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Research Araştırma

Biofilm Formation on Titanium Implants, 24h in situ

Titanyum İmplantlar Üzerinde Biyofilm Oluşumu, in situ 24 saat

ABSTRACT

Aim: Evaluation of the bacteria adhesion on polished and acid etched titanium surfaces at 24 h in situ biofilm formation. According to the nature of Ti implant surface; determine the amount of surface adhesion of microorganisms and evaluate the effect of surface roughness.

Material and Method: A total of 2 different type of Titanium specimens as polished and acid etched surface in nine patients whose healthy that man and woman, aged between 24 and 71 years, were included in our study. All subjects had been on their regular daily diet with no excessive consumption of polyphenolic beverages and foods and had no history of smoking or alcohol. The surface were evaluated by Scannig electron microscopy and Fluorescence microscopy.

Results: Ti surface showed that acid treated surface provide enhanced bacterial adhesion and growth, polished surface decreased enhanced bacterial adhesion and growth 24 in situ which can be interesting for various applications in medical fields as well as in biosciences. At the point when contrast and in vitro and in situ research facility models for explore biofilm arrangement, in situ model is the best as the effect of concoction operators on biofilm advancement.

Conclusion: The modifications of micro topography contribute to an increase in surface area, especially surface roughness. This study has shown that surface roughness improves correctly with bacterial adhesion. Significant surface roughness on Ti implant played an important role in providing effective surface for bacteria-implant contact, biofilm formation, despite having good mechanical properties.

Key words: Titanium, Acid etched, Polished, Biofilm, in situ.

ÖZ

Amaç: Cilalanmış ve asit ile muamele edilmiş titanyum yüzeylere bakteri yapışması ve biyofilm oluşumu değerlendirilmiştir. Ti implant yüzeyin özelliğine göre; mikroorganizmaların yüzeye tutunma miktarını belirlemektir ve yüzey pürüzlülüğünün etkisini değerlendirmektir.

Gereç ve Yöntem: 24- 71 yaş arası sağlıklı erkek ve kadınlardan oluşan, 9 kişilik gönüllü grubu için, cilalanmış ve asit ile pürüzlendirilmiş 2 farklı çeşit Ti numunesi çalışmamızda kullanıldı. Çalışmaya katılan tüm gönüllülerin sigara ve alkol öyküsü olmaksızın, aşırı miktarda polifenolik içecek ve gıdaları tüketmeden, günlük düzenli beslenme yöntemlerine devam etmişlerdir. Yüzey Scannig elektron mikroskobu ve Flouresence mikroskobu ile değerlendirildi.

Bulgular: Ti yüzeyi, asit ile muamele edilmesi; yüzeye bakteri yapışması ve büyümesini arttırdığı, cilalı yüzeyin, bakteri yapışmasını ve in situ büyümeyi azalttığını, bunun tıbbi alanlardaki ve biyosistemlerdeki çeşitli uygulamalar için ilginç olabileceğini gösterdi.

Sonuç: Mikrotopografinin modifikasyonları yüzey alanındaki artışa katkıda bulunur, özellikle yüzey pürüzlülüğü. Bu çalışma, yüzey pürüzlülüğünün bakteri tutunması ile doğru oranda geliştiğini göstermiştir. Yüzey pürüzlülüğü, Ti implantın, iyi mekanik özelliklere sahip olmasına rağmen, bakteri-implant teması, biyofilm oluşumu için etkin yüzey sağlamada önemli bir rol oynamıştır.

Anahtar sözcükler: Titanyum, Asit pürüzlendirilmiş, Cilalı, Biyofilm, in situ.

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INTRODUCTION

The surface control is the most important situation for implant studies. The aim of surface treatment that include topography and coatings for the best osseointegration and compatibility of biomedical implants created to purpose for the lifetime of the individual. There are several papers and patents including in different techniques to reproduce the surface such as roughness, polished, including sandblasting, grinding.

Titanium alloys are mostly preferred materials for dental implants. There were several approaches used for the best functional Ti surface, the approaches were physical or chemical adsorption to the use of covalent bonding or self-organized layers (1). Ti are determined by (American Society for Testing and Materials) as evaluations 1 to 5. Grades 1 to 4 are unalloyed, while review 5, with 6% aluminum and 4% vanadium, is the most grounded. The used specimens are titanium (Ti) discs 5 mm diameter and 1 mm thickness.

Biocompatibility of titanium alloys have been proved in several implant applications. There are several approaches decreased the biofilm formation. Etching, polising, and coating by a functional thin film are some of these preferred methods. For improved osseointegration, etching is accepted as golden standard. On the other hand increased roughens and porosity triggers the bacteria adhesion. In the last reviews, there will be there fundamental systems for adjusted the Titanium and Titanium alloys. The methods are mechanical, manufactured and physical for morphological and changes or for getting unmistakable covering on the objective surface. The mechanical techniques generally used to get an unpleasant and a smooth surface are subtraction or whittling down procedures (2). The guideline focus of using chemical techniques as acids etched is to improve biocompatibility, bioactivity and bone conductivity, disintegration resistance and removal of debasement (3). The pathogens indicate reversible and irreversible examples of adherence (4), implying that bacterial cells can stick to the surface and additionally to segregate and leave the region again before connecting irreversibly and starting the procedure of biofilm development.

The implant surface is defenseless to contamination due to two fundamental reasons, to be specific development of a surface biofilm and bargained insusceptible capacity at the implant/tissue interface. The biocompatibility of titanium embed can be credited to a surface protein layer shaped under physiological conditions (5). This protein layer really makes the surface reasonable for bacterial colonization and biofilm arrangement (6,7,8).

In this study, although the implant has good mechanical properties and biocompatibility, it is exposed to acidic fixation by the adherence of nutrients and microorganisms to intraoral use. Surface roughness occurs in the presence of acid. The effect of acid deformation on the implant surface was investigated. Two different surfaces were formed by polishing and acid etching the surfaces of the implant and the surfaces after 24 hours in situ incubation were compared with the help of microscope. When these comparisons were made, the number of bacteria on the surface, morphology and dead-living conditions were evaluated.

MATERIALS AND METHODS

Nine healthy volunteers that man and woman, aged between 24 and 71 years, who are departmental staffs of the Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, Saarland University, participated in this study. An experienced dentist conducted visual oral examination and found no signs of caries, periodontal pathology or salivary dysfunctions. In other words, the subjects had permanent teeth or sufficient dental fillings, no bleeding on gentle probing and the periodontal pocket depths were between 1 mm and 3 mm. All subjects had been on their regular daily diet with no excessive consumption of polyphenolic beverages and foods and had no history of smoking or alcohol (9). In addition, they had not taken any antibiotic medicine in the past six months. Prior to the experiments, informed written consent about participation in this study was obtained from all volunteers. Clinical Research Ethics Committee Permit Certificate is not required.

Two groups of commercially pure titanium dental implants and disks were studied in the present work. The used specimens are titanium (Ti) discs 5 mm diameter and 1 mm thickness (Friadent Germany). Group 1 consisted of acid etched implants. The other type of dental implant surfaces in Group 2 is polished. All implants were machined from bars of ASTM titanium grade 4. The acid etched dental implants were immersed in a mixture of HNO_3, HCl and H_2SO_4.

Ti specimen was ground away under permanent water-cooling by a polishing machine (Buehler, Düsseldorf, Germany), using silicon carbide grinding paper (P2500-P4000, FEPA-P, waterproof silicon carbide paper, Buehler, Düsseldorf, Almanya). Then the surface was ground plan-parallelly and polished by wet grinding with abrasive paper down to P-grit size of '4.000' (according

to Federation of European Producers of Abrasives (FEPA) standard, mean grain size = 5 μm). The samples were 1 mm thick. Subsequently, a light-microscopic examination (Motic Deutschland GmbH, Wetzlar, Germany) with 10-fold magnification was performed on the surface area of each specimen. Samples with any microscopically visible discolorations, demineralization or surface inhomogeneity were discarded and excluded from the study.

According to Hannig et al. (10), pre-treatment with NaOCl and disinfection with 70% ethanol was adopted. All specimens were firstly cleaned using a 3% NaOCl (Hedinger, Stuttgart, Germany) solution for 10 s and the specimens were washed five times in distilled water (Ecotainer; B. Braun Melsungen AG, Melsungen, Germany), followed by disinfection in 70% ethanol (Hedinger, Stuttgart, Germany) for 10 min and finished with another five washes in double distilled water. Before intraoral exposure, rehydration of specimens took place by storage in distilled water for 24 h at storage at 4 C°.

Individual intraoral fixtures for mounting dentinal specimens were manufactured for all subjects in the form of acrylic appliances in the first and second quadrants of the upper jaw. The impression of the maxilla was taken with alginate impression material (Blueprint, Dentsply DeTrey GmbH, Konstanz, Germany) to produce an elastic mould. Afterwards, a hard plaster model was fabricated, where the individual appliances ('minisplints') were constructed. The minisplints were made of Duran (Scheu-dental GmbH, Iserlohn, Germany) with a thickness of between approximately 0.5 mm and 0.7 mm, covering the molar and premolar teeth on the left and right upper jaw, and extending 3 mm beyond the buccal / palatal marginal sulcus. In order to better stabilize the mounted specimens, minisplints were provided with small perforations.

Specimens were fixed on the maxillary minisplints at a defined position by means of a thin layer of polyvinyl-siloxane impression material (President light-body, Coltene, Altstätten, Switzerland). The specimens were placed on buccal sites of the left and right upper 1st molar (16, 26) and upper 2nd molar (17, 27). The margin was completely covered by the impression material to ensure a save fixation with only the surfaces exposed to the oral environment. The buccally mounted specimens should not be directly in contact to the ductal orifice of the parotid gland. Every 3 or 4 Ti discs were fixed with silicon impression material at the buccal site of the molar and premolar teeth on the custom-made maxillary splints.

In each volunteers, each subject wore four dentinal specimens for 24 h. The splints with mounted dentinal specimens were exposed to the oral environment for periods of 24 h. In the control specimens were not treated with any treated. During the intraoral exposure period, the subjects were instructed to refrain from any consumption of dietary products and drinks. Splints were only removed and stored in 100% humidity environment during meals and daily tooth brushing. After experimental period (24 h), four dentinal specimens were quickly removed from the splints and thoroughly rinsed with sterile water for 5 s to remove non-adherent bacteria and residual saliva, followed by the BackLight™ viability assay (11), SEM-analyses (12) each with two samples.

All analyses (live/dead staining, microbiology and scanning electron microscopy) were assessed using ImageJ software, which is of great help in reducing manual labor and increasing accuracy, objectivity, and reproducibility. For image processing, Image J was used to produce an altered form of the SEM and fluorescence captured images. Picture examination was performed to extract the components of interest from each picture to analyze the microscopic outcome. Computational representation can be considered the reverse of picture examination: it delivers a picture from given inputs, which could be numbers, parameterized shapes, or numerical capacities, to give data about Ti implant surfaces.

Computational representation creates an abnormal state of understanding of what is contained in a picture as a record of microscopic organisms at first glance. This is called picture understanding. The point of perception is to change higher-dimensional picture information into a more primitive representation to encourage investigation of the information. ImageJ software was used to study the data as follows: counting cells, image thresholding, area measurements of cells and subtract background "noise".

RESULTS

Microscopic Observation

All surface observed with Scanning Electron Microscopy such as polished and acid etched surface before *in situ* experiment. (Figure 1). The polished surface looks fairly smooth, while the acid-treated Titanium surface looks rough.

Compared with the two surface, the acid etched surface showed a reduction in bacterial adherence to different levels. After 24-h exposure, it was observed that the

bacteria on the surface were almost eliminated in series of micrographs of volunteers as polished and acids etched surface (Figure 2 and 3).

In both, the control surface as raw Titanium polished have been detected different amount of bacteria according to volunteers (Figure 4 and 5). To compare the all volunteers result show that (Figure 5) polished Titanium surface have not more bacterial attachment.

To compare the all volunteers result show that (Figure 6) acid etched Titanium surface have more bacterial attachment.

Medians and range of the biofilm mass from all groups are summarized, along with the results of the statistical analyses. Image J software indicated that modified surface had significantly different effects on bacterial growth, regardless of biofilm formation time (Figure 6).

The amount of adherence bacteria on both surface by FM

After each surface experiment, the amount of microorganisms detected by BackLight™ viability The evaluation of live and dead bacteria detected is given in Table I. Figure 7 summarized according to Table I.

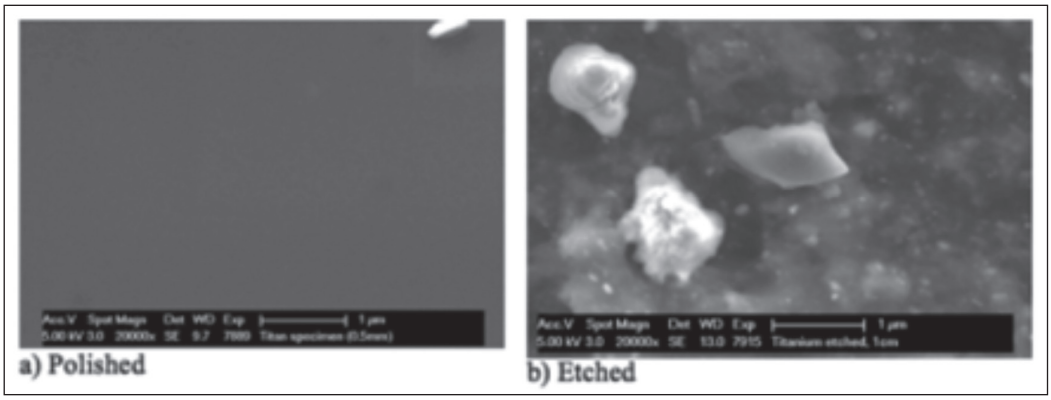

Figure 1: Scanning electron microscopy images of various surface.
A) Ti of polished surface; **B)**; Ti of etched surface; Original magnification: 20.000X.

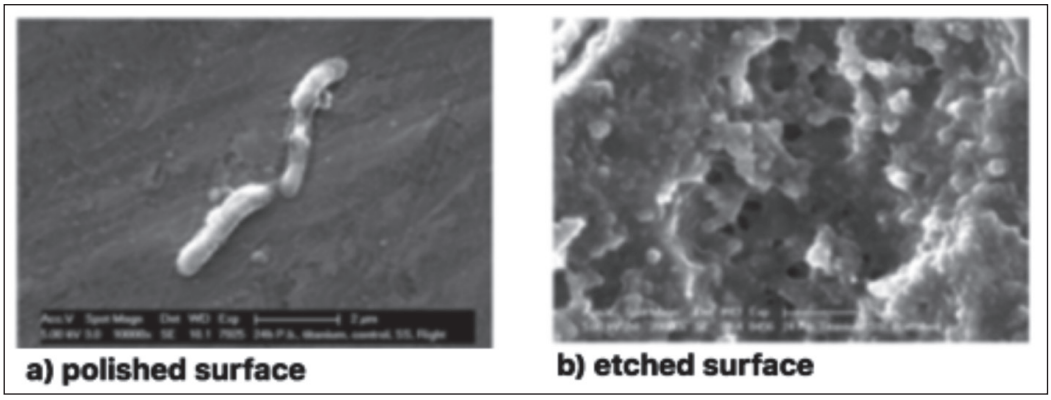

Figure 2: SEM micrographs for volunteers for SS: Biofilm on polished and acids etched samples. Bacteria are adherent to the pellicle layer and embedded in the matrix as well **(B)**. The less bacterial is evident **(A)** 24-h biofilm. Original magnification: 10.000-fold.

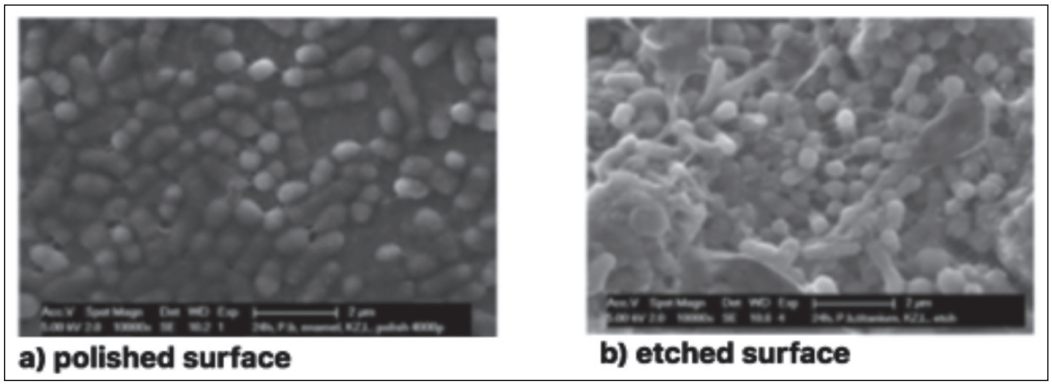

Figure 3: SEM micrographs for volunteers for KZ: Biofilm on untreated and treated samples. Bacteria are adherent to the pellicle layer and embedded in the matrix as well **(B)**. The less bacterial is evident **(A)** 24-h biofilm. Original magnification: 10.000-fold.

Exposition of the dentinal specimens at the buccal sites of the upper 1st and 2nd molars for 24 h. Median scores of biofilm formation analysed by Image J software.

Median scores of biofilm formation analysed by Image J software. Figure 8, summarized according to Table I. A significant difference between the amounts of bacteria was found on Ti surface for each volunteers. The X asixs explain the account for bacteria and Y axis show the properties of surface.

Table I: Live / dead bacteria colonization evaluated by BacLight™ viability assay.

Volunteer /Surface	Polished		Etched	
	Live	Dead	Live	Dead
MHE	196	65	317	158
BK	177	69	297	29
SS	167	70	345	124
KZ	145	98	435	172
SR	201	24	346	253
KL	105	64	364	29
NG	123	24	453	12
JK	185	26	375	52
NL	245	85	468	46
Average	171	58	377	97

Total bacteria from the live / dead bacterial colonization

With BackLight™ viability assay, it is possible to respectively assess live and dead bacterial adherence in the intra-orally formed biofilm with the purpose of estimating the antibacterial property of the tested surface.

The above graphs show that polished surface, the below one show that acid etched surface. Median scores of biofilm formation analysed by Image J software.

Figure 9, summarized according to Table I. A significant difference between the amounts of live and dead bacteria was found on polished and acids etched Ti surface. The X asixs explain the account for bacteria and Y axis show the properties of surface.

The amount of adherence bacteria on both surface by SEM

The surface was examined by SEM to assess the amount of adherent bacteria. Image J software revealed a significant difference among treatments for the experimental periods of 24 h. However, a statistical difference among subjects was observed during the surface of polished rather than the surface of etched biofilm formation time (Table II).

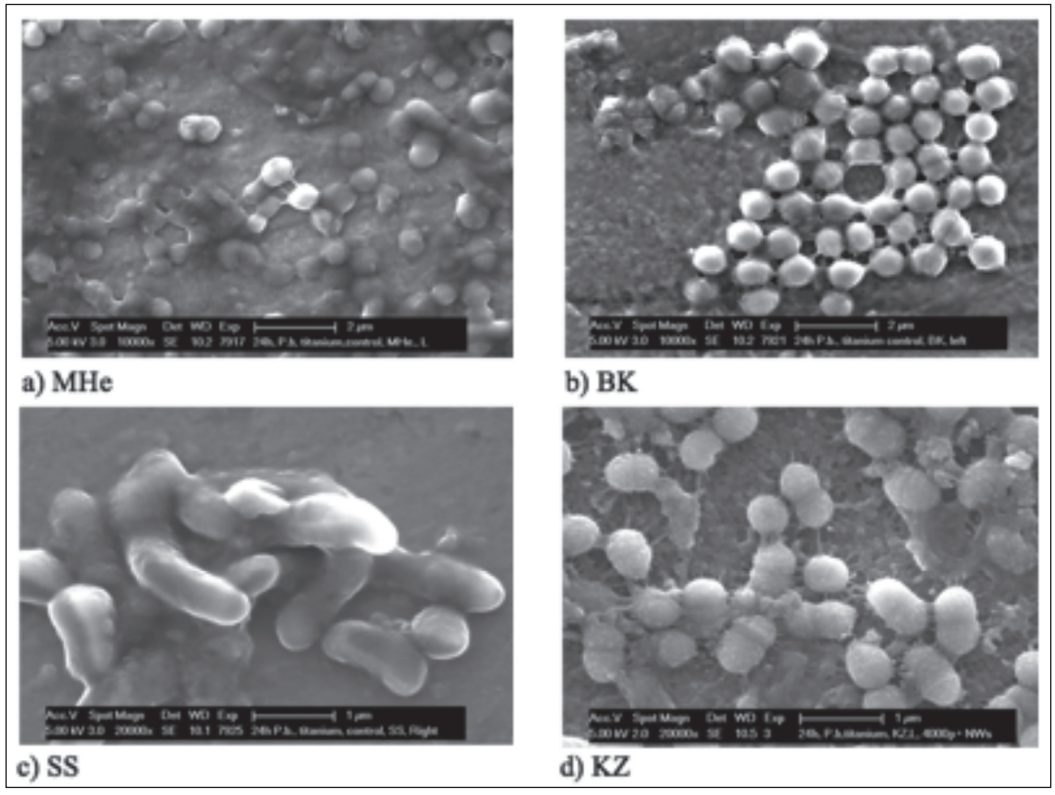

Figure 4: SEM micrographs for volunteers for MHe, BK, SS and KZ: Biofilm on Titanium surface, the surface is polished. Bacteria are adherent to the pellicle layer and embedded in the matrix as well **(A, B and D)**. The less bacterial is evident **(C)** 24-h biofilm. Original magnification: 20.000-fold.

Table II: The bacteria colonization evaluated by SEM.

Volunteer / Surface	Polished	Etched
MHE	21	78
BK	12	45
SS	2	62
KZ	87	129
SR	94	194
KL	37	64
NG	54	175
JK	22	97
NL	32	76

Exposition of the dentinal specimens at the buccal sites of the upper 1st and 2nd molars for 24 h. Median scores of biofilm formation analysed by Image J software. Figure 10 summarized according to Table II. All surface image magnification was 10.000X.

Median values of biofilm scores according to Table II, (by Image J software). A significant difference between the amounts of bacteria was found in the etched surface. There is no significant evidence on the other surface. The Y asixs explain the name of volunteers and X axis show the properties of surface.

DISCUSSION

This study covers basic methodologies of surface treatment and their effects on titanium (Ti) implants.

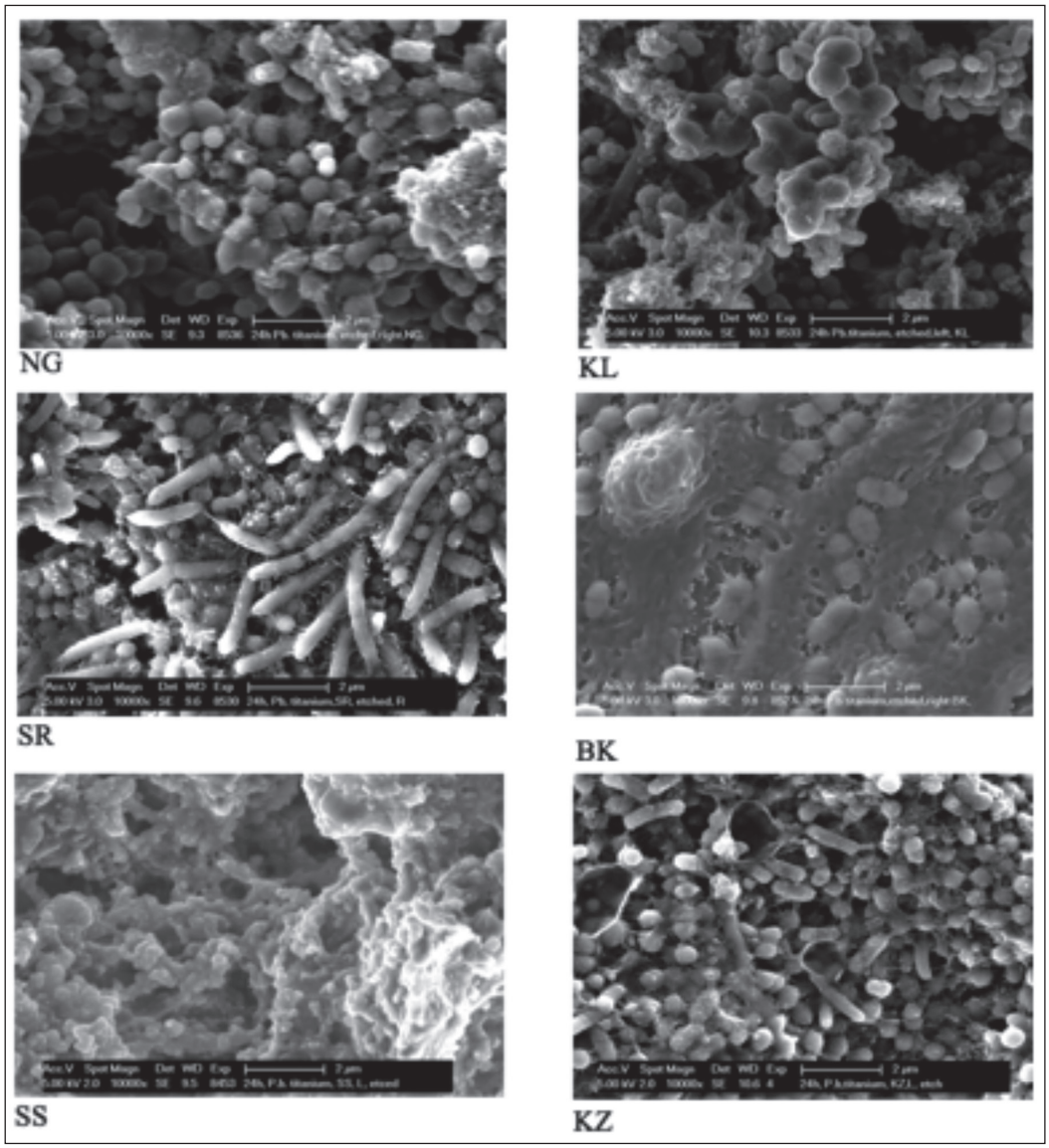

Figure 5: SEM micrographs for volunteers for NG, KL, SR, BK, SS and KZ: Biofilm on Titanium surface, the surface is etched. Bacteria are adherent to the pellicle layer and embedded in the matrix as well all surface on 24-h biofilm. Original magnification: 20.000-fold.

The importance of each treatment as polished and acids etched and its effects were be discussed in detail in order to compare their effectiveness in bacterial adherence. Published literature for the last 20 years was selected with the use of keywords like titanium dental implant, surface roughness, coating, and bacterial adhesion. Microtopography is linked to microroughness on a micrometer scale (1–100 µm) and is modified by manufacturing techniques like machining, acid-etching, anodization, sandblasting, grit-blasting, and different coating procedures. The modifications of microtopography contribute to an increase in surface area.

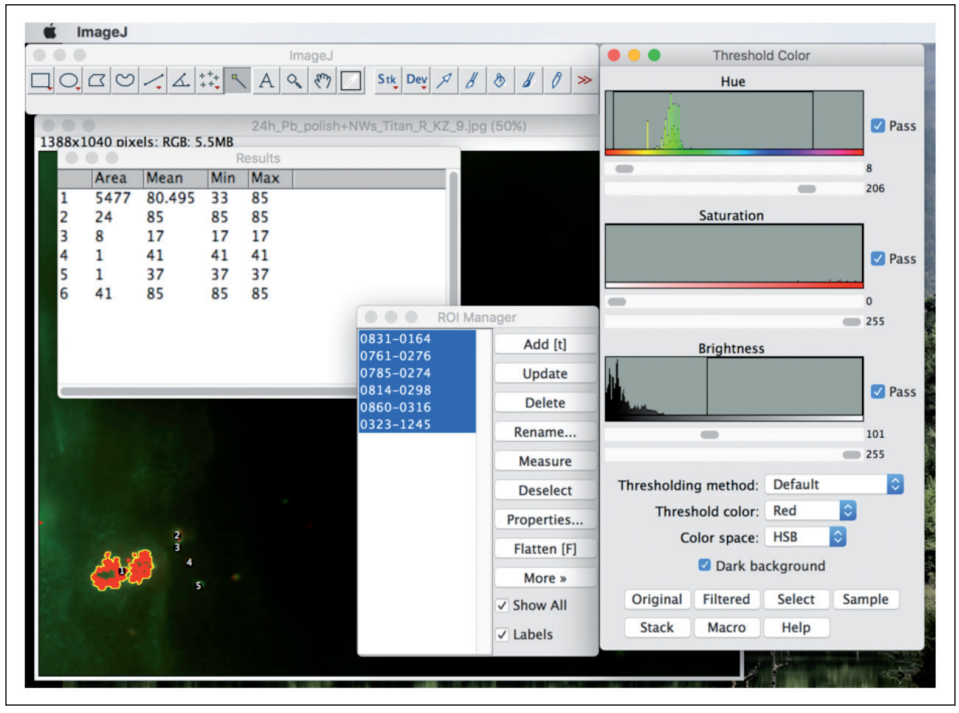

Figure 6: The software of Image J analysed the surface properties as the amount of bacteria and detected minimum and maximum values of biofilm scores. The software treated colour threshold and calculated the means.

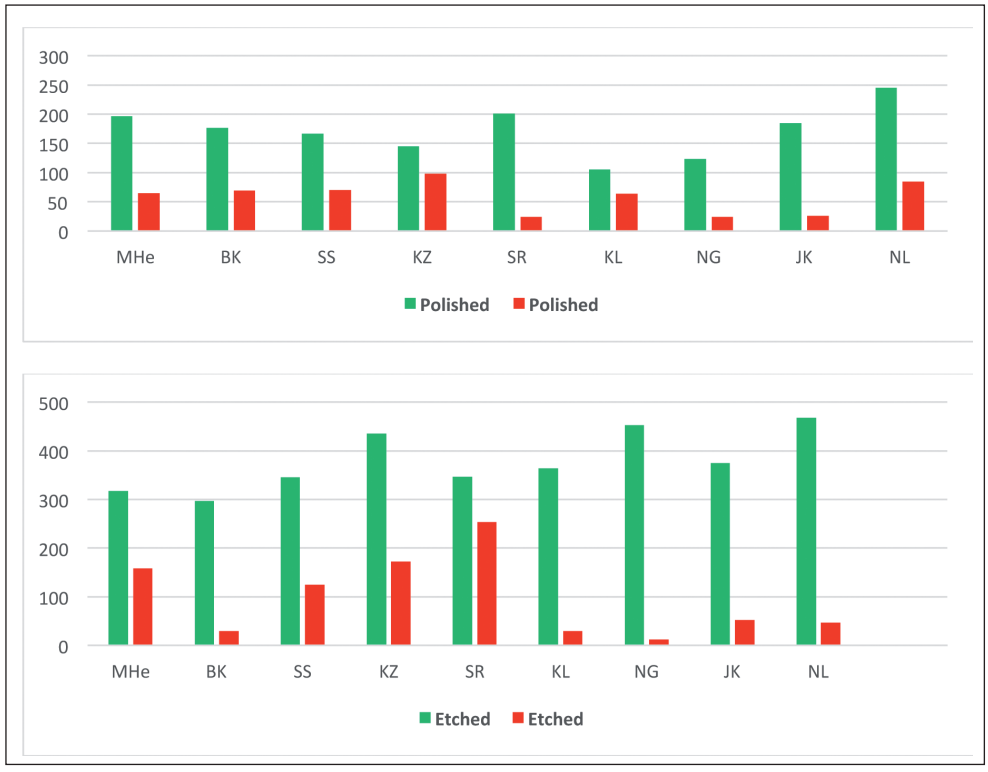

Figure 7: BackLight™ viability assay for determination of live and dead bacteria in 24-h biofilm on different properties of Ti surface.

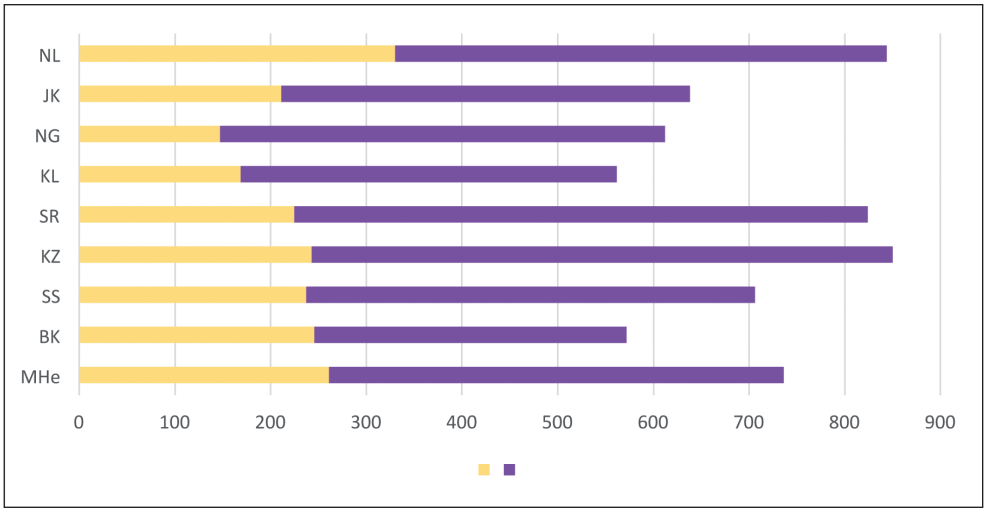

Figure 8: BackLight™ viability assay for determination of bacteria in 24-h biofilm on different properties of Ti surface as polished and etched.

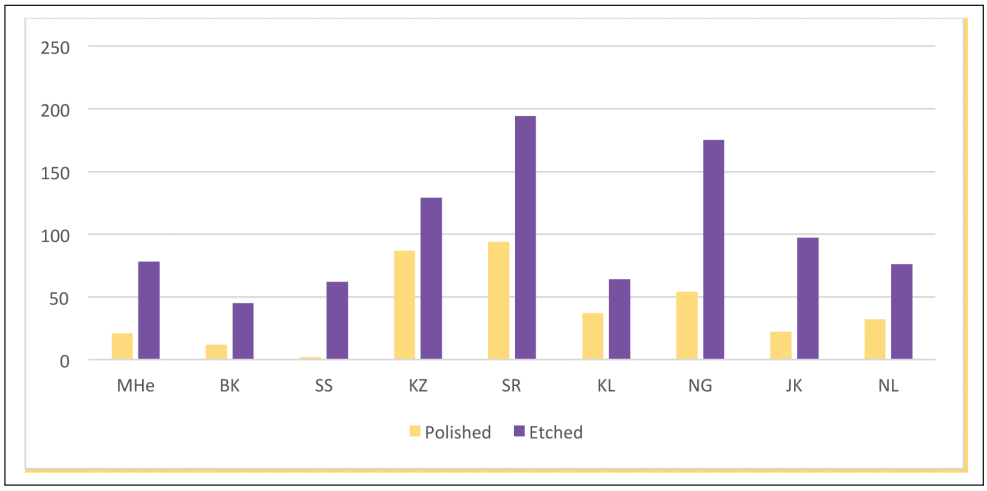

Figure 9: SEM for determination of bacteria in 24-h biofilm on different properties of Ti surface.

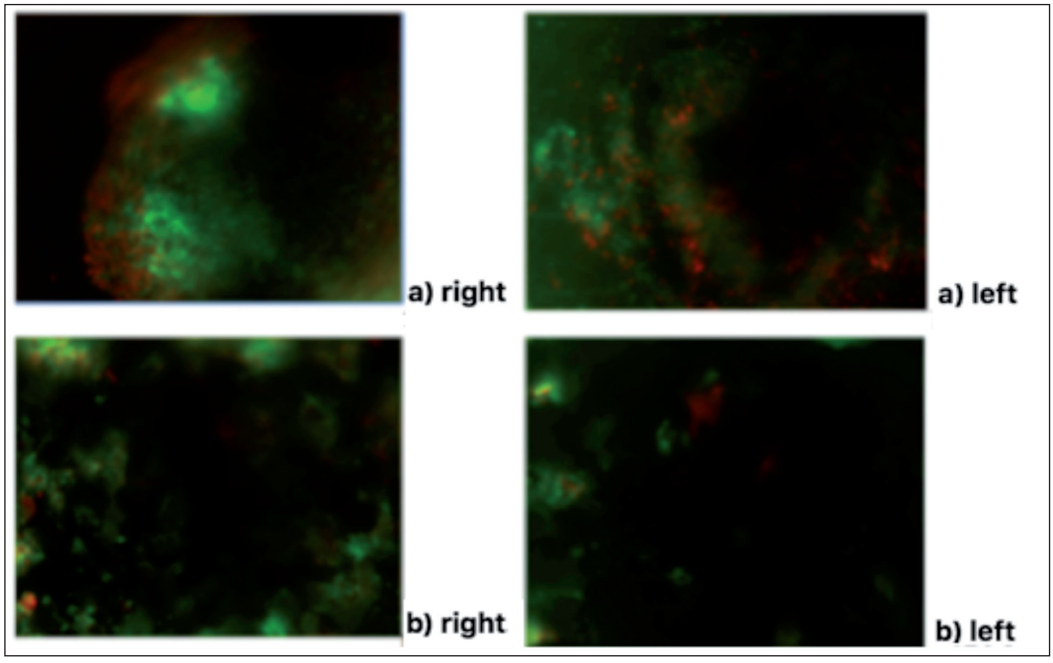

Figure 10: BackLight™ viability assay: Visualization of different arrangements of bacteria in the 24-h biofilm on Titanium surface. The surface was observed for volunteers as KZ (the abbreviation is symbolized the volunteers' experimental name). The bacterial adherence detected using BackLight ™ viability assay for volunteers for each sides was shown as left and right **A)** Ti of polished surface; **B)**; Ti of etched surface.

Studies have shown increased levels of microrough surfaces. Significant surface roughness played an important role in providing effective surface for bacteria-implant contact, biofilm formation, despite having good mechanical properties (13). Overall, published studies indicated that an acid etched surface-modified and a coating application on commercial pure titanium implant was most preferable in producing the good surface roughness (14-16). However, roughness and mechanical properties of titanium could lead to successful dental implants.

The specimens were placed on buccal sites of the left and right upper 1st molar (16, 26) and upper 2nd molar. An overview of bacterial adherence detected using Backlight ™ viability assay for volunteers for each sides was shown in Figure 10. In such a 24-h biofilm, the microbial interaction was evident on the acids treated surface. Bacteria interacted with each other or with pellicle structures through fine fibrils, resulting in bacterial co-aggregation and bacterial adhesion because of surface topology. In addition, bacterial division was apparently detected. The biofilm formed on untreated control samples for a period of 24 h, manifested itself as a microbial community embedded in a polysaccharide matrix.

SEM is the most commonly used microscopic examination technique for biofilms as it permits the immediate recognition of their morphological structure at high magnification. At the smaller scale, the bacterial loads, the biofilm network and the bacterial interactions are strikingly represented. Moreover, the morphogenesis of biofilms at the dentinal surface as opposed to the subsurface and profound parts of the tubules is available. As described above, the fluorescence technology provides insights into the bacterial colonization and viability patterns. Therefore, the combination of the fluorescence technique and the electron/fluorescent microscopic technology allows investigation of the effects of mouthwashes on biofilm quantity and quality, which is of considerable relevance for assessing the efficacy of oral health care products in biofilm management.

Compared with the two surface, the acid etched surface showed a reduction in bacterial adherence to different levels. After 24-h exposure, it was observed that the bacteria on the surface were almost eliminated in series of micrographs of volunteers as polished and acids etched surface (Figure 8- 10 and Table I,II).

Considering the said disadvantages of in vitro models, the in situ show has the favorable position that it can definitely draw the photo of the circumstance in the oral cavity. Hence, the in situ display has been connected in the present examinations.

CONCLUSION

The present study investigation has demonstrated that machined, chemical surface as polished surface is suitable for un-attachment and unfit for bacteria the 24h *in situ* oral biofilms formed on titanium discs, without causing any damage to titanium surfaces. The efficacy of Ti surface was evaluated at two different microscopic observations as SEM and BackLight™ morphological. The polished and etched were used. The untreated surface as polished exhibited great abilities to inhibit bacterial growth on biofilm and resulted in a significant decrease in the total amount of biofilms and the average percentage of viable bacteria, compared with the treated surface as acids etched.

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