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Original paper

Studies regarding salivary total antioxidant activity in different types of orthodontic treatement

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Abstract

The defensive mechanism of the human body includes a wide range of antioxidants (non-enzymatic and enzymatic) that can eliminate reactive oxygen species and prevent their harmful consequences on the host. Total antioxidant capacity (TAC) is a biomarker used to measure the antioxidant potential of body fluids and it may be the most relevant parameter for assessing the defense capabilities. Because of the cumulative effect of antioxidants, it is better to measure the combined activity of all the antioxidants (total antioxidant capacity) instead of measuring the activity of each agent separated. The aim of this study is to investigate the effect of different orthodontic appliances (fixed and removable-aligners) on salivary total antioxidant capacity and if is a correlation between TAC and oral injuries made by them. Three groups of subjects have been tested: one group of ten patients with metallic fixed appliances, one group of ten patients with aligners, and a control group consisting in ten subjects. 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used for TAC assessment and salivary samples were obtained from all individuals before treatment, at two weeks from the beginning, at one and two month of treatment. Also, it was quantified the presence of the injuries in the mucosa (clinical parameter). The result shows that different types of orthodontic appliances increase differently the TAC and seems to be influenced by the injuries.

Keywords Total antioxidant capacity (TAC), DPPH method, orthodontic aligners, fixed appliances.

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Introduction

1. Orthodontic therapy

Orthodontics is a branch of dental medicine. Orthodontic treatment helps in improving facial and dental aesthetics with an increase of self-esteem of the patient. Besides this positive aspects it has some risks: tissue damage (intra-oral or extra-oral), treatment failure or greater predisposition to dental disorders. Patients may get oral ulcers secondary to rubbing of the lips and cheeks on brackets, bands or cleats, long unsupported stretches of wire resting against the lips and also excessive muscular activities of the cheek or tongue that can act as triggers. Occasionally, palatal or lingual arches may cause trauma to the palate or tongue. In a recent study, most of the ulcerations were seen in the first appointment following bonding (MAINALI [1]). Injury to the oral mucosa during orthodontic treatment is a common occurrence, and the most frequent patient complaints relate to brackets irritating the labial/buccal mucosa and distal wires extending into the retromolar area. The damaged epithelium is replaced by a yellow fibrinopurulent membrane surrounded by erythema (and will provoke a painful sensation). The incidence of oral traumatic ulcers ranges between 60% and 81%(RENNICKA & al. [2]).

2. Saliva

Saliva is produced by three pairs of major glands (90% of total saliva secretions is produced by the parotid, submandibular, and sublingual glands) and numerous minor salivary glands located in the oral cavity. In resting position (unstimulated state), two-thirds of the total volume of the whole saliva is produced by submandibular glands. If is stimulated, the parotid glands are responsible for at least 50% of the total volume of saliva from the mouth. A small percentage of the unstimulated or stimulated state is assured by sublingual glands. Because of their high protein content, minor salivary glands contribute significantly to the lubrication of the oral mucosa (IORGULESCU [3]).

It is a body fluid which plays a significant role in the protection of the intraoral structures against injuries caused by various pathogenic microbes, mechanical or chemical irritants. It has some important functions: lubrication of oral tissues, digestion, defensive/buffering capacity, protection of teeth (protects the enamel against demineralization caused by the acids, facilitates the remineralization of incipient caries), restoration of injured soft tissues, washing off food debris and antimicrobial capacity (IORGULESCU [3], HOSSEINI-YEKANI & al. [4]). Saliva can be assessed by some characteristics, such as flow rate, buffering capacity, hydrogen-ion concentration (pH), and its consistency. Saliva can buffer acids, and this ability is essential for maintaining pH values in the oral cavity (HOSSEINI-YEKANI & al. [4]). Its availability and non-invasive collection allow the diagnosis of numerous pathological conditions or diseases in the oral cavity and some systemic disorders (GAWRON-SKARBEK & al. [5]).

Saliva includes a large number of organic and inorganic compounds which influence health status. It possesses a wide range of antioxidants including uric acid, vitamin C, reduced glutathione, oxidized glutathione, and others (PENDYALA & al. [6]). Uric acid (UA) is the most abundant antioxidant, non-enzymatic molecule of plasma origin in saliva, and its concentration in saliva is similar to that in serum (GAWRON-SKARBEK & al. [5]).

3. Total antioxidant activity (TAC)

The defensive mechanism of the human body includes a wide range of antioxidants (non-enzymatic and enzymatic) that can eliminate reactive oxygen species and prevent their harmful consequences on the host. The most important intracellular enzymes which protect cells and tissues from oxygen-derived free radical are:

1. superoxide dismutase (SOD) – that can rapidly transform the superoxide anion to hydrogen peroxide;
2. glutathione peroxidase (GPx) – reduces hydrogen peroxide and/or lipid hydrogen peroxides;
3. S-nitrosoglutathione, and catalase (CAT), which transform hydrogen peroxide by converting it oxygen and water (PUNJ & al. [7]).

Total antioxidant capacity (TAC) has been defined as the moles of oxidants that are neutralized by one liter of solution. It is a biomarker used to measure the antioxidant potential of body fluids and it may be the most relevant parameter for assessing the defense capabilities. A decreased value may raise the susceptibility for oxidative stress (PENDYALA & al. [6], PUNJ & al. [7], ZAMANI-AHARI & al. [8]). Because of the cumulative effect of antioxidants, it is better to measure the combined activity of all the antioxidants or the total antioxidant capacity (TAC) instead of measuring the activity of each agent separated (ZAMANI-AHARI & al. [8]).

The antioxidant capability of the saliva serves as the first line of defense against oxidative stress and is essential for maintaining the oral cavity balance and to avoid the development of local diseases (GAWRON-SKARBEK & al. [5], PORTELLI & al. [9]).

Antioxidants have the capacity to neutralize free radicals and reactive oxygen species, and interfere with oxidation of lipids, nucleic acids and proteins. They inhibit the formation of free radicals, prevent or inhibit the activity of free radicals after they are formed, repair damages inflicted by the activity of free radicals and increase the excretion or absorption of damaged molecules (ZAMANI-AHARI & al. [8]). Antioxidant molecules are present in all body fluids and tissues (PUNJ & al. [7])

The DPPH (an antioxidant assay based on electron-transfer that produces a violet solution in ethanol) method is a promising approach that is being increasingly used in clinical studies for assessing TAC (Total Antioxidant Capacity). This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colorless ethanol solution (GAWRON-SKARBEK & al. [5], GARCIA & al. [10]).

To our knowledge, a comparison between metallic braces and aligners regarding total antioxidant activity and clinical parameters has not been previously described in literature. The aim of this study, therefore, is to investigate the effects of conventional braces and aligners on TAC (as a defense against oxidative stress) and to see if there is a correlation between TAC and oral lesions.

Materials and Methods

Subjects

The study group consisted in 30 subjects divided in 3 subgroups: the control group (A) formed by 6 females and 4 males, aged 18 to 30 (mean 25.6 years), the group with metallic braces (B) consisted in 5 females and 5 males aged 16 to 30 (mean 26.8 years) and the group wearing aligners (C) was formed by 8 females and 2 males aged 18 to 30 (mean 25.4 years).

Inclusion criteria for this study were patients of both genders who were in the age group of 16-30 years (after the growth spurt), with no systemic disease, no antibiotic treatment for the previous six months, no anti-inflammatories one month previous to orthodontic treatment, no oral contraceptives, non-pregnant, with no increased carious activity or active cavities at the start of treatment, with permanent dentition, non-smokers, with no personal history of drug use, candidate for fixed orthodontic treatment with: Skeletal Class I, with no need for extractions, excessive expansion, anchorages auxiliary elements (with mild/moderate crowding exhibiting no greater than 6 mm of space discrepancy per arch), with no history of trauma, bruxism or parafunctions, with no signs or symptoms of clinical or radiological periodontal disease, without large dental restorations (fixed or mobile), without palatinal/lip cleft or anodontia, without any injuries on buccal mucosa at the beginning of the treatment and capable of good collaboration and good oral hygiene.

Approval from the Ethics Committee of "Ovidius" University Constanta was granted in accordance with the principles of Declaration of Helsinki and informed consent was obtained prior to the sample collection.

Types of orthodontic appliances

The conventional metallic braces used in this study were made from high-strength stainless steel (Micro Sprint, Forestadent, Phorzheim, Germany) and the archwires were size 0.12 Ni-Ti (LTS form, American Orthodontics, USA). The aligners were made from Polyethylenterephthalat-Glycol Copolyester (PET-G) (Clear Aligner, Scheu-Dental GmbH, Germany) which fulfills the necessary biocompatibility conditions for medical products. The composite and primer used were purchased from Ormco, USA (Enlight Light Cure Adhesive™ and Ortho Solo™).

After the appliance was fixed in the subject's mouth, anti-inflammatory treatment was strongly discouraged (they were instructed to only use acetaminophen 500 mg in case of marked discomfort).

Saliva collection and storage

On the day the samples were taken, in order to avoid an interaction between any substance contained in the toothpaste and the molecules to be examined, the patients were asked to brush their teeth without toothpaste and to refrain from eating and drinking for at least 30 minutes prior to the visit. Unstimulated whole saliva samples were collected before application (baseline) (T0), at 2 weeks (T1), 1 month (30 days- T2) and 2 months (60 days-T3). For saliva collection, after rinsing their mouth with distilled water, the patients were seated in a relaxed position with the head bent forward to allow saliva to accumulate in the anterior part of the oral cavity. The patients swallowed and saliva was then collected for 5 min into a sterile graduated container. They were asked not to move their tongue or lips during the period of collection. Each specimen was centrifuged at 14000 rpm for 10 min, and the supernatant was used as the saliva sample for analysis of its components. All samples were stored at −20°C until analysis and were used without repeated freeze-thaw cycles.

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method used for assessment of total antioxidant capacity

In this chemical assay, an antioxidant reduces the stable DPPH by donating a hydrogen to it. The radical

scavenging activity of saliva samples against stable DPPH was determined spectrophotometrically. The color of DPPH changed from deep-violet to light-yellow due to its reduction. The color change was measured at 517 nm using a UV/visible light spectrophotometer (Varian Cary 50, Agilient Technologies). Briefly, 3.9 mL of freshly prepared DPPH solution in methanol was added to 0.1mL of centrifuged saliva in disposable microcuvettes and mixed. The samples were kept in the dark for 40 minutes at room temperature. The control used was 0.1 mL of double distilled water.

The scavenging activity percentage (AA%) was determined according to MENSOR & al. [11]

$$AA\% = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

The experiment was done in triplicate for each sample. The results were expressed as percentage decrease with respect to control values and compared.

Assessment of clinical parameters

The presence of injuries in the mucosa were assessed and classified in all clinical visits:

1. Absent
2. 1 or 2 sore spots or ulceration evidenced by visual inspection
3. 3 or Multiple sore spots or ulcerations evidenced by visual inspection

Statistical analysis

For the statistical analysis was used Microsoft Excel 360 with Statistical Analysis Pack (Microsoft Corporation 2018).The results for TAC were expressed as mean ± standard deviation. The data were analyzed using the T-Student test (the P-value with statistical significance was considered 0.05). The Pearson correlation index was calculated with the purpose of determining possible correlations between the variables analyzed (TAC and clinical assessment of injuries) and the valid value was considered 0.95 (at this value there is a strong correlation between the variables).

Results and discussion

Results

The mean age of a total of 30 patients, including 11 males and 19 females (ages between 16 and 30 years) was 25.9 years. In all 3 groups the salivary samples were taken prior to orthodontic treatment (T0), after 2 weeks (T1), 30 days (T2) and 60 days (T2) and for analysis was used the DPPH total inhibitory activity analysis. In control group the TAC variation was minimal (probably because the homogeneity). The group wearing metallic braces has a significant increase of TAC in T1 compared with the control group. In T2 there is an increased value but not statistically significant (Figure 1). All the values decreased progressively close to baseline in T3. For patients treated with aligners, there was an increase in saliva inhibitory activity in T1 but not statistically significant ($p > 0.05$) (probably due to minimal invasiveness of the device and lack of marked lesions). These values decrease to the levels recorded in T0 (Figure 1, Table 1).

Table 1. TAC values compared with T-student with the baseline (T0) of each group

Groups	Inhibitory activity %				P values			
	T0	T1	T2	T3	T0	T1	T2	T3
Control	29.61±4.84	30.24±4.75	29.31±5.08	29.34±5.16	-	0.16	0.41	0.51
Metallic	30.06±2.12	38.72±5.34	32.71±1.75	30.99±2.16	-	0.001	0.079	0.38
Aligner	29.25±4.85	29.91±4.44	30.15±4.16	29.27±4.45	-	0.61	0.84	0.95

*Time (T0, T1, T2, T3) – at the beginning of the treatment (T0), at two weeks (14 days) (T1), at 1 month (30 days) (T2) and after two months (60 days) (T3)
Groups (control (A), metallic (B), aligners (C)) – Control- 10 normal patients that don't require orthodontic treatment; metallic – 10 patients with fixed orthodontic appliance; aligners - 10 patients wearing aligners
Inhibitory activity (%) – The results are expressed as percentages ± standard deviation
P values- results after using T-Student

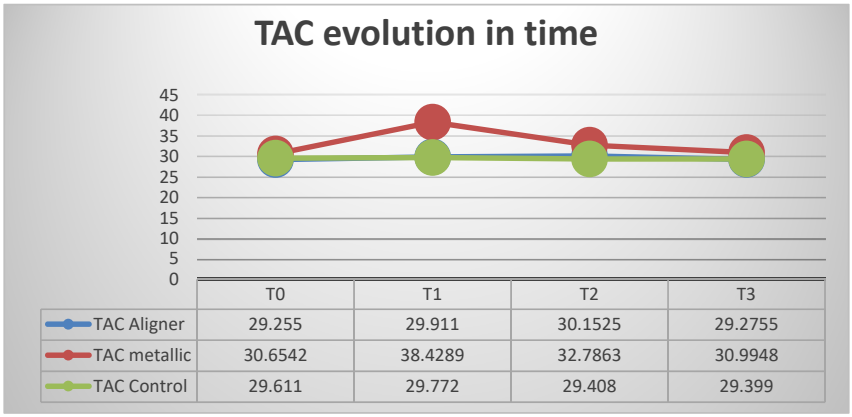

Figure 1. TAC activity in different times

Regarding assessment of injuries, only the metallic braces made some at the beginning of the treatment (in T1, T2) (Table 2).

Table 2. Injuries distribution in different stages of orthodontic treatment

Time / Results / Group	T0			T1			T2			T3		
	1	2	3	1	2	3	1	2	3	1	2	3
Control (A)	10	0	0	10	0	0	10	0	0	10	0	0
Metallic (B)	10	0	0	6	4	0	9	1	0	10	0	0
Aligners (C)	10	0	0	10	0	0	10	0	0	10	0	0

* Time (T0, T1, T2, T3) – at the beginning of the treatment (T0), at two weeks (14 days) (T1), at 1 month (30 days) (T2) and after two months (60 days) (T3)
Group (control (A), metallic (B), aligners (C)) – Control- 10 normal patients that don't require orthodontic treatment; metallic – 10 patients with fixed orthodontic appliance; aligners - 10 patients wearing aligners
Results (1, 2, 3) – 1-absent injuries in the mucosa, 2- 1 or 2 sore spots or ulceration evidenced by visual inspection, 3- 3 or multiple sore spots or ulcerations evidenced by visual inspection

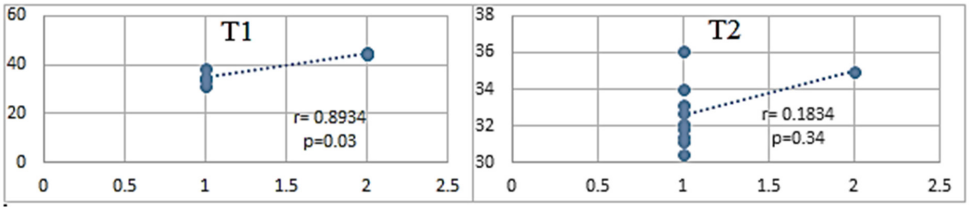

Figure 2. The Pearson correlation in T1 and T2 in fixed orthodontic treatment

The Pearson Index was used in order to establish a correlation between the total antioxidant activity and the presence of oral injuries made by fixed appliances. It seems to be a high correlation between them in T1 (there are 4 patients who have 1 or 2 sore spots or ulceration evidenced by visual inspection) (r=0.8934). In T2 only one patient has one oral injury (r=0.1834) and in T3 there are no injuries (Figure 2).

Discussion

Using saliva as a diagnostic tool is making clinical diagnosis a reality that can be very precise and useful in the assessment of oral health. Newly emerging and fast-growing technologies, such as the latest point systems, RNA sequencing, and fluid biopsy can provide new diagnostic solutions (KUBALA & al. [12]).

The oxidant-antioxidant status changes in the saliva depend on many factors, making the interpretation of occurred changes difficult (REZAEI & SOULTANI [13]).

TAC is a biomarker often used in order to investigate oxidative stress in many pathological conditions (PELUSO & RAGUZZINI [14]). Salivary TAC is affected by diseases, antioxidant foods, supplements, age, gender, lifestyle, oral status and infection; therefore these factors must be taken into account in both case-control and intervention studies. Unstimulated saliva could be the better approach to measure salivary TAC (PELUSO & RAGUZZINI [14], RAHMANI & al. [15]). The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry, so it can be useful to assess various products at a time (GAWRON-SKARBEK & al. [5], GARCIA & al. [10]).

In dentistry, total antioxidant capacity is significantly decreased in healthy subjects with periodontal disease compared with periodontal healthy controls. The alterations in total antioxidant capacity is supported by recent studies indicating that chronic periodontal disease is associated with peripheral neutrophils that are hyper-reactive with respect to the production of reactive oxygen species (ROS). Thus, periodontal disease has been suggested to be associated with reduced salivary antioxidant status and increased oxidative damage within the oral cavity (PENDYALA & al. [6], TRIPATHI & al. [16]). An increase in the antioxidant activity of the saliva may lead to the increase in the suspension of proteins and cariogenic activity (HOSSEINI-YEKANI & al. [4]).

Salivary TAC levels in patients with RAS (Recurrent Aphthous Stomatitis) showed a significant increase from the active lesions phase to the healing time (also been reported after healing of periodontal disease). Rising salivary TAC level from active lesions phase to the healing phase can be related to two probable reasons:

1. like a defense mechanism against the tissue inflammation changes (a compensatory response against oxidative stress caused by periodontal disease)

2. having oral pain change the diet with taking more liquid food and juices that are generally rich in nutrients, vitamins and antioxidant compounds, thereby improving antioxidants status of whole body and saliva (REZAEI & SOULTANI [13]). Some studies have demonstrated the effect of nutrition on antioxidant levels of saliva to support this possibility (green tea intake increase the TAC of whole saliva in adults with significant periodontal problems (BAKHTIARI & al. [17]), *Glycyrrhiza glabra* extracts showed good anti-candida activity and also high anti-oxidant property which reduces the oxidative stress of HIV-infected people (ALUCKAL & al. [18]).

In our study the clinical injuries made by metallic appliances are highly correlated with the increased TAC values.

The biocompatibility, cytotoxicity and genotoxicity of orthodontic materials are important features (considering the physiological or mechanical properties of them). Orthodontic composites were tested and the result showed that there are no cytotoxic effects on children (BAY KARABULUT & ONDER. [19], GULER & al. [20]).

Orthodontic treatment modifies the oxidative–antioxidative balance in the saliva of clinically healthy subjects. Increased nickel concentration in saliva, released from orthodontic appliances, seems to be responsible for

changes in the oxidative status of the saliva (BUCZKO & al. [21]). The salivary level of titanium increased significantly six months after installment of orthodontic appliances (JURELA & al. [22]). VANISHREE & al. [23] used fixed orthodontic treatment and TAC did not show any significant changes during the study period (before application of fixed appliances, at 1 month and after 6 months). After ZOGAKIS & al. [24], oxidant-scavenging abilities was decreased but not statistically significant (at baseline, one-hour after bonding and after 4-6 weeks) and exposure to fixed orthodontic appliances did not show a considerable effect on salivary parameters related to inflammation or stress. YOUNESS & al. [25] compared fixed and removable appliances (not aligners). There was a significant positive correlation between TAC (increase significantly) and active orthodontic treatment. He explained this finding as a result of using orthodontic appliances in which most frequently the application of high intensity forces will give an inflammatory response (with an increased synthesis of free radicals, secondary followed by the oxidative stress) and also because of the metallic ions and monomers released from removable and fixed orthodontic appliances (with chemical alterations in DNA bases, increased lipid peroxidation and altered homeostasis of calcium and sulfhydryls). In our study, the TAC was increased significant for metallic appliances especially in T1 and T2 (compared with control group and aligners) and it seems to be correlated with the presence of injuries.

To our knowledge, this is the first article where is compared the fixed treatment with the aligners regarding total antioxidant activity and clinical parameters. Being so new in orthodontic field, the aligners are not yet well researched. In our latest article regarding oxidative stress released by different types of orthodontic appliances, the metallic one seems to be more invasive then aligner treatment (T0 – at the beginning, T1- after 1 month, T2- after 2 months)(RĂDUCANU & al. [26]). However, future studies are required, with higher sample size and more number of investigated parameters for better exploration of this field.

Conclusion

a) In fixed orthodontic treatment, the materials used, the force, the diet (more liquid) and the injuries that are made so frequently at the beginning of the treatment may have a role in variation of TAC.

b) Aligners do not lead to a change in total antioxidant activity.

c) DPPH method is an easy tool for assessment of TAC. Using unstimulated saliva can reduce time for further research in orthodontic field.

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