to months. Herpetiform ulcers are the least common and appear as small and numerous ulcers (Ship et al. 2000). Due to absence of specific markers of pathogenesis, diagnostic tests usually are not ordered. If suspected, nutritional deficiency, B12, folate/iron levels, and complete blood count may be considered.

In normal conditions, mucous membranes are protected from damage caused by harmful molecules, as well as from free radicals by protective surface phenomena, general host defenses and specific immune responses. The human body has its own natural free radical scavengers, which include the antioxidant vitamins, vitamin A and beta-carotene, B-complex vitamins, vitamin C and vitamin E, the mineral selenium and superoxide dismutase, glutathione peroxidase, reductase and catalase (Arthur 1988; Halliwell 1994). Non-enzymatic antioxidant system, which consists of vitamins A, E, and C and Se, has been shown to react with organic free radicals and protect biomembranes from free radical-induced damage (Cohen 1994; Halliwell 1994).

Malondialdehyde (MDA) is a stable end-product of peroxidation of membrane lipids by reactive oxygen species, and is widely used as an indicator of increased lipid peroxidation (Nielsen et al. 1997). It is well established that interactions between MDA and membrane components result in disturbed structure and function of cell membranes.

There have been studies linking increased oxidative stress and impaired antioxidant capacity in patients with RAU. Cimen et al. (2003) reported increased oxidative stress and decreased antioxidant defenses in mucosa of patients with RAU. Since free reactive oxygen radicals cause destruction of biomembranes by oxidizing unsaturated fatty acids, and oxidative stress suppresses the immune system's ability to protect and restore affected cells, anti-oxidative status of patients suffering from RAU is among to the potential determinants of susceptibility to this disorder. Furthermore, an increased level of lipid peroxidation and a decreased level of tocopherols in experimental gastric mucosal injury (Kwiecien et al. 2002; Nafeeza et al. 2002) and also in plasma of patients with gastric ulcer has been reported (Tandon et al. 2004).

Saliva is increasingly used and well validated in diagnosing, monitoring systemic health and disease states, and predicting disease progression and detecting biomarkers in a wide range of fields including clinical biochemistry, immunology, clinical pathology, forensic medicine and sports medicine (Tabak 2001). A growing number of hormones and drugs can be reliably monitored in saliva which can be easily collected even by the subjects at repeated intervals; it requires no spe-

cial collection or storage equipment (Lawrence 2002; Tabak 2001). However, the use of saliva in determination of possible indicators of RAU is not a common practice. Since human saliva contains a large number of enzymes derived from the salivary glands, epithelial cells, and other sources, and ulcerations of oral cavity is the major sign of RAU, we wanted to determine the level of nonenzymatic antioxidants and MDA in saliva in comparison with serum in the same patients.

Hence, the aim of the present study was to assess the level of lipid peroxidiation and levels of antioxidant vitamins in both saliva and blood of patients with RAU.

Subjects and Methods

All the patients admitted to the Department of Dermatology outpatients clinic of the Firat University Faculty of Medicine with diagnose of recurrent aphthous ulceration from June 2003 to June 2004, were included in this prospective, controlled study. The protocol for this study was approved by the local Ethics Committee of the Firat University Faculty of Medicine.

A total of 30 consecutive patients with RAU (16 female, 14 male) and 20 healthy controls were recruited after giving informed consent. Following a detailed medical history, physical examination and laboratory assessments at the beginning of the study, only patients with a typical history of at least three recurrences per year and clinical findings of RAU were considered. History of smoking habits and alcohol consumption, respiratory or cardiovascular disorders, any chronic illnesses and Behçet's disease were among to the exclusion criteria. Patients with symptoms and signs of any active inflammation were also excluded from the study. Complete comprehensive history of present illness, severity, frequency, duration of lesions and responses to past treatments were recorded.

The mucous membranes of the oral cavity were examined to determine localization, size, and number of the ulcers. Dermatological examination also involved screening for any other skin problems.

For laboratory analysis, blood samples and saliva of the patients and controls were taken after overnight fasting (at least 8-hour) between 8 and 9 in the morning. For isolation of serum, a 4 ml blood sample was centrifuged at 3,000 rpm at 4°C for 5 min and aspirated into tubes and stored at –20°C until analyzed for MDA and vitamins A, E and C.

The saliva samples were always collected in restful and quiet circumstances. Following flushing of mouth with a 100 ml of distilled water, 2 - 3 drops of 2% acetic acid was dropped into mouth, and whole saliva was collected for 5 min by the subject leaning forward and spitting saliva into test tubes that were kept in crushed ice, and immediately after collection the samples were stored at –20°C until analyzed.

Determination of Vitamin A and E levels in serum and saliva

Status of vitamin A was determined by measuring serum retinol and beta-carotene concentrations while vitamin E status was determined by measuring serum alpha-tocopherol levels. The quantification was made according to Miller et al. (1984) utilizing absorption spectra of 326 and 296 nm for vitamin A and E, respectively.

HPLC separations were accomplished at room temperature with a Cecil liquid chromatography system (Series 1100, Cambridge, England) consisting a sample injection valve (Cotati 7125, Rheodyne, Cotati, CA, USA) with a 20 μl sample loop, an ultra-violet (UV) spectrophotometric detector (Cecil 68174, Cambridge, England), integrator (HP 3395, Avondale, PA, USA) and a Techsphere ODS-2 packed (5 μm particle and 80Å pore size) column (250 × 4.6 ID) with a methanol: acetonitril: chloroform (47: 42: 11, v/v) as mobile phase at 1 ml/min flow rate. All procedures were performed under light-protected conditions.

Determination of Vitamin C and MDA levels in serum and saliva

Status of vitamin C was derived from serum ascorbic acid measures. The extraction of vitamin C and MDA were performed according the method described by Miller et al. (1984). The supernatant was filtered and the vitamin C level was determined using the method of Tavazzi et al. (1992) and MDA levels by Karatas et al. (2002). The Supelcosil LC-18-DB HPLC reversed-phase column (3 μm particle size and 250 × 3.9 ID, Hertfordshire, UK) was utilized for the detection of vitamin C and MDA levels. The mobile phase used for vitamin C detection was 3.7 mM phosphate buffer (pH 4.0) with a flow rate of 1 ml/min, while the MDA level was determined with mobile phase consisted of 30 mM KH_2PO_4 buffer (pH = 4 with H_3PO_4) and methanol (65 - 35%, v/v) at 1.5 ml/min flow rate. All chemicals and reagents used were of analytical grade and were purchased

from Merck Chemical Co. (Darmstadt, Germany). Bidestilled water was used for the all procedures.

Laboratory assessments also involved vitamin B12 levels. Serum vitamin B12 concentrations were determined using commercial kits (Vitamin B12 Elecsys reagent kit, Roche Diagnostics, Lewes, East Sussex, UK) by the electrochemiluminescence method.

Statistical analysis

The data were statistically analyzed using SPSS statistical package (SPSS, version 9.0, Chicago, IL, USA). Data are expressed as mean ± S.D. Differences between the groups were analyzed for significance using one-way ANOVA and Tukey's HSD tests. Correlation assessments were performed by using the Spearman correlation analysis. Statistical significance was defined as $p < 0.05$.

Results

The present study included a total of 30 patients (ages ranged from 17 to 59 years, mean 35.07 ± 11.4 years), including 16 (51%) female and 14 (49%) male patients. Duration of illness ranged from 3 to 180 months (mean: 50.29 ± 48.75 months). Control group consisted of 20 healthy individuals (ages ranged from 23 to 39 years, mean 32.27 ± 7.25 years), including 11 (54.5%) female and 9 (45.5%) male subjects. There was no significant difference between the control and patients group with respect to the mean age.

The values of the serum antioxidant vitamins and MDA are given in Table 1. Serum vitamin A and E levels were significantly lower in patients with RAU as compared to the controls ($p < 0.005$). Likewise, serum vitamin C and B12 levels were significantly lower in patients with RAU ($p < 0.05$ and $p < 0.003$, respectively). Conversely, serum MDA levels were higher in patients with RAU in comparison with control subjects ($p < 0.005$).

We then compared the saliva levels of vitamins A, E and C and MDA (Table 2). The patients with RAU had significantly lower vitamins A and E compared to the control subjects ($p < 0.005$). Saliva vitamin C level was also significantly lower in patients with RAU ($p < 0.05$). In contrast, salivary levels of MDA were significantly higher in the patients than in the control group ($p < 0.005$).

Serum-saliva correlation analysis revealed

Table 1. *Serum levels of vitamins A, E, and C and MDA in the control and patients with aphthous ulceration*

	Control ($n = 20$)	Patients ($n = 30$)
Vitamin A (μg/ml)	0.72 ± 0.14	0.60 ± 0.13 **
Vitamin E (μg/ml)	8.27 ± 1.60	6.92 ± 1.30 **
Vitamin C (μg/ml)	8.95 ± 1.66	7.68 ± 1.43 *
MDA (nmol/ml)	1.68 ± 0.44	3.31 ± 0.70 **
Vitamin B12 (pg/ml)	339.8± 31.4	229.2 ± 22.2 ***

* $p < 0.05$, ** $p < 0.005$, *** $p < 0.003$.

Table 2. *Saliva levels of vitamins A, E, and C and MDA in the control and patients with aphthous ulceration*

	Control ($n = 20$)	Patients ($n = 30$)
Vitamin A (ng/ml)	28.5 ± 0.21	16.80 ± 5.08 **
Vitamin E (μg/ml)	0.40 ± 0.11	0.26 ± 0.10 **
Vitamin C (μg/ml)	1.04 ± 0.11	0.83 ± 0.26 *
MDA (nmol/ml)	0.28 ± 0.12	0.48 ± 0.16 **

* $p < 0.05$, ** $p < 0.005$.

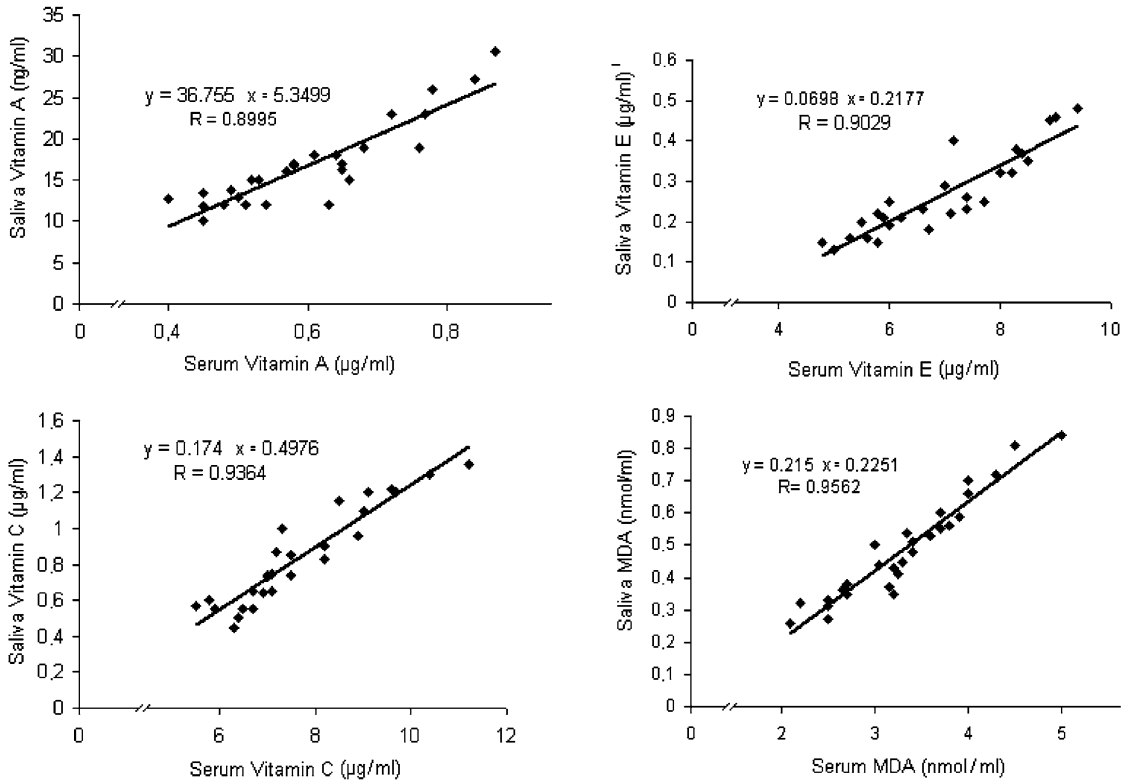

Fig. 1. Scatterplot of saliva-serum levels of vitamins A, E and C, and MDA in 30 patients with recurrent aphthous ulceration (RAU). There was a significant correlation between saliva-serum levels of vitamin A (upper left, $r = 0.91$, $p < 0.001$), E (upper right, $r = 0.90$, $p < 0.001$), C (lower left, $r = 0.94$, $p < 0.001$), and MDA (lower right, $r = 0.96$, $p < 0.001$) among all RAU patients.

strong and highly significant correlation for vitamins A, E, and C and MDA in patients with RAU, respectively ($r \geqq 0.899$, $p < 0.0001$, Fig. 1). Highly significant serum-saliva correlations were also evident for vitamins A, E, and C and MDA in control subjects ($r \geqq 0.864$, $p < 0.001$, Fig. 2).

Discussion

In the present study, we observed that serum and saliva levels of some non-enzymatic indicators of antioxidant protective system (vitamins A, C and E) and levels of MDA (an indicator of lipid peroxidation) was higher in patients with RAU than in a matched group of healthy control subjects. The differences in the level of lipid peroxidation and antioxidant vitamins in serum were consistently reflected in saliva, suggesting that the saliva can be used as biological sample in monitoring the lipid peroxidation and levels of antioxidant vitamins in patients with RAU in active stage of the disease.

Several parameters, including serum levels of IL6 and IL-8 (Sun et al. 2004), and tumor necrosis factor (Taylor et al. 1992) have been considered as sensitive markers in monitoring the disease activity of RAU with limited specificity. But, determination of specific laboratory indices for diagnoses and clarification of ethiopathogenesis of the RAU are yet to be discovered.

In addition to being important in speaking and swallowing, saliva has a part of the defense system of human body, by containing immunoglobulin and non-immunological (lysozyme, lactoferrin, histatins and salivary peroxidase systems) protecting factors (Nagler et al. 2002). Since RAU involves damage of the oral mucosa, level of lipid peroxidation and antioxidant vitamins in saliva as well as in serum were evaluated in this study. Salivary concentrations of vitamins C, and E and MDA were several-fold lower than

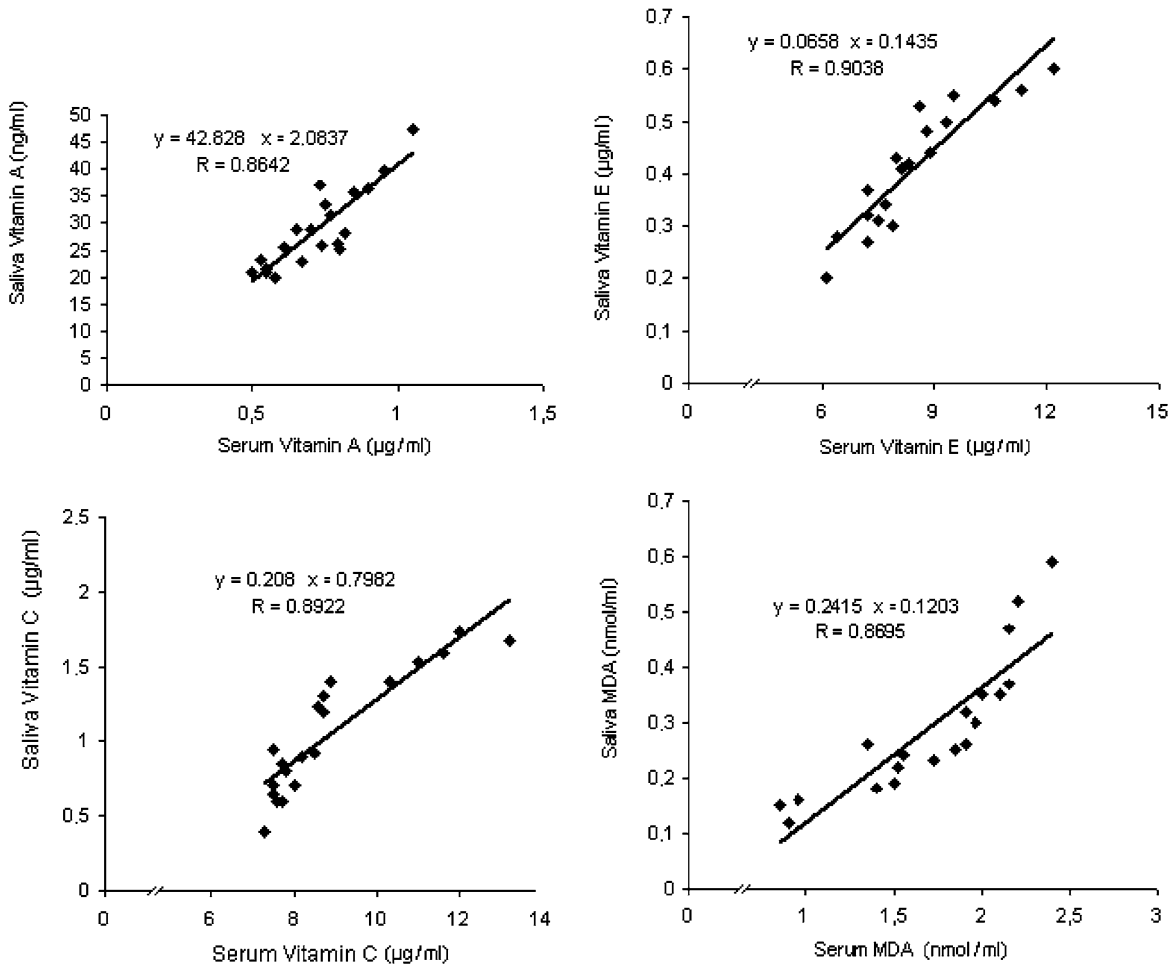

Fig. 2. Saliva-serum correlation of vitamins A, E and C, and MDA in 20 control subjects. There was a significant correlation between saliva-serum levels of vitamin A (upper left, $r = 0.86$, $p < 0.01$), E (upper right, $r = 0.89$, $p < 0.01$), C (lower left, $r = 0.90$, $p < 0.01$), and MDA (lower right, $r = 0.87$, $p < 0.01$) among all control subjects.

the respective values in serum but a positive correlation was found between their salivary and serum levels (Tables 1 and 2, Fig. 1). In contrast, salivary vitamin A concentrations were significantly higher than the respective values in serum in both control and patients with RAU. This could be due to the method employed which directly measured vitamin A in saliva, whereas serum measurements excluded the fraction of the vitamin tightly bound to serum proteins.

Previously published studies showed a decline in circulating enzymatic antioxidant capacity and increase in free radical production in patients with RAU (Cimen et al. 2003). However, they have not examined the non-enzymatic antioxidant capacity, especially the possible correlation between plasma and saliva concentrations of these parameters (Ogura et al. 2001; Cimen et al. 2003). In agreement with those, our study showed a decline in serum antioxidant capacity. But, measuring saliva levels of vitamins A, C and E and MDA in the same patients represents novel sites of this study, indicating correlation between serum and saliva levels of these measured parameters.

There are evidence that there may be a relation between the inflammatory process and free radical metabolism (Winrow et al. 1993; Köse et

al. 1995). Lipid peroxidation has a free radical chain reaction which causes degeneration of cell membranes. Lipid peroxides are disintegrated quickly and form reactive carbon compounds. Among these, MDA is an important reactive carbon compound which is used commonly as an indicator of lipid peroxidation (Köse et al. 1995; Jacob and Burri 1996).

Cimen et al. (2003) reported that plasma MDA levels in patients with RAU was higher than that in the control group ($p < 0.01$). Consistently, in our study serum and saliva MDA levels in patients with RAU were significantly higher than in the controls ($p < 0.005$). Cimen et al. (2003) have reported a decrease in activity of enzymatic antioxidant capacity in patients with RAU as indicated by GSH-Px, SOD and CAT but to our knowledge this is the first study evaluating vitamin A, C and E levels together with lipid peroxidation in both serum and saliva in the same patients with RAU.

Serum and saliva vitamin A levels in patients with RAU were significantly lower when compared to the controls ($p < 0.005$). Vitamin A functions as catalyzer of removal of singlet oxygen and as a result vitamin A inhibits singlet oxygen-dependent reactions (Jound 1986).

Serum and saliva vitamin E levels in patients with RAU were also significantly lower when compared to the controls ($p < 0.005$). Serum vitamin C levels in our patients with RAU were significantly lower when compared to the controls ($p < 0.05$) (Table 2). Vitamin C also has a role in activating vitamin E when it loses its antioxidant capacity by turning into tocopherol (Levine 1997). As a free radical scavenger, ascorbate works directly in the watery environment of the cells and in the lipid rich areas of the cell, interacting with vitamin E in the later medium. The same property of vitamin C also prevents the formation of nitrosamines from nitrites and nitrates (Lu et al. 1986). Vitamin C inhibits division and growth of cell through the production of hydrogen peroxide, which damages the cells probably through an unidentified free radical(s) generation (Maramag et al. 1997). It has been shown that Fe, Ca, and vitamins B12 and C were lower (Ogura et al. 2001; Piskin et al. 2002) and supplementation with vitamin B12 led to marked improvements in patients suffering from RAU initially deficient in vitamin B12.

Salivary concentrations of antioxidant vitamins and MDA also represented their respective concentrations in serum with good correlations (Figs. 1 and 2). The findings reinforce the concept that saliva contains the same biomarkers for disease that are found in the blood. Although the levels of most biomarkers in saliva are considerably lower than the levels in the general circulation, in the present study vitamin A results indicated the reverse. These results also highlight the potential clinical value of saliva as a valid and convenient diagnostic biofluid in patients with RAU. This novel approaches to harness the potential of saliva for diagnostics in RAU may provide additional advantages in illumination of pathogenesis of this oral disorder.

In conclusion, results from this study indicate that there is an increased oxidative stress and antioxidant vitamins (A, E and C) levels are significantly decreased in patients with aphthous ulceration. For a conclusive statement, further studies on large series should be performed to clarify the importance and role of antioxidant vitamins in recurring oral aphthae.

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