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IMPACT OF DIFFERENT ACID CONCENTRATIONS ON BOND STRENGTH OF METALLIC BRACKETS AND ENAMEL DAMAGE - an in vitro study

ПРОЦЕНА НА ВЛИЈАНИЕТО НА РАЗЛИЧНИ КОНЦЕНТРАЦИИ НА СРЕДСТВО ЗА НАГРИЗУВАЊЕ НА ЕМАЈЛОТ ВРЗ ЈАЧИНАТА НА ВРСКАТА ПОМЕЃУ ЕМАЈЛОТ И МЕТАЛНИТЕ БРЕКЕТИ И ОШТЕТУВАЊЕ НА ЕМАЈЛОТ - ин витро студија

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Abstract

Introduction: Enamel etching is an important and necessary phase of bonding protocol. Different etching duration and different acid concentration can be used to prepare the tooth surface for bonding. Generally, the longer the etching duration and the higher the acid concentration, the stronger the bond strength between bracket and tooth surface. However recent studies are focused on lowering the etch concentration during bonding of brackets. **Aim of the study:** To evaluate the enamel damage caused by different acid concentrations during enamel etching; to determine the impact of acid concentration during enamel etching on the bond strength of orthodontic brackets; to determine the etch concentration which will achieve the optimal bond strength while minimizing enamel damage. **Material and methods:** The material for our In Vitro study consisted of 30 intact premolar teeth, extracted for orthodontic reasons. The teeth were categorized in three groups. Each group of teeth was etched with different acid concentration (3.7%, 18.5% and 37%). Subsequently, shear bond strength of orthodontic brackets was tested in Universal Testing Machine. **Results and discussion:** The lowest mean bond strength (7.59 MPa) was recorded in the teeth etched with 3.7% phosphoric acid. While the highest mean SBS was found in the group of teeth etched with 37% phosphoric acid (10.35). The group of teeth etched with 18.5% phosphoric acid represented intermediate SBS value, which was 9.16 MPa. Teeth etched with 37% phosphoric acid showed higher adhesion at the enamel/resin interface, while teeth etched with 3.7% phosphoric acid had higher adhesion at the resin/bracket interface. Findings for the group of teeth etched with 18.5% phosphoric acid were in between. **Conclusions:** We consider that lower concentrations of acid should be used during bracket bonding.

Апстракт

Вовед: Нагризувањето на емајлот на забот е важна и неопходна фаза во протоколот на поставување на фиксниот ортодонтски апарат. За подготовка на емајловата површина и врзување на брекетот за забот може да се користи различното време на нагризување и различната концентрација на киселина. Општо земено, колку е подолго времето на нагризување и поголема концентрацијата на киселина, толку е појака врската меѓу брекетот и емајлот на забот. Сепак, неодамнешните студии се фокусираат на намалување на концентрацијата на киселината за нагризување. **Цел на студијата:** да се процени штетата предизвикана од различни концентрации на киселина на емајлот, да се утврди влијанието на концентрацијата на киселината врз јачината на врската меѓу забот и брекетот, да се одреди концентрацијата на киселината со која би се постигнала оптимална јачина на врската со минимално оштетување на емајлот. **Материјал и метод:** Материјалот за оваа Ин Витро студија го сочинуваат 30 здрави премолари (екстрахирани од ортодонтски причини, во цел на екстракциона ортодонтска терапија), поделени во 3 групи. Кај секоја група беше извршено нагризување со три различни концентрации на киселина (3,7%; 18,5% и 37%). Последователно, јачината на врзување беше тестирана во машината за универзално испитување (Universal Testing Machine). **Резултати и дискусија:** Најниска средна јачина на врската (7,59 MPa) регистриравме кај забите нагризувани со 3,7% фосфорна киселина, додека највисока (најака) просечна јачина на врската е регистрирана кај групата заби кои беа нагризувани со 37% фосфорна киселина (10,35 MPa). Групата заби нагризувани со 18,5% фосфорна киселина покажа средни вредности на јачина на врската (9,16 MPa). **Заклучоци:** Од добиените резултати изведовме заклучок дека е поцелисходно да се користат пониски концентрации на киселина за нагризување при поставувањето на фиксниот ортодонтски апарат во секојдневната пракса.

Introduction

Enamel etching is an important and necessary phase of bonding protocol. Different time of etching and different acid concentration can be used to prepare the tooth surface for bonding. Generally, the longer is the etching time and the higher is acid concentration, the stronger is the bond strength between bracket and tooth surface. But

not necessarily. For example, some studies demonstrate poorer adhesion when enamel is over etched. Especially, etching time beyond 90 seconds resulted in significantly lower bond strengths than all other etching times[1].

The idea of etching enamel surfaces with orthophosphoric acid was first introduced by Buonocore in 1955. He used 85% orthophosphoric acid for 30 seconds and discovered that the bond strength of acrylic restorative resins was significantly increased by etching of the enamel surface[2].

However, at that time it was not intended to be used for bonding orthodontic brackets. It was Dr. George Newman who introduced the idea of bonding different plastic attachments to the tooth surface, hence initiating a very important transition in orthodontic fixed treatment, transition from banding of the teeth to direct bonding of brackets.

Bracket failure is a common problem during fixed orthodontic treatment. It is annoying for an orthodontist and can prolong and complicate the treatment plan. Therefore, creating a good bond strength is necessary for a successful fixed orthodontic treatment.

We also have to mention that many studies report that there is a significant difference of shear bond strength of brackets bonded to enamel comparing to brackets bonded to teeth with cavities that have been filled with different composites[3].

However, enamel etching can be harmful for enamel surface. Different acid concentration can have a different impact on enamel surface. The best choice would be to achieve the best bond strength with lowest acid concentration.

Iatrogenic effects of etching were listed as below[4]:

- Fracture and cracking of enamel upon debonding
- Increased surface porosity – possible staining
- Loss of acquired fluoride in outer of 10mm of enamel surface
- Loss of enamel during etching
- Resin tags retained in enamel – causing discoloration of resin
- Rougher surface if over etched

Considering all those risk factors, some studies were conducted in order to evaluate the bond strength of orthodontic brackets without enamel etching. The conclusions of those studies were astonishing suggesting that the application of filled adhesive without acid etch not only provides sufficient bond strength for bracket bonding, but also results in minimum resin remnants[5]. However, it was remarked by the researchers of the study itself that the same has limitations and that those results may not yet be applicable in general.

Another reason to reduce the acid concentrations used during orthodontic etching is the problems that acid can cause on oral mucosa. It was reported that inadequate rinsing of dental acid etchants or remaining dental acid etchants can cause problems, including chemical burning, irritation and inflammation, intra and extra orally[6].

Etching enamel surfaces with phosphoric acid (H3PO4) is an accepted and widely applied technique to improve bonding of dental resins to enamel in restorative dentistry, in preventive dentistry and for direct bonding of orthodontic attachments[7].

The depth of etch and the amount of enamel surface loss depends on many factors, such as: the type of acid used, the concentration of acid, etching duration and the chemical composition of the enamel[8]. According to Silverstone et al.[9], three types of etching patterns can be revealed when examining with scanning electron microscopy after acid etching:

Type 1: Generalized roughening of the enamel surface, with preferential dissolution of the prism centers, resulting in a “honeycomb” appearance.

Type 2: preferential dissolution of prism peripheries resulting in a “cobblestone” appearance.

Type 3: Combination of type 1 and type 2 patterns (some regions resembling hollowed prism centers adjacent to areas where the prism peripheries appeared to be removed)

Galil and Wright[10] described two more types of etch patterns located in the cervical third of the buccal surfaces of the teeth:

Type 4: pitted enamel surfaces, as well as structures, which look like unfinished puzzles, maps or network.

Type 5: flat, smooth surface

A review of the literature concerning the relationship between the type of etching pattern and bond strength seems to indicate that regular and distinct type 1 and type 2 patterns provide maximum adhesion[11].

Buonocore used phosphoric acid to obtain as effective an adhesion on enamel surface as on metal surfaces. The concentration of the first phosphoric acid solution used by Buonocore was 85%, and it was applied for 30 seconds[2]. Buonocore adhered acrylic materials on the non-etched teeth surfaces and etched surfaces. Although acrylic materials adhered on the etched surfaces were bonded with enough strength that they needed debonding procedures, failures were observed on non-etched surfaces[12]. This technique was an important advance in directly bonding orthodontic attachments to the tooth surface by means of micro-retention. However, honeycomb structures were not obtained in enamel prisms after etching with 85% phosphoric acid, and successful results in terms of retention were not achieved.

Many researchers reported that a 35-38% concentration of phosphoric acid is effective in terms of optimum bond strength; however, 5-10% concentration changes did not have negative effects on bond strength[13,14,15,16,17].

Today, 35-38% orthophosphoric acid is effectively used to change enamel surface characteristics and to provide micro mechanic bond strength.

Etching procedures with phosphoric acid differ in terms of microtopographic etching patterns over enamel surfaces[18,19]. The intended etching pattern was only observed in 1/20 of enamel etched with phosphoric acid. This was attributed to the presence of aprismatic enamel and partial contact between phosphoric acid and the enamel surface[20]. Microtopographic evaluation, depending on the etching procedure, revealed a non-uniform depth. It was reported that a depth of 3-15 µm or more is necessary to provide optimum shear bond strength and penetration[21]. However, in literature, surface depths between 10 µm and 175 µm were presented. The difference in the depth is thought to be caused by aprismatic enamel and remineralization of Ca-P to enamel surface[21,22].

The concentrations of phosphoric acid recommended for clinical use in dentistry range from 30% to 60%[7]. However, many studies report that the higher the concentration - the bigger the enamel surface loss. Consequently, concerning the acid concentrations, it is preferable to use low concentrations causing minimal loss of enamel while securing an adequate bond.

The dilemma of the high bond strength of brackets, which is achieved after etching an enamel with 37% acid was also reported, since debonding these brackets often leads to enamel cracks and fractures[23].

Lately, safer enamel etching and bracket debonding is being promoted in order to enhance enamel resistance to demineralization during orthodontic treatment.

Aim of the study

Enamel loss and bracket failure are one of the essential complications during the fixed orthodontic treatment.

Hence, the aims of our study were:

1. To evaluate the enamel damage caused by different acid concentrations during enamel etching
2. To determine the impact of acid concentration during enamel etching on the bond strength of orthodontic brackets
3. To determine the etch concentration which will achieve the optimal bond strength while minimizing enamel damage

Material and methods

The material for our In Vitro study consisted of 30 intact premolar teeth, extracted for orthodontic purposes. The criteria for tooth selection were as follows: no caries, no enamel cracks or fractures, or any kind of enamel defect. The extracted teeth were collected at the University Clinical Dental Center of Kosova, Department of Oral Surgery and Dental Office "Donident" in Prishtina.

Until the beginning of the research, the teeth were stored in 0.9% NaCl solution.

The teeth were mounted in self-cured acrylic resin and were randomly categorized/divided in 3 groups, each group containing 10 teeth.

First group of teeth was etched with 3.7 percent phosphoric acid.

Second group of teeth was etched with 18.5 percent phosphoric acid.

Third group of teeth was etched with 37 percent phosphoric acid.

Etching duration for all groups was 30 seconds. Dentaurum ConTec Go 37% phosphoric acid etching gel was used for etching the third group of teeth, while for the first and second group of teeth, 37% acid was diluted with distillated water.

All etched teeth were washed by air water spray for 15 seconds, and dried with oil free air syringe.

Brackets were bonded on the labial surface of the teeth according to general rules of bracket placement. One component "no mix" bracket adhesive in syringes will be used. Orthodontic bonding system Dentaurum ConTec Go adhesive was used. The type of brackets used for this study was Dentaurum "discovery" brackets, System Roth 22.

The samples were tested for shear bond strength (SBS) with universal testing machine. Shear bond strength values were expressed in MPa. The de-bonded teeth were examined under 10x magnification microscope for the amount of remnant left and were scored according to Adhesive Remnant Index (ARI). The tests were performed at the Faculty of Mechanical Engineering – University of Prishtina.

Results and discussion

Table 1 contains calculated bond strength for each tooth, ARI score of each tooth, the mean bond strength for specific group of teeth and the ranges for each group. The lowest mean bond strength (7.59 MPa) was recorded among teeth etched with 3.7% phosphoric acid. While the highest mean SBS was found in the group of teeth etched

with 37% phosphoric acid (10.35). The group of teeth etched with 18.5% phosphoric acid showed intermediate SBS value, which was 9.16 MPa.

Based on the results of some studies, the clinically acceptable range of shear bond strength for bonding of orthodontic brackets is 5.9-7.8 MPa[24]. According to these results, two groups of teeth in our study, etched with 18.5 and 37% phosphoric acid showed sufficient SBS. Regarding the group of teeth etched with 3.7% phosphoric acid, the mean SBS was satisfying, however few teeth among that group had lower SBS than the one considered as clinically acceptable value in the literature.

Similar with our study, it is the goal of many researchers to study the bond strength of orthodontic attachments when using lower concentration of acid in order to minimize enamel damage. Thus, it was reported that a 25% of phosphoric acid concentration with 60 sec etching duration marked sufficient bond strength[25].

Furthermore, some studies suggest that even 15% of phosphoric acid, in both 5 and 15 second duration, create strong enough bond between brackets and tooth surface[26].

The effect of variations in acid concentration (5% and 37% H3PO4) and duration of etching (15 and 60 seconds) on the shear bond strength of an orthodontic bonding system to etched enamel was studied and it was reported that the shear bond strength was not significantly different[27].

Many studies suggest that the concentration of acid can be reduced clinically without having an adverse effect on the retention of bonded brackets[28]. A certain study found out that reducing the phosphoric acid concentration from 37% to 15% and applying it for 60 seconds had no significant increase in the failure of bonded attachments[16,29].

However, whenever we discuss acid concentration regarding a shear bond strength, we have to take into consideration that, lately, orthodontic treatments have been more prevalent among adults. This has been emphasised because in such situations brackets and different fixed orthodontic attachments often are to be bonded to different materials, like metals, ceramics or composites. Many studies report that there is a significant difference of shear bond strength of brackets bonded to enamel compared with brackets bonded to teeth with cavities that have been filled with different composites[3].

The ARI scores of teeth included in our study were in correlation with the acid concentration used during etch-

Table 1. Shear bond strength between bracket base and enamel in groups with different acid concentration (3.7%, 18.5%, 37%) and ARI sores for each tooth

Tooth	3.7%		18.5%		37%	
	MPa	ARI	MPa	ARI	MPa	ARI
1	8.0	1	9.8	2	10.2	2
2	6.7	0	8.8	1	9.4	1
3	7.3	0	8.9	1	9.7	1
4	9.0	1	10.4	3	9.9	1
5	5.2	0	10.1	2	11.0	3
6	6.4	0	9.9	2	11.7	3
7	7.7	0	8.4	0	11.2	2
8	8.7	1	8.7	1	9.9	2
9	9.0	1	8.6	1	9.7	2
10	7.9	0	9.0	2	10.8	2
X	7.59		9.16		10.35	
Range	5.2-9.0		8.4-10.4		9.4-11.7	

ing. Hence, the highest amount of adhesive left on the teeth was recorded in the group etched with 37% phosphoric acid. While decreasing the etch percentage resulted with lower ARI scores, these results correspond with literature findings[20].

Table 2 represents adhesive remnant index among groups of teeth etched with different acid concentrations. According to the results of our study, the highest ARI scores were found among teeth etched with higher concentration of acid. This means that teeth etched with 37% phosphoric acid showed higher adhesion at the enamel/resin interface, while teeth etched with 3.7% phosphoric acid had higher adhesion at the resin/bracket interface. Findings for the group of teeth etched with 18.5% phosphoric acid were in-between. Similar conclusions regarding the correlation between the etch concentration and ARI index were reported in literature. Hence, Niaki[30] reports that when applying 15% phosphoric acid, more than 50% of the resin remained on the tooth surface, while all the resin remained on the tooth surface when applying 37% phosphoric acid.

Table 2. Adhesive remnant index (ARI) scores in groups according to different acid concentration (3.7%, 18.5%, 37%)

ARI	3.7%	18.5%	37%	Total
0	6	1	0	7
1	4	4	3	11
2	0	4	5	9
3	0	1	2	3

Another finding that has been reported when comparing phosphoric acid concentrations was that etching with 37% acid resulted in higher amounts of adhesive left on the teeth than when etching with 2% acid (according to ARI scores)[16]. These findings are also in line with the results of our study regarding the ARI index, and they draw the conclusion that when using 37% acid, the bond strength between the enamel and the resin is often higher than the one between the resin and the bracket. Alternatively, after etching with 2% acid the adhesion between the enamel and the resin appears to be lower than the adhesion between the resin and the bracket. This is particularly important when using the ceramic bracket, since it has been reported that the bond strength of the first ceramic brackets was very high compared to metal brackets[31].

Conclusion

- Lower concentrations of acid during etching provided sufficient shear bond strength of orthodontic brackets
- The highest ARI scores were found in teeth etched with higher concentration of acid

We believe that further clinical studies should be conducted regarding this issue.

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COMPARATIVE ANALYSIS OF THE USE OF ANB ANGLE AND WITS APPRAISAL FOR DETERMINATION OF THE SAGGITAL JAW RELATIONSHIP

КОМПАРАТИВНА АНАЛИЗА НА УПОТРЕБАТА НА ANB АГОЛОТ И WITS АНАЛИЗАТА ЗА ОДРЕДУВАЊЕ НА САГИТАЛНИТЕ МЕЃУВИЛИЧНИ СООДНОСИ

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Abstract

Introduction: Finding a simpler and more accurate approach for the analysis of sagittal relations of jaws is a big challenge. The choice is not simple, due to the variability of the points on which the ANB angle is based, which leads to the usage of an additional linear parameter such as Wits. **Objective:** To observe the daily involvement of the Wits appraisal and its correlation with the ANB angle, as well as to show the justification of the usage of the Wits appraisal, its stability, and modifications for obtaining a comprehensive diagnostic image. **Material and methods:** Literature reviews and analysis were performed by the research network ResearchGate, Academia.edu and PubMed, by searching for papers not older than 2000, with the keywords Wits appraisal, Steiner cephalometric analysis, Orthodontic cephalometry and literature from books and contemporary journals publication. **Results:** The variable position of the points that determine the ANB angle showed the justification for the additional use of the Wits appraisal, and a correlation was observed in normodivergent patients. The influence of the occlusal plane position on the Wits noted its disadvantage and its modification requirement in patients with increased lower face height and high FMA angle. **Conclusion:** Sagittal jaws relations analysis should take into consideration additional factors such as the size of the FMA angle, the inclination of the occlusal and the mandibular plane, the vertical growth. If necessary, the modification of the Wits appraisal can be used. **Keywords:** Wits appraisal, Steiner cephalometric analysis, orthodontic cephalometric analysis.

Апстракт

Вовед: Пронаоѓањето на поедноставен и попрецизен пристап за анализа на сагиталните вилични соодноси претставува голем предизвик. Изборот не е едноставен, поради променливоста на точките врз коишто се темели ANB аголот, што довело до употреба на дополнителен линеарен параметар како што е анализата според Wits. **Цел:** Да се воочи секојдневното вклучување на Wits проценатa за анализа на сагиталните меѓувилични односи и корелацијата со ANB аголот. Истовремено да се прикаже оправданоста на употребата на Wits проценатa, нејзината стабилност и модификации на истата за добивање на сеопфатна дијагностичка слика. **Материјал и методи:** Беше извршен преглед и анализа на литература од академската мрежа ResearchGate и Academia.edu и од PubMed, преку пребарување трудови не постари од 2000 година, со клучните зборови Wits appraisal, Steiner cephalometric analysis, Orthodontic cephalometry и литература од книги и трудови со најнови сознанија на испитуваната тема. **Резултати:** Променливата положба на точките кои го одредуваат ANB аголот ја прикажа оправданоста на дополнителната употреба на Wits анализата, а корелација е забележана кај нормодивергентни пациенти. Влијанието на положбата на оклузалната рамнина врз Wits анализата го нотираше недостатокот на оваа анализа, а со тоа ја нагласува потребата за нејзина модификација кај пациентите со зголемена долна лицева висина и висок FMA агол. **Заклучок:** При сагитална анализа на меѓувиличните односи треба да се земат во предвид и дополнителните фактори како што се големината на FMA аголот, инклинацијата на оклузалната и на мандибуларната рамнина, вертикалниот раст. Доколку е потребно дополнително може да се примени и модификацијата на Wits анализата. **Клучни зборови:** Wits процена, Steiner кефалометриска анализа, ортодонтска кефалометриска анализа.

Introduction

The analysis of cephalometric images plays an important role in orthodontic diagnosis and in the analysis and monitoring of treatment as well. Recognizing the importance of cephalometry, dates back to 1931 when the cephalometer was first introduced by Broadbent in the United States[1] and Hofrath in Germany[2]. X-ray cephalometric analysis allows monitoring of changes related to growth, diagnosis, and classification of malocclusions through a number of developed analysis techniques[2]. In addition to the analysis of sagittal relation-

ships, cephalometric images can also be used to analyze facial symmetry. The manner of recording the patient is determined depending on whether analysis of the sagittal or frontal ratios will be performed, i.e., the position of the head in the cephalostat. Profile analysis recordings are more widely used[3]. The large number of available cephalometric analyses use different anatomical and constructed points that are drawn on paper on the cephalogram if the standard analysis technique is used, which then results in a number of different reference planes, lines and angles. Hence the numerous techniques for orthodontic treatment analysis and planning[4], Cavdar et al.[5] point out the weakness of the standard technique in two aspects: the longer time required for analysis, and the possibility of errors in drawing points and lines. On the other hand, no differences were observed in the analysis, according to Steiner, with the conventional method in relation to the computer method with CephNinja[6]. In 1948, Down developed a method for analyzing cephalometric images based on a study of 20 individuals with excellent occlusion, and over the years it has been proven that the recommended standard values are almost constant, despite the numerous variations in the study. As in all spheres, orthodontics still strives for a technique that will meet the requirements for a good analysis, and at the same time, is simple to use. This led to the development of the Steiner analysis in 1953, where the SN plane is the reference plane (as opposed to the Frankfurt reference plane of the Down technique)[7]. Steiner, in his method recommended an evaluation of the analysis of skeletal, dental, and soft tissues. Skeletal analysis shows the relationship of the upper and lower jaw to the base of the cranium, but also the relationship of the jaws to each other. Dental analysis includes the relationships of the upper and lower incisors respectively to the jaw in which they are located, but also their relationship to each other. Soft tissue analysis plays a role in contributing to the balance and harmony of the lower facial profile[8]. In order to determine the skeletal sagittal relationship, he used the angles SNA to determine the relation of the upper jaw to the cranial base, and SNB angle for the relation of the lower jaw to the base[9]. In 1953, Riedel introduced the ANB angle for the relationship between the two jaws, which Steiner accepted and popularized later in his analysis as an excellent indicator[10]. The average value for the SNA angle is 82 degrees; higher values of this angle indicate prognathism of the maxilla to the cranial base, and lower values of the same indicate retrognathism of the maxilla relative to the cranial base. The average value for the SNB angle was defined by 80 degrees, and a higher or lower value of this angle would mean consequent mandibular prognathism or retrognathism of the mandible relative to the cranial base. While SNA and SNB have precisely defined mean values, it has been accepted by many authors[5,6,9,10] that the ANB angle is in the range of 2 to 4 degrees. A value between 2 and 4 degrees indicates Class I of skeletal intermaxillary relation, a lower grade means Class III, and a larger grade Class II[4]. But in addition to the sagittal relationship, Steiner in his analysis also gave guidelines for analyzing the vertical relation by determining the angle between the occlusal plane and the SN line = 14 degrees; larger angle indicates vertical growth and skeletal open bite, while smaller angle indicates horizontal growth and deep bite. For dental relations, he recommended measuring the distance and angle of the most labial point of the incisors to the NA line for the upper and to the NB line for the lower incisors, as well as the interincisal angle, and additionally introduced the Holdaway ratio = 4mm. As a base for the position of the lips, he presented the S'line, which determines protrusion or retrusion if the lips are in front of or behind the line itself[9].

However, studies have shown that Steiner angular measurements are quite sensitive, especially for the ANB angle which depends on the position of the nasion point (N) and sella turcica (S), the length of the anterior cranial base, and the type of vertical growth. To overcome these sensitivities, Jacobson A.[11] proposed, as an addition, the Wits appraisal where the occlusal plane passing through the cusps of the first premolars and molars is taken as the reference plane. Regarding the overlap and / or the existence of the distance between the normal AO and BO lowered from points A and B for each jaw respectively in the occlusal plane, additional information is obtained for defining the sagittal jaw relationship[10].

Aim

The aim of this paper is to observe the daily increasing involvement of the Wits appraisal in Steiner analysis of cephalometric images, due to the variability of points used in Steiner analysis, the correlation between the ANB angle, and the Wits appraisal. At the same time to present the need as well as the validation for including the Wits appraisal in the Steiner analysis, but also the stability and modulations of the eponymous estimation.

Material and methods

In order to achieve the set goal, a review of literature available on the academic network ResearchGate, Academia.edu and the medical network PubMed was performed. The PubMed search included the following keywords: Wits appraisal, Steiner cephalometric analysis, Orthodontic cephalometry. Searching for literature with the abovementioned keywords was limited by the time

frame of papers published from 2000 to the present. An additional filter was a search for complete free texts, but also abstracts. The papers presented as abstracts were then taken from the original journal where they were published in full. The search by the above filters generated a multitude of papers, of which 13 papers were used as a literature review for this paper.

In addition to searching these three networks, 6 papers published in scientifically accredited journals (listed in the references) were used due to the intrigue of the content of the papers themselves.

For the aforementioned purpose, a review of literature from the books with relevant themes and from novel paper facts was included.

Results and discussion

The modern and fast-paced way of life imposes a search for better methods for more successful diagnosis and treatment, which has led to the inclusion of the Wits appraisal in the Steiner analysis. However, a large part of the scientific community in their research seeks to find the correlation between the ANB angle and the Wits appraisal, in order to justify the additional use of this method which prolongs the analysis. The study by Ahmed M. et al.[10] showed a significant positive correlation between the Wits analysis and the ANB angle (r = 0.831). They further state that hyperdivergent or hypodivergent vertical growth affects sagittal relationships, as well as the accuracy of determining a relevant anteroposterior parameter. Therefore, they emphasize that determining the ANB angle as the most relevant parameter in their study refers to normodivergent patients, and that this should be taken into consideration in everyday practice. By stating that the study refers to normodivergent patients, the authors distance themselves from the relevance of ANB as a standalone parameter for other groups of patients.

Correlation testing is also reported in the study by Jan A. et al.[12], which again confirmed a significant correlation between the ANB angle and the Wits appraisal in determining sagittal jaw relationship (r = 0.469). Knowing the weakness of the ANB angle, however, they state that other studies require a connection between the ANB angle and the SN line, which actually leads to a prediction of the Wits appraisal. In addition, this study did not take into account the effects of the position of the palatal plane, the occlusal plane, and the horizontal overlap of the incisors. According to the above, relying only on the ANB angle and neglecting other parameters can lead to incompletely accurate diagnosis.

Furthermore, Jacobson A.[11] points out the instability of the ANB angle for two reasons: the variation of the anteroposterior relation of the point nasion (N) to the jaws, and the rotation of the jaws towards the cranial reference plane (SN plane). According to Wits' findings, individuals had severe malocclusion and moderate malocclusion, but according to the ANB angle, all individuals would be characterized with the same degree of malocclusion. Thus, Jacobson A.[11], in his study, confirms the need to supplement the analysis with the Wits appraisal to obtain a more comprehensive and more detailed picture not only of the type but also of the severity of the anomaly, which significantly affects the overall treatment that would be undertaken, and its outcome, i.e., that the diagnosis is one of the guiding factors for success.

In addition to Jacobson A.'s[11] assertions of ANB angle deficiency, due to the position of the point (N) and the rotation of the jaws towards the SN line, it also depends on the vertical dimensions, especially on the anterior vertical dimension. Furthermore, the mean values refer more to dolichocephaly, than to brachiocephalic or mesocephalic individuals. However, with the Wits appraisal, the vertical dimension also has a large effect, as does the position of the occlusal plane[13].

The examination of the connection between these two methods goes so far that some of the authors analyze it through computed tomography, making it clear that both parameters coincide only in patients with Class III malocclusion. Hence, the question of the influence of the position of the occlusal plane and the Wits appraisal arises again, and the position of the mandibular plane that showed a correlation with the Wits estimate (r = 0.242) must not be neglected. The previously obtained findings are in favor for the use of both parameters for the analysis of sagittal relationships, but without neglecting the other factors that affect them[14].

To make the picture more complete, Jabbar A. et al.[15] analyzed the relationship between ANB angle, Wits appraisal, and horizontal overlap in individuals with Class I malocclusion, with Class II division 1 malocclusion and Class III malocclusion. There was a strong statistical correlation between the ANB angle and the horizontal overlap only in individuals with Class III malocclusion, and the same significance was observed only in individuals with Class III in analysis with the Wits appraisal. Hence, new views are opened which indicate that, in addition to these two analyses, the horizontal overlap can also be a good indicator of sagittal skeletal Class III malocclusion, but certainly not the only and pathognomonic indicator.

Another factor to consider when analyzing these two parameters is their comparison in different populations and genders. This is shown in the retrospective study of Duran SG. et al.[16] of the Turkish population. The inconsistency between the two parameters was considered for each gender separately. Again, the conclusion is that both parameters should be used together and simultaneously,

but also that the shortcomings of the ANB cannot be corrected only with the use of Wits appraisal because it is once again confirmed that Wits has its drawbacks, especially dependent on dental points, as well as from the vertical growth of the alveolar ridge. Furthermore, there is a difference between these two parameters and their correlation between the different genders. It is again proved that the position of the mandibular and occlusal planes affects the inconsistency between these two parameters. Increasing the SN-MP angle leads to a greater discrepancy towards these two parameters. Additionally, the ANB angle can 'overestimate' the sagittal relations of the jaws as the occlusal plane increases, while the Wits can give lower values at the same occlusal plane increase, hence the discrepancy between these two parameters occurs as a result of changing the position of the occlusal plane.

In addition to comparing these two parameters, the scientific community also compared only the Wits parameter in different ethnic groups, showing that the Wits appraisal is ethnically specific and once again proved to be gender-dependent as in the previous study[17].

There is no doubt that the examinations have deepened and even go so far as to compare the two parameters with dental parameters such as the position of the molars and its impact on them. In the study of Aldrees AM. et al.[18] the correlation between the dental relation of the molars and the ANB angle was 57.7%, while in the study of Zhou L. et al.[19] the correlation was 61%. The difference is explained with the age of the patients who were included in both studies, i.e., the presence of patients with early permanent dentition where the molars are still in the tuber-tuber position before their transition to Class I. However, the coherence of the ANB angle and Angle molar classification is greater in Class I dental malocclusions than in other dental classes of malocclusions. While the Wits appraisal showed a greater correlation with Class III dental malocclusions, which is thought to be due to the increased mandibular angle that affects the position of the lower first molars. The dependence of both parameters on other factors arises again.

Iwasaki H. et al.[20], gave special attention to the correlation of these two parameters in Class III malocclusion. The anteroposterior relationship between the upper and lower dental arch during adolescence is quite susceptible to change, therefore it is important to determine the anteroposterior relationship of the jaws for a proper and accurate diagnosis. Of course, they recommend using the ANB angle or the Wits appraisal for this purpose, but they stated that given the results it is difficult to evaluate which parameter would be more relevant. Therefore, the motive was to examine the differences in the assessment of jaw relationships with the two parameters and to make a recommendation for their combined synergetic use.

The analysis confirmed that using the ANB angle, the jaw discrepancy is more severe or more pronounced than the same jaw relationship to be analyzed with the Wits appraisal in patients with Class III dental malocclusion.

Furthermore, they concluded that in case of greater retro inclination of the incisors and a larger negative horizontal overlap, the ANB angle is more relevant for assessing anteroposterior jaw relation than Wits appraisal. Due to the fact that the rotation of the jaws affects the values of the ANB angle, the inclination of the mandibular plane was analyzed as well and was stated that the mandibular plane is not a geometric factor that affects the "overestimation" of the value of the ANB angle.

According to the results obtained from the study, it can be noticed that in patients with a large horizontal negative overlap, flat occlusal plane, and rotation of the mandible counterclockwise, the use of the ANB angle to assess the anteroposterior relationship of the jaws is justified.

Interest in the influence of occlusal plane inclination on ANB angle values and the Wits appraisal to assessing the anteroposterior jaw relationship, was also shown in the Santo DM study[21]. He estimated that the angular measurements are geometrically quite sensitive and cannot show completely accurate results, in addition, that they are dependent on the age of the patients. Due to these deficiencies of the angular measurements, and cited in previous studies, Santo DM.[21] also confirms the complementary use of Wits appraisal as a linear measurement to partially eliminate the shortcomings of the angular measurements, but at the same time taking into account that linear measurements are dependent on other factors, including the position of the occlusal plane. Although the ANB angle and the Wits appraisal evaluate skeletal anomalies in the same direction — anteroposterior, they would be expected to coincide to a large extent, but this is not always the case. In this study, it was challenging to show what other parameters may be a key factor for their mismatch. Santo DM[21] thought that this could be as a result of the variation in the position of the occlusal plane as well as the facial height. According to the analysis, some of the patients had a high occlusal plane, and some a low position of the same plane.

In patients with low occlusal plane (almost straight occlusal plane) consistency was observed, i.e., almost equal classification of the severity of sagittal malocclusion, but in contrast, in highly placed occlusal plane (inclined cranial), difference, in the classification of the severity of sagittal malocclusion between the ANB angle and the Wits appraisal, was confirmed in the majority of patients except one. The analyses in this study confirm the fact that the inevitable use of both parameters in the analysis of the sagittal jaw relationship, but without excluding other factors from the analysis that affect the parameters

themselves, and can lead to crucial errors from the start of the treatment plan and diagnosis of malocclusion. Equally important is the influence of the dental parameters that actually define the occlusal plane, not just the point nasion (N).

The findings suggest that the Wits appraisal has also disadvantages and therefore cannot fully cover the imperfections of the ANB angle for sagittal jaw analysis. Therefore Hayes L. et al.[22] proposed a modification of this appraisal for patients with a high FMA angle or with increased lower anterior face height.

In a perfect world, the use of sagittal relationship analysis only by defining the values of the ANB angle, and only the Wits appraisal without modifying it, would be sufficient. However, as the variations between the units become higher, these two basic parameters cannot be a fixed relevance for defining the sagittal jaw relations.

In accordance with this, Hayes L et al.[22] concluded that the value of the ANB angle cannot be used on its own to diagnose the type and severity of the anomaly. Namely, the ANB angle was the same for both patients, while the values of the Wits appraisal were quite different for the two patients in the same group. They noted that Wits appraisal depends on the position of the occlusal plane, which could affect patients with a high FMA angle and an increased lower face height. They therefore proposed a modification of this appraisal by forming the so-called newly constructed "normal" occlusal plane, i.e., an angle between the "normal" occlusal and mandibular plane NOP-MP of 17 degrees. This would actually mean replacing the present acute angle in certain situations. The advantages of this method are in the evaluation of patients with high FMA angle and increased lower face height, where the ANB angle and the classic Wits appraisal are not the most relevant for analysis in these patients. Another advantage of this modification is that it can predict unwanted vertical skeletal growth on the anterior part of the mandible. In addition, this proposed modification of the Wits appraisal makes it possible to predict the construction of the occlusal plane in patients with an anterior open bite. However, it should also be noted that this modification is only an addition to the use of the other two parameters and does not mean their exclusion from the analysis. On the contrary, he adds that in the presence of normal values of the FMA angle, and when the analysis of the angles SNA and SNB are within the normal standard values, the analysis with both the ANB angle and the conventional Wits appraisal and the modified Wits appraisal is equally valid for defining anteroposterior relations of the jaws. All this again emphasizes the fact that many factors affect the values of these two parameters and that if there are factors such as high FMA angle and increased lower face height, the supplementary use of the modified method with the other two previous parameters gives a more realistic picture of the jaw position[22].

According to the abovementioned analyses, with the covered advantages and disadvantages of the parameters for analysis of the sagittal jaw relations, the question arises why not look further, why use only these two parameters and their modification, and not introduce a completely new method that might provide even better and more relevant values for diagnosis[23].

Therefore, Nagar et al.[23] in their study propose a completely new method for sagittal analysis of the jaw relation, although that method can actually be presented as another modification of the Wits appraisal.

This new modification was represented by the line drawn perpendicular to the vertical dimension in profile radiographs taken in the natural position of the patient's head in the cephalostat – called the new reference extracranial line - true horizontal reference (HOR). The advantage of this extracranial line is that it is common for both dental bases. By obtaining this horizontal line, it was now possible to exclude the occlusal line and its defects from the analysis and obtain the so-called occlusal plane named - "Horizontal appraisal". This appraisal was used to analyze sagittal relationships in addition to the ANB angle.

In order to understand the relevance of this horizontal appraisal, a correlation was made with the ANB angle, performed for the entire sample and the horizontal appraisal showed a higher correlation coefficient than the conventional Wits appraisal. However, like the inadequacies of the abovementioned appraisals to analysis, this horizontal appraisal has its drawbacks. One of them is that the fixed vertical line necessary for the performance of the horizontal line (HOR) may vary during the recording, due to the position of the cephalometric film, weakened and unstable mechanical part of the cephalometer, etc. The dependence of the horizontal line (HOR) on obtaining a previously fixed vertical line during recording does not fully justify this appraisal as a substitute for the previous parameters, it also designates as an additional factor that can be taken into account in the analysis only. But, unlike other parameters for analysis, the author states that with this appraisal the time of analysis is extended, and that with this appraisal the number of possible mistakes is high, not only by the doctor who performs the analysis, but also by the radiologist who does the imaging. Although this appraisal correlates well with the ANB angle, due to the manner in which it is performed, many doctors consider it complicated, and it is therefore considered that this horizontal appraisal is not widely accepted. Although alternatives were sought to simplify it and minimize the possibility of human error, the same did not produce results for its wider use[23].

Conclusion

An increasingly dynamic and faster life leads doctors to strive for simplified and faster techniques of cephalometric analysis in everyday work. This is a big challenge in everyday life because the simple cephalometric technique does not always mean a reliable analysis technique. The desire to find a quick and accurate analysis was a leading factor in making all these studies, so that the best solution could be found. Knowing the key role of diagnosis in introducing good treatment and its outcome, the authors have conducted numerous studies. From the previously mentioned researches and analyses of the same, it can be seen that the analysis of the ANB angle is inevitable for the assessment of the sagittal relationship of the jaws. Although, the angle itself has many disadvantages such as dependence, and its variability on the age of the patient; the position of the nasion (N) point in both the antero-posterior and vertical directions; rotation of the SN plane; rotation of the maxilla or the mandible, or both jaws; change of SN-OP angle; as well as the degree of facial prognathism; it was not excluded from the daily analysis of the sagittal skeletal relations of the jaws. All this confirms that the ANB angle can be considered as a starting point and analysis point, which due to its shortcomings should be supplemented with the Wits appraisal. Although a correlation of these two parameters has been shown - the ANB angle and the Wits appraisal, we should take into account that in the same studies the analysis was performed on normodivergent patients, without vertical deviations in growth and development. This can confirm the justification for including the Wits appraisal as a supplementary analysis. The role and significance of the inclination of the occlusal plane is special, as well as its influence in the Wits appraisal. The dependence of this plane on dental parameters makes it quite unstable, but there is an optional solution for that, i.e., the inclusion of a modified Wits appraisal, especially in the group of patients with high FMA angle and increased lower anterior facial height. From all the above it can be seen that the sagittal relations are also influenced by the relations of the jaws in other planes. This should guide the therapist not to look at the analysis of these sagittal relationships in isolation, and therefore not to reduce it only to cephalometric sagittal analysis at the ANB angle, but after considering the other factors and analyses - the value of the FMA angle, the position of the occlusal plane, of the mandibular plane, the vertical growth of the face, etc., to include other parameters of the analysis of the sagittal relations - Wits appraisal or its modification, to obtain a complete comprehensive picture of the type and severity of the sagittal skeletal anomaly, thus enabling the establishment of a correct diagnosis, and planning an appropriate orthodontic therapy.

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EVALUATION OF CARCINOMA - ASSOCIATED FIBROBLASTS (CAFS) AND TUMOR - ASSOCIATED MACROPHAGES (TAMS) IN ORAL SQUAMOUS CELL CARCINOMA (OSCC): AN IMMUNOHISTOCHEMICAL STUDY - case report

ЕВАЛУАЦИЈА НА ФИБРОБЛАСТИ - АСОЦИРАНИ СО КАРЦИНОМ (CAFS) И МАКРОФАГИ - АСОЦИРАНИ СО ТУМОР (TAMS) ВО ОРАЛНИОТ ПЛАНОЦЕЛУЛАРЕН КАРЦИНОМ (OSCC): ИМУНОХИСТОХЕМИСКА СТУДИЈА - приказ на случаи

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Abstract

Stromal cells in the tumor microenvironment (TME) of oral squamous cell carcinoma (OSCC) interact closely with tumor cells and influence their behavior in many different ways by promoting tumor growth, cancer cell invasion, and metastasis, thus influencing the prognosis of the disease also. This study shows the presence of CAFs (cancer-associated fibroblasts) and TAMs (tumor-associated macrophages) in TME of three patients diagnosed with OSCC and discusses the association of the density of CAFs and TAMs with the clinicopathological parameters of presented OSCC. The presentation of these 3 cases is important because the density of CAFs and TAMs in the tissue samples correlate with the clinicopathological parameters of the disease and may be used as potential prognostic factors in OSCC.

Апстракт

Стромалните клетки од туморската микросредина кај оралниот планоцелуларен карцином комуницираат со канцерските клетки и влијаат на нивното однесување на различни начини промовирајќи го растот на туморот, инвазијата на канцерските клетким метастазирањена карциномот, и на тој начин влијаат и на прогнозата на пациентот со оваа болест. Овој труд преку прикажување на случаите на 3 пациенти со орален планоцелуларен карцином имунохистохемиски го потврдува присуството на фибробласти-асоцирани со карцином (CAFs) и тумор асоцирани макрофаги (TAMs) и ја дискутира корелацијата на нивната густина со клиничкопатолошките параметри. Кај прикажаните три случаи дензитетот на CAFs и TAMs корелира со клиничкопатолошките параметри и можеби би можеле да се искористат како потенцијални прогностички фактори кај оралниот планоцелуларен карцином.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the six most common malignancies in the world with a very high incidence and mortality. Treatment of choice is surgery with adequate margins, and radiotherapy and / or chemotherapy, as well as targeted therapy are complementary to surgery. Despite great advances in modern radiotherapy, chemotherapy and targeted therapy as well, the prognosis is poor. Many prognostic factors affect the oncological outcome in the form of 5-year survival and overall survival.

Clinical and pathological parameters are crucial when planning treatment and determining the prognosis of patients with this type of malignancy. Significant clinicopathological parameters are TNM status, location of the primary tumor, depth of tumor invasion (DOI), margin status following tumor resection, lymphovascular,

perineural and bone invasion, number and location of positive lymph nodes for metastasis, extranodal extension of metastatic tumor from the lymph nodes into the surrounding tissues, and presence of distal metastases are accepted criteria. Taking the biological nature of cancer cells into account, all of these parameters are interrelated and cannot be independent prognostic predictors[1].

Recent studies focus on the effects of the components of tumor microenvironment (TME) on the initiation and progression of OSCC. The components of TME, including cancer-associated fibroblasts, vascular and lymphatic endothelial cells, the extracellular matrix (ECM) and inflammatory immune cells are the most important modulators of the primary OSCC behavior. Stromal cells in the tumor microenvironment (TME) interact closely with tumor cells and influence their behavior in different ways, for example, promoting tumor growth, invasion, and metastasis, as well as resistance to anticancer treatment. So far, the complex relationship between TME and the immune system is still unclear.

The large number of immune cells that infiltrate tumor tissue show functional plasticity and at some point may acquire pro-tumor or anti-tumor activity. There is evidence that stromal cells, including fibroblasts, endothelial cells, and mesenchymal cells, play a key role in shaping the tumor immune environment. One of the most active cell types in tumor stroma are fibroblasts who have the potential for transdifferentiation into an activated-myofibroblast phenotype known as cancer-associated fibroblasts (CAFS). CAFs play an important role in growth and development of malignant epithelial tumors e.g. promoting tumorigenesis, invasion, and metastasis by stimulating angiogenesis and reconstituting the extracellular matrix thus preparing the territory for metastasis in the early stages of the disease[2]. Several studies have suggested that CAFs may be used as an important prognostic factor in a variety of tumors[3,4], although their prognostic role in OSCC is rarely published.

The tumor-fighting host organism sends a variety of immune cells to the tumor tissue, including dendritic cells and macrophages to induce and direct the anti-tumor immune response. Of these, macrophages are the most common type of tumor-infiltrating immune cells and are called tumor-associated macrophages (TAMs)[5]. There are two paradoxical types of TAMs: M1 and M2 macrophages. M1 macrophages are responsible for the anti-tumor response through tumoricidal activity and the production of pro-inflammatory cytokines[6]. In contrast, M2 macrophages have an immunosuppressive (pro-tumor) function by stimulating angiogenesis, and supporting tumor cell invasion. Numerous microenvironmental factors released by CAFs and tumor cells in the TME may be major regulators of TAM polarization[6].

Objectives

This study shows the presence of CAFs and TAMs in the oral squamous carcinoma tumor microenvironment by reporting 3 (three) characteristic cases; and confirms the association between the density of CAFs and TAMs with the clinicopathological parameters of OSCC. Additionally, we assessed the relation between the number of TAMs in TS (tumor stroma) and TN (tumor nest) with the OSCC parameters. We hope that this publication will give a contribution to the literature published so far.

Material and methods

Patients and tissue samples

Tissue samples were obtained from 3 patients with histopathologically confirmed OSCC, with no history of radiation or chemotherapy. They underwent surgery at the University Hospital for Maxillofacial surgery within the "Mother Teresa" University Clinical Center in Skopje, in 2020. Patient data were collected from hospital histories. The primary tumors were localized on the tongue and the sublingual region. As a control sample for the presence/absence of CAFs and TAMS, a tissue section with incisional surgical biopsy of a clinically healthy mucosa was taken from a patient with previously confirmed OSCC in the bilateral sublingual region.

The stage of the disease was classified according to the criteria of the AJCC TNM classification system of the OSCC. Tumor differentiation was classified into 4 levels: good, moderate, poorly differentiated, and undifferentiated (anaplastic) according to Broder's histological classification of tumor cell differentiation in oral squamous cell carcinomas (Broder's grading descriptive system)[7].

Immunohistochemistry (IHC) and IHC evaluation

Tissue samples were processed and analyzed at the Institute of Pathology, within the "Mother Teresa" University Clinical Center in Skopje. Immunohistochemistry was performed using Dako EnVision flex system.

The following primary mouse monoclonal antibodies were used: anti-α-SMA and CD68. Nikon

Patologist use Nikon 80 digital microscope in the analysis and for taking microphotographs.

CAFs and TAMs Assessment

CAFS were generated from the samples and evaluated by immunohistochemistry. Positive or negative fibroblasts were identified on the basis of α-SMA expression. This confirmed the identity of the CAFs for further research.

So, CAFs are defined as large spindle-shaped fibroblasts that express α-SMA. First, by selecting 4 typical visual field of tumor stroma in every slice by high-power magnification of the microscope, we counted the area of α-SMA-positive fibroblasts (CAFs) and calculated the CAFs density (dCAFs) = the positive staining area/tumor stromal area (%) of every visual field. CAFs density is the mean value of the 4 fields. The cut-off value for CAFs is 10%[2].

CD68 expression was determined by counting the number of CD68 positive macrophages (TAMs). Macrophages were defined as stromal cells larger than 10 μm in diameter which express CD68; and the same were counted in the invasive regions of the specimens. Each section was displayed on a low power magnification (low-power field with 100x magnification) to identify the areas with the highest macrophage density, then the macrophages were counted in three fields on high power magnification (high-power field on 400x magnification) and the average number of macrophages was obtained[8]. Additionally, we assessed the number of CD68+ macrophages in TS (tumor stroma) and TN (tumor nest).

Case reports

Clinico-histological features with results (Table 1)

Case No. 1. A patient with a primary OSCC in the tongue, 58 years old. The pathology findings confirm a moderate degree of tumor differentiation (G2); AJCC TNM classification pTNM = pT3, pN2b, pM0, pL1, pV0 and IVA-stage disease. α-SMA staining shows a high percentage of CAFs (> 50% of the mean value) (Figure 1). CD68 marks high density of TAMs in the tumor stroma counting 100-150 TAMs and high density in tumor nests (TN) counting 30-50 TAMs (Figure 2).

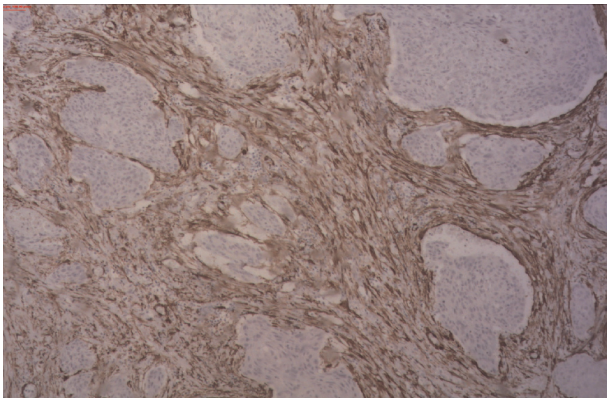

Figure 1. *Immunohistochemical staining of CAFs. CAFs are α-SMA-positive (original 100x magnification). A typical "network" pattern that forms when there is an abundance of CAFs that occupy almost the entire tumor stroma.*

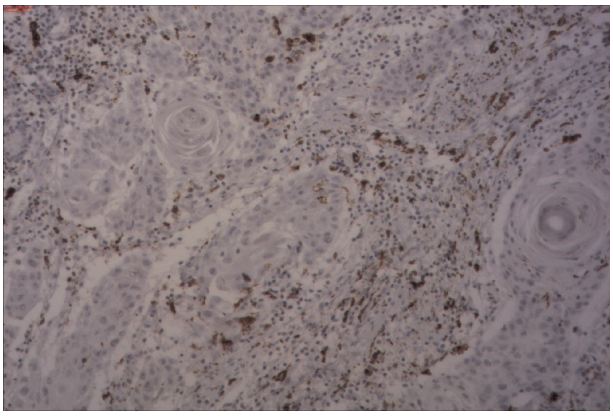

Figure 2. *Immunohistochemical staining of CD68-positive TAMs. Representative example of positive expression of CD68 in TAMs in tumor nests (TN) and tumor stroma (TS) of OSCC (original 100X magnification). CD68 cytoplasmic staining in cells identified as TAMs.*

Table 1.Clinicopathological parameters and results of immunostaining. OSCC, oral squamous cell carcinoma; T, tumor stage; N, node stage; G, Broder's grading system (G1(well-), G2 (moderately-), G3 (poor differentiation) and G4 (undifferentiated, anaplastic). L,V, lymphatic /or vascular infiltration; α-SMA-alpha smooth muscle actin. CD68+ TAMs, CD68-positive tumor-associated macrophages; TN, tumor nest; TS, tumor stroma.

OSCC	Case 1	Case 2	Case 3
T	T3	T1	Normal mucosa
N	N2b	N0	N0
Clinical stage (I-IV)	IVA	I	I
G (1,2,3,4)	G2	G1	Carcinoma was not identified
L,V	L1	L0	L0
α-SMA (%)	>50	>10	>1
CD68+ TAMs (TN)	30-50	9-10	No detected
CD68+TAMs (TS)	100-150	30-40	20-30

Case No. 2. Patient with OSSC in the oral sublingual region, aged 60 years. The finding is consistent with well-differentiated oral squamous cell carcinoma (G1). AJCC classification corresponds to first clinical stage of disease pTNM = pT1, Nx, pMx, pL0, pV0.

Immunohistochemistry showed the following results: low density of CAFs was found around arterial blood vessels (<10%). 9-10 TAMs were detected in tumor nests, and 30-40 cells in the tumor stroma.

The Case No. 3. presented with histopathologic identification of small focus of Human Papilloma Virus (HPV) positive oral squamous cell carcinoma, moderately differentiated (G2) and localized in the sublingual region billateraly. AJCC TNM classification was: pT1, N0, M0, L0, V0; and the patient was in the first clinical stage of the disease. Lymphovascular invasion was not detected. As a control sample, an extra material of a clinically healthy oral mucosa close to the primary tumor was additionally taken. Microscopic analysis of the control sample showed the presence of a regular oral mucosa. Immunohistochemistry was undertaken only on the control sample. α-SMA accounts for less than 1% of myofibroblasts. (Figure 3), and 20-30 CD68-positive macrophages (Figure 4).

Clinico-histological features with results are shown in Table 1.

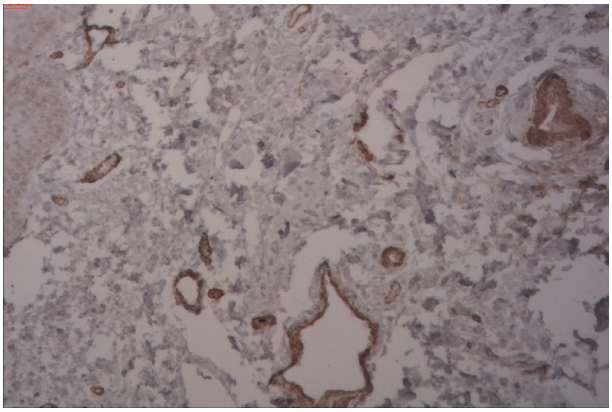

Figure 3. *Immunohistochemical staining of CAFs. Low density of α-SMA positive myofibroblasts (original 100x magnification*)

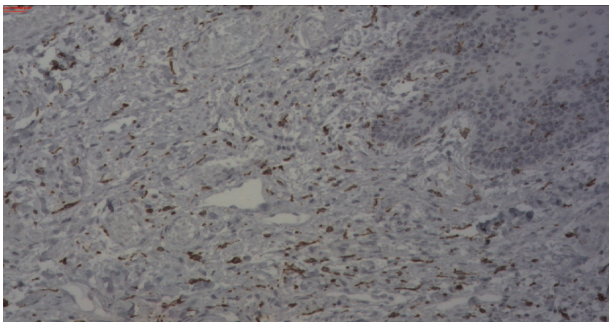

Figure 4. *Immunohistochemical staining of CD68 + TAMs. Low density of CD68 positive tissue macrophages (original 100x magnification).*

Discussion

Significance of infiltration with CAFs and TAMs

The molecular mechanism leading to the expression of immune antigens by the tumor cells is still poorly understood. Several immunohistochemical markers are available for evaluation of TAMs. CD68 is known as a pan-macrophage marker and facilitates the identification of both types of polarized macrophages (M1 and M2)[9]. This cytochemical marker is used for immunostaining of monocytes / macrophages in histochemical analysis of inflamed tissue, tumor tissue, and other immunohistopathological uses because macrophages and other mononuclear phagocytes exhibit strong CD68 expression.

CD68+ expression in TN and TS was found in the first two patients, but a very small number of CD68+ macrophages were found in the third patient who had no cancer in the control sample. The high CD68+ count in patient number one is associated with a higher stage of disease, a larger tumor diameter, poor tumor differentiation, lymphatic invasion, and lymph node metastasis. According to these clinical parameters, poor prognosis for the patient is expected. In the Jeong et al. study for tumor-associated macrophages as potential prognostic biomarkers in invasive breast cancer, the high number of CD68+ macrophages in TN and TS was associated with higher histological grade, larger tumor diameter and metastases. In breast cancer, the high number of CD68+ infiltration is associated with a poorer prognosis, also[8].

CD68 alone, or in combination with other cellular markers, is widely used as a cancer-associated diagnostic and prognostic marker[9]. Tumor cells used to express CD68 also, because metastatic tumor cells express immune markers to avoid macrophage-mediated phagocytosis and cell damage by cytotoxic CD8 + T-cells during invasion of normal, non-tumor tissue. Excessive expression of macrophage antigens in tumor tissue may indicate a pro-metastatic condition and may be associated with a poor prognosis[10], which is consistent with the prognosis of case number one, according to the clinico-histopathological prognostic parameters[7,11].

In cases 1 and 2, CD68 marks a lower density of TAM in the tumor nest (TN) and a higher density in tumor stroma (TS). According to the results of the literature, in endometrial cancer, infiltration with CD68 + TAMs in TN shows a positive correlation with reduced recurrence, while in invasive breast cancer suggests an unfavorable prognosis. If these results are taken into account, recommendations are given to examine TAMs localization in both TN and TS of the malignant tumors[8].

α-SMA reflects the expression of CAFs in the tumor mesenchyme and is the most common marker of CAFs[12].

Normal mesenchymal fibroblasts do not express α-SMA. Our results showed a very high density of SMA+ fibroblasts (CAFs) in case number one, with moderately differentiated squamous cell carcinoma (G2), large tumor size, tumor progression through lymphatic permeation and metastasis, and high degree of malignancy. According to the clinico-pathological parameters, the patient has unfortunately a very poor prognosis[7,11]. A small percentage of CAFs is seen in a patient number two, with a smaller tumor size, absence of lymphovascular permeation and metastases, good tumor differentiation (G1) and the same was in the earlier stage of disease. We found almost no CAFs in healthy oral mucosa in case number three, but their minimal presence may be attributed to a pre-existing cancer in this patient. Identical to our result, the results of the study by Chen et al. indicate a statistically significant difference in the density of CAFs in tissue with nasopharyngeal carcinoma (NPC), normal nasopharyngeal mucosa, and NPC metastases, indicating that CAFs are an important component of tumor stroma that play a role in the growth and development of malignant epithelial tumors and participate in the early stages of tumor preparations for future metastases[2]. This study suggests that the occurrence of α-SMA+ myofibroblasts precedes the onset of invasion and contributes to tumor growth and progression[2,12]. Ibrahim O. Bello's[13] research on tongue cancer has shown that the density of CAFs is associated with the degree of tumor malignancy, tumor growth and progression[13]. Similarly, some studies have shown that stromal CAFs are associated with a higher risk of recurrence and poor prognosis in colorectal and breast cancer[14]. Kellerman's findings are similar, confirming that the higher degree of infiltration with CAFs is associated with more advanced TNM stage and lymph node metastasis[15]. Also, the study by Takahashi et al.[16] reveals that the high rate of infiltration with CAFs correlates with lympho-vascular invasion[16].

CAFs were hardly found in the normal mucosa sample in patient number three, which is consistent with the finding of Fujii et al.[17], where α - SMA+ cells were not found in the stroma of normal oral mucosa specimens or premalignant lesions, except in the smooth muscles of the vessel wall that were used as internal positive control. Findings from his study also show that approximately 60% of OSCCs contain a significant proportion of myofibroblasts and many of them contain myofibroblasts in the deep invasive front of the tumor. Maybe the absence of α - SMA + myofibroblasts in the stroma of the normal oral mucosa in our study is giving us the long-expected answer suggesting that close contact with cancer cells is required to induce myofibroblast transdifferentiation in the invasive tumor front. Most importantly, the study demonstrates that an abundance of myofibroblasts leads to a more aggressive type of SCC including increased proliferative potential[17], which is consistent with our finding in case number one.

The abundant presence of myofibroblasts, especially in the invasive tumor front, is significantly associated with shorter overall survival, so that increased myofibroblast counts may be useful in predicting the prognosis of oral SCC. The study by Sobral et al.[18] demonstrates that myofibroblasts in OSCC stroma are associated with increased tumor aggressiveness, and thus a shorter survival time. CAFs directly facilitate tumor invasion by producing proteases that digest the extracellular matrix (ECM) and produce a variety of pro-invasive molecules. These findings suggest that CAFs promote cancer invasion, resulting in a poor prognosis of OSCC patients. These claims cannot be convincingly refuted in our report because a large number of cases and their follow-ups are required to prove these suggestions wrong or false.

CAFs play a very important role in recruiting and polarizing TAMs[16]. Many studies that have examined the relationship between CAFs and TAMs in the OSCC have found that CAFs primarily induce the pro-tumoral and immunosuppressive phenotype of macrophages[16]. Moreover, the infiltration of CAFs into tumor tissue correlates not only with the number of CD68 + macrophages but also with CD163 + macrophages, indicating CAFs tilt toward the M2 macrophages in the TME. The results of the study provide new insights of the role of CAFs in the immunosuppressive microenvironment of these tumors. CAFs promote immunosuppressive microenvironment by induction and accumulation of pro-tumoral macrophages[16]. In order to increase the effectiveness of immunotherapy, therapeutic strategies that will alter CAFs-mediated immunosuppressive microenvironment should be considered. TAMs that promote key processes in tumor progression such as angiogenesis, immunosuppression, invasion, and metastasis, may potentiate or antagonize the efficiency of anti-tumor cytotoxic chemotherapy, target-antibodies against cancer cells, and immunotherapies. TAMs are also responsible for the reparative mechanisms in the tumor after radiotherapy or treatment with agents that target vascularization[5]. Some studies discuss the biological significance and clinical implications of these findings and emphasize the need of novel therapeutical approaches that effectively target TAMs and improve the outcome[5].

Conclusion

With this report, we determined the presence of CAFs and CD68-positive TAMs in the early and advanced stages of OSCC and determined their absence in the regular oral mucosa. We also pointed out the inter-

connectedness of the CAFs and CD68 + TAMs density with the clinicopathological features of OSCC.

It is becoming increasingly clear that researching the components in TME will help in better understanding of the different responses to antitumor therapy and thus in more precise defining of the target cells for anticancer therapy.

So, as we realize that CAFs and TAMs play a key role in shaping the tumor immunosuppressive microenvironment, and in order to increase the effectiveness of conventional therapies as well as immunotherapies, we suggest that novel cancer therapies in which CAFs and TAMs would be potential targets, should be considered.

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EVALUATION OF ENAMEL SURFACE AND BOND STRENGTH DEPENDING ON THE ETCHING DURATION - an in vitro study

ЕВАЛУАЦИЈА НА ПРОМЕНИТЕ НА ПОВРШИНАТА НА ЕМАЈЛОТ И ЈАЧИНАТА НА ВРСКАТА ПОМЕЃУ ЕМАЈЛОТ И МЕТАЛНИОТ БРЕКЕТ ВО ЗАВИСНОСТ ОД ВРЕМЕТРАЕЊЕТО НА СРЕДСТВОТО ЗА НАГРИЗУВАЊЕ НА ЕМАЈЛОТ – ин витро студија

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Abstract

Introduction: Etching in orthodontics is the most important phase of brackets bonding, and failure to achieve proper bonding may result in poor orthodontic results. Thus, for many years, many attempts have been made to achieve the best etching method in order to maximize orthodontic therapy effect, and on the other hand, to minimize unnecessary re-bonding of failed brackets during orthodontic therapy. Failure to do so leads to prolonged orthodontic therapy including complications like unnecessary checkups at orthodontic office. **Aim of the study:** The impact of different etching duration on enamel surface; the evaluation of bond strength regarding different etching duration; the optimal etching duration for bond strength. Establishing laboratory evidence of the minimum etching duration of enamel to provide effective retention of orthodontic brackets. **Material and methods:** Thirty sound premolar teeth (extracted for orthodontic reasons) were divided into three groups. Each group of teeth was etched for 10, 30 and 60 seconds. Shear bond strength and ARI Index were evaluated. **Results and discussion:** The highest bond strength was found among the group of teeth etched for 30 seconds (9.89 MPa). However similar results were found in the group of teeth etched for 10 seconds (9.44 MPa). The group of teeth etched for 60 seconds exhibited the lowest SBS (8.16 MPa). The larger number of teeth exhibited ARI index 1 (10 teeth). Followed by the equal number of teeth belonging to ARI index 0 and 2. (7 each). Lowest score was recorded with ARI index 3. **Conclusions:** We consider that 10 seconds of etching is suitable in daily orthodontic clinical practice.

Апстракт

Вовед: Нагризувањето на емајловата површина е многу значајна фаза во поставувањето на брекетите од фиксниот ортодонтски апарат, и може да биде причина за неуспех во ортодонтскиот третман поради воспоставување на слаба врска меѓу забот и брекетот и да влијае на јачината на силата. Затоа низа години наназад се прават обиди да се постигне најдобриот метод за нагризување со цел да се подобри ефектот од нагризувањето и да се минимизира непотребното повторување на постапката за врзување на брекетот со емајлот на забот со што се успорува и компромитира текот на ортодонтскиот третман, што вклучува и дополнителни прегледи и пролонгирање на времетраењето на третманот. **Цел:** Да се испита влијанието на различно време на нагризување на емајлот, да се изврши проценка на јачината на врската меѓу брекетот и емајлот на забот во зависност од различното време на нагризување и да се утврди оптималното време на нагризување, преку лабораториски докази за минимално потребно време од нагризување на емајлот како би се обезбедила ефикасна врска помеѓу брекетот и емајлот на забот. **Материјал и метод:** Испитувањето е извршено на 30 здрави премолари (екстрахирани од ортодонтски причини, во цел екстракциона ортодонтска терапија) поделени во три групи. Кај секоја група беше изведено нагризување во траење од 10, 30 и 60 секунди. Извршена е проценка на трите различни времетраења на нагризување на емајлот врз јачината на врската на емајлот и брекетот и беше одреден ARI индексот. **Резултати и дискусија:** Најака врска помеѓу емајлот на забот и брекетот е пронајдена кај втората група на заби нагризувани со киселина 30 секунди (9,89 MPa). Сепак слични резултати најдовме кај првата група заби нагризувани 10 секунди (9,44 MPa). Групата заби нагризувани 60 секунди покажа најнизок степен на јачина на врската (8,16 MPa). Кај поголемиот број на заби ARI индексот беше 1 (10 заби). Проследено со ARI индекс од 0 до 2 по 7 заби. Најмал број на заби покажаа резултат на индексот ARI 3. **Заклучоци:** Според добиените резултати најдобро и оптимално време на нагризување е 10 секунди кое е истовремено и најсоодветно за примена во секојдневната ортодонтска пракса.

Introduction

Etching in orthodontics is the most important phase of brackets bonding, and failure to achieve proper bonding may result in poor orthodontic results. Thus, for many years many attempts have been made to achieve the best etching method in order to maximize orthodontic therapy effect and in other hand to minimize unnecessary re-bonding of failed brackets during orthodontic therapy. Failure to do so leads to prolonged orthodontic therapy including complications like unnecessary checkups at orthodontic office.

The enamel is the hardest and most highly mineralized substance in the human body. The makeup of enamel (by weight) consists of 96% minerals, 3% water, and 1% organic material, such as proteins. The majority of the mineral content is calcium phosphate in carbonated hydroxyapatite crystals. These highly oriented crystals are extremely long and contain over 1000 times the volume of similar crystals in bone, dentin, and cementum. The crystals are organized into bundles known as prisms, about 4μm in diameter, and extend outward from the dentin surface.

The process of creating enamel is called amelogenesis. Amelogenesis is genetically controlled process, therefore the size, shape, caries susceptibility, and even shade can vary from person to person.

The creation of the enamel etch pattern, rendering a tooth more susceptible to adhesion, requires a strong acid. The acid removes a small amount of interprismatic enamel creating a porous surface, thus increasing the total bonding surface area and allowing adhesion promoters to penetrate into enamel pores and ultimately results in secure micromechanical retention.

Etching dissolves hydroxyapatite crystals and provides micromechanical retention by allowing penetration of adhesion promoters and development of resin tags during bonding[1].

Etching enamel surfaces with phosphoric acid (H3PO4) is an accepted and widely applied technique to improve bonding of dental resins to enamel in restorative dentistry, in preventive dentistry, and for direct or indirect bonding of orthodontic attachments[2].

The topography of the etched surface enamel, the etching duration, and the concentration of the etchant could also be important factors influencing bond strength.

Many studies report that there is a significant difference of shear bond strength of brackets bonded to enamel comparing to brackets bonded to teeth with cavities that have been filled with different composites[3].

The chemical etching of tooth enamel with phosphoric acid was discovered by Buonocore in 1955[4]. He demonstrated increased adhesion produced by acid pretreatment of enamel using 85% phosphoric acid for 30 seconds. This led to dramatic changes in the practice of orthodontics[5].

In 1973, Retief reintroduced the idea of etching and bonding teeth with improved composites that significantly reduced shrinkage and microleakage[6]. This new method of bonding provided strength suitable for bonding orthodontic brackets to teeth.

Despite the discovery of different etchant materials, phosphoric acid remains the gold standard[7].

Good orthodontic practice is essential to correct malocclusion and the use of safe and reliable orthodontic accessories are desirable. Accidental bracket debonding is a frustrating inherent aspect of orthodontics, resulting in a longer treatment and additional cost for materials and service[8].

Besides the optimal acid concentration, etching duration plays a major role in effectively bonding brackets. Considering the fact that the acid applied to tooth causes the roughening of the enamel surface, it is very important to achieve ideal etching duration which will produce maximum bonding strength of bracket and, on the other hand, cause minimum damage to the etched enamel surface.

According to previously reported literature, adequate shear bond strength for orthodontic bonding should be from 5.6 to 7.8 MPa[9].

Etching time should never be underestimated as one of the major facts in orthodontic treatment.

The initial recommended acid etching duration was 60 seconds[10]. Further research demonstrated etching duration of 15 to 20 seconds was equally effective. Etching duration should vary according to the clinical situation.

Buonocore (1955) recommended etching for 30 seconds, but after the detailed fissure sealant studies of Silverstone, (1974) a one-minute exposure to acid became the accepted duration for all applications of acid etching technique. More recent studies of bond strengths, particularly in relation to orthodontic brackets, have suggested that the failure rate of bonds is unaffected by reducing the etching time.

Barkmeier et al. found neither qualitative differences in the enamel surface structure nor differences in the bond strength after etching for 15 or 60 seconds with a 50% phosphoric acid. However, the study did not use wide range of etching duration nor did it count the percentage of bond failure interface distributions.

Many people need to wear fixed orthodontic devices, such as braces, to correct problems with teeth and jaw (e.g. overcrowding or front teeth that stick out (protrude) or go too far backwards (retroclined)). The manner in which these braces are fixed in place will be of interest to them. In order to attach an orthodontic device, such as a brace, to a tooth, the surface of the appropriate tooth first needs to be prepared so that it can retain the glue or bond-

ing agent used to for safely attaching the device. For the past 50 years, the usual manner of doing this was etching (roughening) the surface of the tooth with acid, commonly phosphoric acid, although maleic acid or polyacrylic acid are also used sometimes. Possible harms of etching include permanent loss of enamel (hard surface) from the surface of the tooth, making it more likely for it to lose calcium or weaken during and after treatment. Recently, to reduce the length of time and complexity of the process, a technique using self-etching primers (SEPs) has been developed as an alternative to conventional etchants or acids. However, it remains to be determined whether SEPs or conventional etchants are better, and which is the best SEP, acid, concentration and etching duration[11].

Cleaning procedure of tooth surface before etching and bonding orthodontic elements

Organic pellicle layers on the enamel surface cannot be completely removed by brushing. This organic pellicle layer has been reported to reduce the bond strength between the adhesive resin on the base of the bracket and the tooth[12]. To prevent this, polishing is recommended before performing the bonding procedure. For the polishing procedure, using polishing brushes, disposable rubber cups or sterilization available with low-speed (lower than 20000 rpm) micromotors and non-fluoride pastes for 10 seconds, is recommended[13]. Enamel loss of 5-14 μm in depth was reported as a result of the type and application time of the rubber cups or polishing brushes.

Phosphoric acid application on the enamel surface of the tooth

Phosphoric acid is used to eliminate oxidation of metal surfaces and enhance adhesion of dyes to metal surfaces in metal and dye industry[14]. Regarding this information, the first steps in etching tooth surface where made, in order to gain better conditions for bonding of orthodontic brackets.

Etching for 15-30 seconds is accepted as the optimum working time by manufacturers and clinic[15,16,17,18,19,20,21].

Aim of the study

The orthodontic treatment depends on many factors. Proper bonding of orthodontic brackets is one of the most important stages during orthodontic treatment. Enamel etching and shear bond strength of orthodontic brackets are the main objective of our research.

Therefore, the aims of our in vitro study will be:

- The impact of different etching duration on enamel surface
- The evaluation of bond strength regarding different etching duration
- The optimal etching duration for bond strength

Material and methods

Thirty sound premolar teeth (extracted for orthodontic reasons) were included in this study. The premolar teeth can be from the upper or the lower jaw, first or second ones, randomly selected. The selection criteria for the collected teeth will be as follows: complete root development, caries free, no fractures or enamel hypoplasia. The teeth will be collected from University Clinical Dental Center of Kosova – Department of Oral Surgery, and Dental Office "Vitadent".

Collected teeth were properly stored until the research. Teeth were stored in 0.9% NaCl.

Teeth were placed into silicon cups of chemical-cure acrylic resin. The container was filled with acrylic up to the cemento-enamel junction to simulate bony support of natural teeth.

Premolar teeth were divided into three groups, each group containing ten premolars. First group of teeth was etched for 10 seconds, the second one for 30 seconds and the third one for 60 seconds. Etching was conducted with 37% phosphoric acid used for standard etching procedure in orthodontics when brackets are bonded. Dentaurum ConTec Go 37% phosphoric acid etching gel was used for etching.

All etched teeth were washed by air water spray for 15 seconds, and dried with oil free air syringe.

The brackets were fixed on the labial surface of the teeth. The brackets were fixed with one component "no mix" bracket adhesive in syringes. Orthodontic bonding system Dentaurum ConTec Go adhesive was used. The type of brackets used for this study was Dentaurum "discovery" brackets, System Roth 22.

Shear bond strength of orthodontic brackets was tested on universal testing machine. The tests were performed at the Faculty of Mechanical Engineering – University of Prishtina. Shear bond strength was calculated in Mega Pascals (MP). After debonding the orthodontic bracket, the remaining resin on the buccal surface was evaluated for Adhesive Remnant Index (ARI).

Results and discussion

Shear bond strength (SBS) and ARI scores are presented in Table 1. The highest bond strength was found

among the group of teeth etched for 30 seconds (9.89 MPa). However similar results were found in the group of teeth etched for 10 seconds (9.44 MPa). The group of teeth etched for 60 seconds exhibited the lowest SBS (8.16 MPa).

According to previously reported literature, adequate SBS for orthodontic bonding should be from 5.6 to 7.8 MPa[22]. According to these findings, all three groups of our study showed sufficient shear bond strength. Similar findings were reported by Barkmeier et al. (1985) that tested shear bond strengths on human premolars after etching for either 15 or 60 seconds. No significant differences were found between the treatment groups. Although interpretation is being complicated somewhat by the fact that the enamel surfaces were ground-flat before etching. Braannstrom and Nordenval found no apparent difference between 15 and 120 second etching duration with 37% phosphoric acid; however, the effect of a shorter etching time was not thoroughly investigated. Nordenvall et al. conducted serial studies of different etching duration on deciduous and young, and old permanent teeth, and found than 15 seconds of etching created a more retentive condition than 60 seconds in young permanent teeth. They used the degree of surface irregularities as an indicator for the quality of mechanical retention. Therefore, it did not indicate the absolute bonding strength. Carstensen (1986) studied the clinical failure rate of mesh-backed metal brackets on 1134 anterior teeth, after etching for 30-35 seconds with 37% phosphoric acid. Only 10 brackets were lost during the 16-month study period. The failure rate was twice as high in the maxilla as in the mandible, although the difference was not statistically significant. In a second study, a comparison was made between the effects of etching for 15-20 and 30-35 seconds. Only two brackets failed out of the 90 bonded after each etching time, these were both in the maxillary arch and after 15 seconds of etching. It was concluded that a 15-second etching time was sufficient for bracket bonding on anterior teeth[23].

It has been previously reported that etching of less than 10 seconds and more than 60 seconds do not produce enough shear bond strength[24,25]. Ten seconds of etching does not produce enough tagged areas on the enamel, and etching of 60 seconds or more than 60 seconds impairs the integrity of honeycombed prismatic structures on the enamel, which negatively affects bond strength. For the protection of dental structures, a topical fluoride application is generally preferred. It is reported that no additional etching time is required for fluoride applied teeth before treatment[26,27,28]. These results does not match our findings, since in our study 10 seconds of etching provided sufficient bond strength.

However, it is well known fact that the longer etching duration, the higher enamel damage is recorded. This means that the most favorable etching duration in our study was the shortest time of etching (10 seconds) since it provided suitable SBS (9.44 MPa) with minimum enamel damage. According to literature, reports regarding

Table 1. Representing bond strength (MPa) and ARI scores of the tested teeth

Tooth	10 sec		30 sec		60 sec	
	MPa	ARI	MPa	ARI	MPa	ARI
1	8.0	0	10.5	3	9.2	1
2	9.4	1	9.2	2	8.4	1
3	9.9	2	11.4	3	6.9	0
4	10.2	3	12.1	3	8.4	0
5	9.5	1	10.4	3	7.3	0
6	9.7	2	8.0	1	9.4	2
7	10.4	3	9.3	1	8.3	1
8	8.9	0	9.9	2	7.7	0
9	9.4	1	10.4	2	6.9	0
10	9.0	1	7.7	1	9.1	2
X	9.44		9.89		8.16	
Range	8.0-10.4		7.7-12.1		6.9-9.2	

the optimal shear bond strength of the bracket to the enamel which is expected to prevent bracket debonding during treatment, while not causing enamel damage during debonding and keeping the enamel intact after treatment[29].

Regarding ARI score findings in Table 1 among groups of teeth with different etching duration, results revealed that there was a correlation between SBS and ARI score. This means that the highest ARI scores were recorded in teeth with high bond strength. This is in line with literature references which demonstrated that higher shear bond strength values are associated with high amounts of remnant adhesive on enamel surface[30].

Table 2 represents ARI (Adhesive Remnant Index) according to different etching duration. The larger number of teeth exhibited ARI index of 1 (10 teeth). Followed by the equal number of teeth belonging to ARI index of 0 and 2. (7 each). Lowest score was recorded with ARI index of 3.

Similar reports with findings of our study regarding ARI index can be found in the literature review. Hence, the majority of the ARI scores were 0 and 1, with brackets presenting a greater number of bond failures at the enamel/adhesive interface. Although this interface is considered dangerous due to the risk of damaging the enamel surface, no damage in teeth was observed after debonding[31].

Table 2. ARI scores according to different etching duration (10 sec, 30 sec, 60 sec)

ARI	10 sec	30 sec	60 sec	Total
0	2	0	5	7
1	4	3	3	10
2	2	3	2	7
3	2	4	0	6

Conclusion

Sufficient shear bond strength was found among the group of teeth etched for 10 seconds only. Also, the lowest ARI Index was recorded at the same group of teeth. Therefore, we consider that etching for 10 seconds is suitable in daily orthodontic clinical practice.

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THE CONNECTION BETWEEN ORAL HEALTH AND SALIVARY LEVELS OF TOTAL PROTEINS, CALCIUM AND PHOSPHATES

ПОВРЗАНОСТ НА ОРАЛНОТО ЗДРАВЈЕ И САЛИВАРНИТЕ НИВОА НА ВКУПНИТЕ ПРОТЕИНИ, КАЛЦИУМОТ И ФОСФАТИТЕ

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Abstract

Aim of the study: To determine the association between oral hygiene and the values of total protein, calcium. and phosphate in the saliva in young, healthy population. **Material and methods:** The research was conducted on 41 respondents of both sexes, aged 19-24 without pathological changes in the oral cavity. Unstimulated saliva was collected using the spitting method into graduated test tubes for 5 min. The samples were then centrifuged at 4000 rpm for 20 minutes. The level of protein in the samples was determined using the Biuret method, the concentration of Ca was determined with Calcium-cresolphthalein, and phosphate concentration was determined with phosphomolybdate/UV. The concentrations were determined spectrophotometrically with UltroSpec 1000. The index of dental plaque, gingival inflammation and dental calculus were clinically noted, indicating the level of oral hygiene in each individual. The obtained data from the examinations were statistically processed using the Pearson's correlation coefficient. **Results:** We detected a weak correlation between the index values of the dental plaque and total salivary protein p=0.000 and p=0.003, respectively. Weak positive correlation was noted between index values of dental calculus and salivary calcium p=0.000, and of calculus and salivary phosphate p=0.015. **Conclusion:** The results showed that there is a connection between gingival inflammation, dental calculus and values of salivary protein, calcium, and phosphate in the saliva of this group of respondents. **Keywords:** saliva, salivary proteins, calcium, phosphates, oral health.

Апстракт

Цел: да ја утврдиме поврзаноста помеѓу оралното здравје (хигиена) и вредностите на вкупните протеини, калциумот и фосфатите во плунката кај млада, здрава популација. **Материјал и метод:** спроведено е испитување на 41 испитаник од двата пола, на возраст од 19 до 24 год. без патолошки промени во оралната празнина. Колекционирана е нестимулирана плунка со методот на исплукување во градуирана епрувета во тек на 5 мин. Примероците беа центрифугирани на 4000 вртежи во тек на 20 минути. Нивото на вкупните протеини во примероците беше одредувано со помош на Модифицирана Биуретска метода. Концентрацијата на Са во плунката ја одредувавме со Calcium-cresolphthalein. Саливарните фосфати ги определувавме со phosphomolybdate/ UV. Концентрациите беа одредени со спектрофотометарот UltroSpec 1000. Клинички ги нотиравме индексот на дентален плак, гингивална инфламација и забен камен, кои укажуваат на степенот на орална хигиена кај секоја индивидуа. Добиените податоци од лабораториските и клиничките испитувања беа статистички обработени, беше користен Pearson-овиот коефициент на корелација. **Резултати:** Утврдивме позитивна корелација помеѓу индексните вредности на ГИ и денталниот плак со вкупните саливарни протеини за p=.000 и p=.003 соодветно. Слаба позитивна корелација нотиравме помеѓу индексните вредности на калкулусот и вкупниот саливарен калциум за p=0.000, како и на калкулусот со саливарните фосфати p=0,015. **Заклучок:** резултатите покажаа дека постои поврзаност помеѓу гингивалната инфламација и денталниот калкулус и вредностите на саливарните протеини, калциумот и фосфатите во плунката кај оваа група испитаници. **Клучни зборови:** салива, саливарни протеини, калциум, фосфати, орална хигиена.

Introduction

The oral cavity is a specific ecosystem with many functions, where saliva as a biological fluid plays a major role in maintaining oral homeostasis and oral health. Saliva, as a secretory product of the large and small salivary glands, modulates this ecosystem, primarily through constant rinsing of the teeth and oral mucosa, protection against microorganisms, participation in mastication, taste perception, prevention of oral infections and dental caries[1,2,3]. Saliva consists of organic, inorganic material and macromolecules. Inorganic substances, such as calcium and phosphate ions, are involved in the formation of the dental tissues, dental calculus, but also in the protection against caries. Salivary proteins have a protective, antimicrobial and lubricating function (mucin) that provides a

barrier between toxins and soft oral tissues. Salivary proteins modulate the activity of calcium and phosphate ions. Saliva is an ionic reservoir of calcium and phosphates necessary for mineralization of initial carious lesions[4]. Through constant rinsing of the oral cavity, saliva plays a huge role in maintaining oral hygiene and oral health.

This study aims to determine the connection between oral health (oral hygiene) and salivary levels of total proteins, calcium, and phosphate in young healthy population.

Material and methods

The research was conducted with 41 young healthy respondents of both sexes, aged 19-24, students at the Faculty of Dental Medicine within UKIM, without pathological changes in the oral cavity. The study included those without systemic diseases and those who had not used certain medications in the last three months. Unstimulated saliva was collected from all respondents using the spitting method according to the recommendations proposed by Navazesh[5]. The subjects were spitting in polypropylene, graduated test tubes for 5 min. The tubes were placed in an ice-filled glass container to prevent protein breakdown. Students were advised not to consume food and drinks for at least 90 minutes before saliva collection. Before collecting, they first thoroughly rinsed their mouths with deionized water, then they were comfortably placed in a sitting position with their head slightly tilted forward and proceeded to spit. The collected saliva samples were briefly vortexed in a Vortex apparatus for about 1 min and then centrifuged at 4000 rpm for 20 minutes.

The level of total protein in the samples was determined using the Modified Biuret method, by measuring the absorbance of the test sample. The concentration of Ca in saliva was determined with Calcium-cresolphthalein. The principle is based on the reaction of the calcium from the analytical sample with o-cresolphthalein from the Biuret solution forming a red colored-complex that is measured spectrophotometrically[6].

Salivary phosphates were determined with phosphomolybdate/UV because inorganic phosphates from the saliva samples react with molybdate from the solution in acidic pH, thus forming phosphomolybdate-complex, which can be measured spectrophotometrically. The concentrations were determined with the UltroSpec 1000 spectrophotometer apparatus.

The index of dental plaque, gingival inflammation and dental calculus were clinically noted, indicating the level of oral hygiene in each individual. The obtained data from the laboratory and clinical examinations were statistically processed using Pearson's correlation coefficient.

Results

41 respondents participated in the study, 12 (29.27%) of the respondents were male, while 29 (70.73%) were female. Regarding the representation of gender, we registered a statistically significant difference of $p<0.05$ between the representation of males in relation to females (Table 1).

Table 1. Representation of the respondents in relation to gender

	n	Percent %
female	29	70.73171
male	12	29.26829

Table 2. Average age of respondents included in the study

	Average age	Min.	Max.	Std.Dev.
Age	19.95122	19.00000	24.00000	0.835201

Table 4. Distribution of the respondents according to smoking habits

	n	%
Non - smokers	26	61.90476
Smokers	15	35.71429

Table 3. Average values of the examined salivary components

	average	min.	max.	Std.Dev.
Amount of saliva excreted (ml/min)	0.358537	0.150000	0.700000	0.150276
Proteins (mmol/L)	1.902927	0.100000	3.960000	0.922392
Calcium (mmol/L)	1.092439	0.210000	6.710000	1.035939
Phosphates (mmol/L)	3.110976	1.250000	6.440000	1.241102

Table 5. Distribution of respondents according to the values of the indices of oral hygiene and gingival inflammation

	Dental calculus		Gingival inflammation		Dental plaque (biofilm)	
Index values	n	Percent %	n	Percent %	n	Percent %
0	9	21.95122	4	9.75610	/	/
1	15	36.58537	14	34.14634	22	53.65854
2	13	31.70732	18	43.90244	17	41.46341
3	4	9.75610	5	12.19512	2	4.87805

Table 6. Average values of total salivary proteins in smokers and non-smokers

mmol/L	Non-smokers	Smokers	t-value	df	p
Proteins	2.144231	1.484667	2.323465	39	0.025459

Table 7. Difference between average values of total salivary proteins in males and females

mmol/L	female	male	t-value	df	p
Proteins	1.942069	1.808333	0.418025	39	0.678221

Table 8. Difference between average salivary calcium values in smokers and non-smokers

	Non-smokers	Smokers	t-value	df	p
Calcium	0.832308	1.543333	-2.21817	39	0.032436

The average age of the respondents was 19.95 ± 0.83. The youngest respondent was 19 years old, and the oldest respondent was 24 years old. (Table 2)

In our study, most of the respondents 26 (61.9%) were non-smokers and 15 (35.7%) were smokers. (Table 4)

The obtained results regarding the presence of dental calculus showed that in 9 respondents was noted index 0 (21,95%), in 15 was noted index 1 (36,58%), in 13 respondents was present index 2 (31,70%), and in 4 subjects was observed index 3 (9,75).

Regarding the presence of gingival inflammation, the results showed that in most of the participants, 18 (43,90%) was present index 2, and in 14 (34,14) index 1 of gingival inflammation. In only 4 subjects (9,75%) we did not register inflammatory changes in the gingiva, and in 5 (12,2%) we noted index 3 of gingival inflammation.

Regarding the obtained results for presence of dental plaque, we noticed that none of the participants in the study had an index 0, in 22 (53,65%) subjects we registered index 1, in 17 index 2 (41,46%), and only in 2 participants (4,88)- index 3 dental plaque. (Table 5.)

We registered a statistically significant difference for p=0.025459 between the average values of total salivary proteins in smokers and non-smokers. (Table 6.)

We did not register a statistically significant difference (p=0.678221) between the average values of total salivary proteins between male and female respondents. (Table 7.)

We registered a statistically significant difference for p=0.032436 between the average values of calcium in smokers and non-smokers. (Table 8.)

Table 9. Difference between average salivary calcium values in males and females

	male	female	t-value	df	p
Calcium	0.89069	1.58000	-2.01097	39	0.051278

Table 10. Difference between average salivary phosphate values in non-smokers and smokers

	Non-smokers	Smokers	t-value	df	p
Phosphates	2.906923	3.464667	-1.40268	39	0.168624

Table 11. Difference between average salivary phosphate values in males and females

	male	female	t-value	df	p
Phosphates	3.030345	3.305833	-0.641916	39	0.524683

There was no statistically significant difference between average salivary calcium values in males and females (p= 0,051) (Table 9.)

We did not observe a statistically significant difference for p = 0.168 between the average salivary values of phosphates in smokers and non-smokers. (Table 10.)

We have not determined a statistically significant difference between the average values of phosphates in saliva in male and female respondents. (Table 11.)

Table 12. Correlation between index values of examined clinical parameters and total salivary proteins

	Total proteins
Dental calculus	r = - 0.1873
	p = 0.241
Gingival inflammation	r = 0.7691
	p =.000
Dental plaque (biofilm)	r = 0.4482
	p =.003

The results show presence of positive correlation between total salivary proteins and index of gingival inflammation (r= 0,76 p =0.000), and presence of weak positive correlation between index of dental plaque and salivary proteins (r =0,44; p =0,003). (Table 12.)

The Pearson correlation index indicates that there is a positive correlation between salivary calcium and index of dental calculus (r =0,53 p =0,000). (Table 13.)

Table 13. Correlation between index values of clinical parameters and salivary calcium

	Calcium
Dental calculus	r = 0.5258
	p = 0.000
Gingival inflammation	r = 0.2333
	p = 0.142
Dental plaque (biofilm)	r = 0.2386
	p = 0.133

Table 14. Correlation between index values of clinical parameters and salivary calcium

	phosphates
Dental calculus	r = 0.3763
	p = 0.015
Gingival inflammation	r = 0.1893
	p = 0.236
Dental plaque	r = 0.1333
	p =.406

We found the presence of a positive correlation between dental calculus index and salivary phosphates (r=0,36; p=0,015) (Table 14.)

Discussion

The average amount of saliva excreted in our subjects is 0.358 ml /min, which is within the normal values of salivation in most of the population 0.33-1.42ml/min[7]. The results showed that there were no significant differences in the amount of unstimulated saliva between smokers and non-smokers. We believe that this is due to the age of the respondents, young individuals, short "experience" of smoking and preserved function of the salivary glands, as well as the small number of smokers who participated in the study – 15 of the subjects are smokers[8].

Regarding the total salivary proteins, we noticed a significant difference between smokers and the group of non-smokers for p = 0.02, i.e. higher protein concentration in the saliva of non-smokers 2.144 mmol /L. In terms of gender, there was no significant difference between the two groups of respondents (p = 0.678221). (Table 6)

The concentration of the various components of saliva is significantly affected by variations in saliva secretion. Hormonal factors, external influences and systemic diseases have a significant influence on these variations in the composition of saliva. Literature data indicate that smokers have a change in saliva quality. Most of them have thick, sticky saliva. It has been proven that due to the harmful effects of nicotine, the parotid salivary glands that secrete serous saliva suffer first, and the loss of their function is compensated by the submandibular and sublingual salivary glands that secrete predominantly sero-mucous and mucous secretions. This explains the presence of thick saliva in smokers. Harmful substances from cigarette smoke destroy the protective macromolecules of saliva, enzymes, and proteins, therefore it loses its protective role[9,10].

The results showed that there is a significant difference in the presence of calcium in the saliva between smokers and non-smokers (p=0.032), i.e. more Ca was found in the saliva of smokers (1,543 mmol / l) (tab.8). We did not determine a significant difference in the presence of Ca in the saliva of the respondents in terms of gender (p=0,051) (Table 9).

Based on the obtained results, we did not find significant differences in the average values of phosphates in saliva, neither between the group of smokers and non-smokers (p=0.168), nor in terms of gender (p =0.524683). (tab. 10 and tab.11)

We found a positive correlation between the level of total protein in the saliva of our subjects and gingival inflammation r=0.76 (p=0.000), as well as a positive correlation between total protein and dental plaque r=0.44 (p=0.003) (Table 12). We believe that the presence of gingival inflammation causes an increase in total protein in saliva. Gingival inflammation causes vasodilation and increased transudation of fluid and proteins from the blood vessels of the gingiva into the gingival fluid, thus increasing the amount of protein in the saliva. Some studies suggest that total salivary protein levels increase in individuals with gingivitis and periodontitis[11]. The main factors influencing the concentration of protein and the protein composition of the saliva are salivary flow, the presence of proteins originating from the salivary glands themselves, as well as those originating from the gingival fluid. The increased protein levels may be due to local synthesis and secretion by the glands themselves. Additionally, glandular-derived proteins, Cystatin C as well as amylase, which are significantly increased in patients with periodontitis, confirm the glandular origin of these proteins[12].

The increase of salivary albumin also plays an important role in the increase of total proteins. Albumin is detected as a minor component in whole saliva (parotid, submandibular and sublingual), but a significant increase in its concentration has been observed in patients with gingivitis and periodontitis[11]. Changes in the concentration of endogenous immuno-reactive proteins such as cystatin and secretory IgA, as well as increased gingival fluid secretion, have been reported in these patients[12,13]. This may indicate that gingival bleeding, and the amounts of GCF derived proteins or immuno-reactive proteins may be one of the causes of oral malodor, because of increased levels of sulfur components[14]. It was reported that the decrease in salivary flow could also result in increased protein concentration. Lower flow rate of saliva secretion could affect oral conditions by leading to increased accumulation of dental plaque and bacteria[15] which can affect oral health.

Gingival fluid is a physiological fluid, but also an inflammatory exudate originating from the blood vessels of the gingival plexus in the gingival corium. As it traverses through the inflamed periodontal tissues to the sulcus, important biological molecular markers are gathered from the surrounding tissues and dissolved into the saliva[16].

The hypothesis that periodontal pathogens induce inflammatory responses resulting in increased levels of salivary albumin and total protein is well known. P. gingivalis, P. intermedia and T. denticola are predominantly present in patients with periodontitis. T. denticola increased the levels of salivary albumin and total proteins as the proteins existing in the periodontal pocket, including immunoglobulins and albumin as potential energy sources for T. denticola. Hollman and Van Der Hoeven[18] have reported that degradation of albumin by T. denticola was not detected but suggested that T. denticola occurs in close association with strong proteolytic bacteria such as P. gingivalis and T forsythia in the subgingival plaque. Thus, controlling the microbes in turn decreases the

inflammatory response, which in turn decreases the plasma leakage in the saliva through GCF.

The presence of Ca, phosphates, and other inorganic ions, especially fluoride in saliva, provides an important protective role and maintains the integrity of dental tissues. The medium rich in Ca and phosphates allows the mineralization of the initial carious lesions and the demineralized areas of the enamel. The balance between demineralization and mineralization depends on the saturation of saliva with calcium and phosphates, saliva flow, its pH, stimulated or unstimulated saliva, and the level of salivary alkaline phosphatase. Salvolini[19] studied 100 healthy subjects of both genders, aged 10-80 years, and suggested that the concentrations of calcium and phosphorus were not affected by age.

The average values of the concentration of calcium in saliva in physiological conditions at healthy individuals is 8.8 - 10.5 mg/dL, but these values vary in stimulated and unstimulated saliva. Gauri, Nagarajappa et al.[20] in their studies found a low mean Ca value of 5.87 mg/dL in unstimulated saliva, and after stimulation, the Ca concentration values increased to 7.17 mg /dL. Jarvinen[21,22] describes similar findings in his study, low values of calcium and phosphate concentrations in unstimulated saliva, which he explains with the presence of a small amount of unstimulated saliva.

Vogel[23] indicates that unstimulated saliva is well-saturated with phosphates, but a rapid decrease in their concentration is observed during stimulated secretion (eg. chewing gum).

In our study, we found the presence of a moderate positive correlation between Ca in saliva in our subjects and dental calculus r=0.52 (p=0.000). (Table 13). We also found presence of a weak positive correlation between salivary phosphates and dental calculus in our subjects r=0.37 (p=0.015) (tab 14).

This means that increased concentrations of Ca and phosphates in saliva lead to increased presence of dental calculus. We believe that the saturation of saliva with these ions causes apposition of these minerals in the present dental biofilm, especially in patients who do not maintain proper oral hygiene, which leads to calculus formation. Mineralization can begin as early as 24-48 hours. Calcification starts in separate foci on the inner surface of plaque, these foci of mineralization gradually increase in size and coalesce to form a solid mass of calculus[24]. It is a well-known fact that dental calculus itself is not an including agent for pathological changes that occur in gingival tissues; instead, it is covered with a layer of unmineralized plaque which is proven to be the key etiological agent involved in these pathogenic mechanisms. Dental calculus plays an instrumental role in further deterioration of the periodontal disease and oral health disorders. On the other hand, reduced concentration of calcium ions can cause demineralization and carious lesions of the hard tooth structures and the possibility of accumulation of dental plaque in those places[24].

Phosphorus is the second most important mineral in the human body after calcium. About 80%-90% of phosphorus is present in the bones and teeth in the form of hydroxyapatite and the rest is present in the extracellular fluid (ECF), soft tissue and erythrocytes. In living tissue, phosphorus exists in the form of phosphate (PO4-3). According to the theory of mineral precipitation on the formation of calculus, calcification will occur when the pH, calcium, and phosphate saliva concentrations are high enough to cause precipitate calcium phosphate salts[25]. Prashaanthi N. et al in their study report that patients with periodontitis have higher level of calcium and phosphates in saliva than healthy subjects[25]. Other authors[26] in their study report that supersaturation of saliva with respect to calcium phosphate salts is the driving force of calculus formation. They found a positive correlation between the mean values of salivary calcium concentration with the amount of calculus formation. However, the increased concentration of phosphate ions and the presence of bicarbonate in the stimulated saliva have an influence on the regulation of acid-base balance and may stimulate the process of mineralization of tooth structures.

Conclusion

The results of this study show that there is a connection between gingival inflammation, dental calculus and the values of salivary proteins, calcium, and phosphate in saliva in this group of respondents. Saliva is the most available and non-invasive bio-fluid of the human body, which permanently rinses the oral cavity while trying to cope with an ever-changing milieu. Saliva is critical to the preservation and maintenance of oral health, and any changes in its amount or quality may alter the oral health status.

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