Ph.D. Thesis

Effects of Chemical and Prophylactic Agents Used in Dentistry on Titanium Implant Surfaces

Krisztina Ungvári DMD

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Szeged

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PUBLICATIONS RELATED TO THE THESIS

ARTICLES

I. A. Stájer, **K. Ungvári**, I. K. Pelsőczi, H. Polyánka, A. Oszkó, E. Mihalik, Z. Rakonczay, M. Radnai, L. Kemény, A. Fazekas, K. Turzó: Corrosive effects of fluoride on titanium: investigation by X-ray photoelectron spectroscopy, atomic force microscopy and human epithelial cell culturing. *J Biomed Mater Res A* 2008; 87:450-458.

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II. **K. Ungvári**, I. K. Pelsőczi, B. Kormos, A. Oszkó, Z. Rakonczay, L. Kemény, M. Radnai, K. Nagy, A. Fazekas, K. Turzó: Effects on titanium implant surfaces of chemical agents used for the treatment of peri-implantitis. *J Biomed Mater Res B Appl Biomater* 2010;94:222–229.

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IV. K. Turzo, A. Stajer, **K. Ungvari,** I. K. Pelsoczi, H. Polyanka, A. Oszko, D. Matusovits, E. Muhalik, Z. Rakonczay, M. Radnai, A. Fazekas: Investigation of the corrosive effects of flouride on titanium. 0289 (85184)IADR PEF Dublin September 13-16, 2006 *J Dent Res* 85; p. 183 PEF, 2006 (abstract IF: 3.475)

V. I. Pelsőczi-Kovács, **K. Ungvári**, H. Polyánka, Z. Tóth, B. Hopp, C. Gergely, Z. Rakonczay, F. G. J. Cuisinier, A. Fazekas, K. Turzó: Human Fibroblast Cell Culturing on Surface Modified Titanium Implants. 0378 (96424)Annual Meeting IADR-Continental European and Isreeli DivisionThessaloniki, Greece September 26-29, 2007 *J Dent Res* 86; Spec. Iss. B 0378, 2007 (abstract: IF: 3.496)

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VIII. **K. Ungvári,** I. Pelsőczi, A. Oszkó, Z. Rakonczay, A. Fazekas, K. Nagy, K. Turzó: Effects of cleansing solutions on titanium surfaces: tissue culture study. Abstr. 13811288th General Session & Exhibicion of the IADR Barcelona, Spain July 14-17, 2010 *Journal of Dental Research*, Vol. 89, Special Issue B, No 4209, 2010 (abstract IF:: 3.773)

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Contents

Abbreviations and glossary

AFM: Atomic force microscopy (AFM) or scanning force microscopy (SFM) is a very high-resolution type of scanning probe microscopy, with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. *http://en.wikipedia.org/wiki/Atomic_force_microscopy*

ASTM: ASTM International, formerly known as the American Society for Testing and Materials (ASTM), is a globally recognized leader in the development and delivery of international voluntary consensus standards. Today, some 12,000 ASTM standards are used around the world to improve product quality, enhance safety, facilitate market access and trade, and build consumer confidence. *http://www.astm.org/ABOUT/aboutASTM.html*

BSA: Bovine serum albumin (also known as BSA or "Fraction V") is a serum albumin protein derived from cows. It is often used as a protein concentration standard. *http://en.wikipedia.org/wiki/Bovine_serum_albumin*

CAD/CAM dentistry: (Computer-Aided Design and Computer-Aided Manufacturing in dentistry), is an area of dentistry using CAD/CAM technology to produce a range of dental restoration including: crowns, crown lays, veneers, inlays and on lays, fixed bridges, dental implant restorations and orthodontic appliances. *http://en.wikipedia.org/wiki/CAD/CAM_Dentistry*

CHX: Chlorhexidine digluconate is an antibacterial solution, effective against a wide variety of gram-negative and gram-positive organisms; used also as the acetate ester, as a preservative for eyedrops, and as the gluconate or hydrochloride salt, as a topical anti-infective. *http://medical-dictionary.thefreedictionary.com/chlorhexidine+digluconate*

CP: Commercially pure by ASTM. Grade 1-4 are unalloyed and considered commercially pure or "CP". Generally the tensile and yield strength goes up with grade number for these "pure" grades. The difference in their physical properties is primarily due to the quantity of interstitial elements. They are used for corrosion resistance applications where cost and ease of fabrication and welding are important. *http://en.wikipedia.org/wiki/Titanium_alloy*

FBS: Fetal bovine serum or FBS (or fetal calf serum) is the portion of plasma remaining after coagulation of blood, during which process the plasma protein fibrinogen is converted to fibrin and remains behind in the clot. Fetal bovine serum comes from the blood drawn from a bovine fetus via a closed system of collection at the slaughterhouse (aka abattoir). Fetal bovine serum is the most widely used serum-supplement for the *in vitro* cell culture of eukaryotic cells. This is due to it having a very low level of antibodies and containing more growth factors, allowing for versatility in many different cell culture applications. *http://en.wikipedia.org/wiki/Fetal_bovine_serum*

FPDs: A bridge, also known as a fixed partial denture, is a dental restoration used to replace a missing tooth by joining permanently to adjacent teeth or dental implants. *http://en.wikipedia.org/wiki/Bridge_(dentistry)*

H_2O_2: Hydrogen peroxide (H_2O_2) is an oxidizer commonly used as bleach. It is the simplest peroxide (a compound with an oxygen-oxygen single bond). Hydrogen peroxide is a clear liquid, slightly more viscous than water that appears colorless in dilute solution. It is used as a disinfectant, antiseptic, oxidizer, and in rocketry as a propellant. The oxidizing capacity of hydrogen peroxide is so strong that it is considered a highly reactive oxygen species. *http://en.wikipedia.org/wiki/Hydrogen_peroxide*

KSFM: Keratinocyte Serum Free Medium for epithelial cells. Contains L-glutamine. Keratinocyte-SFM is supplied with prequalified human recombinant Epidermal Growth Factor 1-53 (EGF 1-53) and Bovine Pituitary Extract (BPE) in separate packaging. Components are not sold separately. *http://products.invitrogen.com/ivgn/product/17005042*

MES (buffer): MES is the common name for the compound 2-(N-morpholino)ethanesulfonic acid. MES is used as a buffering agent in biology and biochemistry. *http://en.wikipedia.org/wiki/MES_(buffer)*

Micro BCA™ Protein assay: The Micro BCA Protein Assay Kit is a specialized version of the popular Pierce BCA Protein Assay for determining the protein concentration of dilute samples. Mixing together the three Micro BCA Reagents results in a working solution that is sufficiently concentrated to measure protein when mixed with an equal volume of sample. The result is an assay for accurately measuring 0.5 to 20 µg/mL protein solutions. The assay is exceptionally linear and exhibits very low levels of protein-to-protein variability. *http://www.piercenet.com/products/browse.cfm?fldID=02020102*

MTT: Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength by a spectrophotometer. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. *http://www.mnstate.edu/provost/MTT_Proliferation_P...*

OD: Optical Density. In spectroscopy, the absorbance A (also called optical density) is defined as: $A_\lambda = \log10(I_0/I)$, where I is the intensity of light at a specified wavelength λ that has passed through a sample (transmitted light intensity) and I_0 is the intensity of the light before it enters the sample or incident light intensity (or power). Absorbance measurements are often carried out in analytical chemistry, since the absorbance of a sample is proportional to the thickness of the sample and the concentration of the absorbing species in the sample, in contrast to the transmittance I / I_0 of a sample, which varies exponentially with thickness and concentration. *http://en.wikipedia.org/wiki/Absorbance*

PBS: Phosphate buffered saline (abbreviated PBS) is a buffer solution commonly used in biological research. It is a water-based salt solution containing sodium chloride, sodium phosphate, and (in some formulations) potassium chloride and potassium phosphate. The buffer helps to maintain a constant pH. The

osmolarity and ion concentrations of the solution usually match those of the human body (isotonic). *http://en.wikipedia.org/wiki/Phosphate_buffered_saline*

PFM: Porcelain-fused-to-metal dental crowns (PFMs) have a metal shell on which is fused a veneer of porcelain in a high heat oven. The metal provides strong compression and tensile strength, and the porcelain gives the crown a white tooth-like appearance, suitable for front teeth restorations. *http://en.wikipedia.org/wiki/Crown_(dentistry)*

RPMI: Roswell Park Memorial Institute medium, commonly referred to as RPMI, is a form of medium used in cell culture and tissue culture. This medium contains a great deal of phosphate and is formulated for use in a 5% carbon dioxide atmosphere. *http://en.wikipedia.org/wiki/Roswell_Park_Memorial_Institute_medium*

SEM: A scanning electron microscope (SEM) is a type of electron microscope that images a sample by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition, and other properties such as electrical conductivity. *http://en.wikipedia.org/wiki/Scanning_electron_microscope*

SDS: Sodium dodecyl sulfate (SDS or NaDS), sodium laurilsulfate or sodium lauryl sulfate (SLS) ($C_{12}H_{25}SO_4Na$) is an anionic surfactant used in many cleaning and hygiene products. The salt consists of an anionic organosulfate consisting of a 12-carbon tail attached to a sulfate group, giving the material the amphiphilic properties required of a detergent. *http://en.wikipedia.org/wiki/Sodium_dodecyl_sulfate*

Ti: Titanium is a chemical element with the symbol Ti and atomic number 22. It has a low density and is a strong, lustrous, corrosion-resistant (including sea water, aqua regia and chlorine) transition metal with a silver color. *http://en.wikipedia.org/wiki/Titanium*

TMA: β-titanium orthodontic alloy (TMA) contains primarily titanium and molybdenium. *http://en.wikipedia.org/wiki/Beta-titanium*

XPS: X-ray photoelectron spectroscopy (XPS) is a quantitative spectroscopic technique that measures the elemental composition, empirical formula, chemical state and electronic state of the elements that exist within a material. *http://en.wikipedia.org/wiki/X-ray_photoelectron_spectroscopy*

1. Introduction

An **alloplastic material is biointegrated** if it functions in intimate contact with the surrounding tissues for any period of time and does not have any adverse/damaging effect on the body as a whole. The successful biointegration of biomaterials depends on several factors related to the material, like the **bulk and surface characteristics of the material**, the **design** (construction) and the **biocompatibility of the material**. Naturally, the **applied surgical technique and the general health condition and life-quality of the patient** are also important features. Biocompatibility is defined as the acceptance of an artificial implant by the surrounding tissues and by the body as a whole.[1]

The main research topics of our group are to investigate the biointegration of alloplastic materials (replacement of missing parts of human body) and how the chemical and surface microstructural modifications of the dental implants made of titanium influence their biointegration. Our studies relate to replacements of body structures in case of which the biological function requires significant load-bearing capability. Example for that are dental implants and artificial hip-joint replacements. These biomaterials have several common aspects: in general they are made from titanium (Ti), and their biological integration depends on -among others- the surface structure of the metal (Figure 1). These studies have a general interest/value as basic research which is highly applicable for biomedical and industrial uses as well.

Figure 1. Titanium: silvery grey-white metallic (http://en.wikipedia.org/wiki/Titanium)

The importance of bio- and alloplastic materials' knowledge in dentistry is evident as the goal of dentistry is to maintain and improve the health of the human teeth (oral cavity) in order to improve the quality of life of the dental patient. All these activities require the replacement or alteration of the existing tooth structure and also the development of auxiliary dental appliances using alloplastic materials.

As healthcare improves and people tend to live longer, materials with specific biomedical applications become more important. In dentistry the main challenges for

centuries have been the development and selection of biocompatible prosthetic materials that can withstand the adverse conditions of the oral environment. The oral cavity represents a multivariate external environment with a wide range of circumstances, like foods, abrasion, acidic pH, temperatures from 5 to 55 ^{o}C, high masticator forces, bacteria, etc. There is also an increasing need to develop materials that can be implanted into the maxillofacial area in order to rehabilitate the damaged chewing apparatus due to loss of natural teeth. The multiplicity of dental applications requires more than one type or class of material because no one material has yet been developed that can fulfill the varying requirements.

1.1. Characteristics of titanium and its alloys

In the early 1950s, titanium (Ti) was often referred to, by those working for the aerospace industry, as „the wonder metal".[2] Nowadays it has several application fields in industry, like alloying element in steel, and about 95% of titanium or extracted from the Earth is refined into titanium dioxide (TiO_2), a white permanent pigment used in paints, paper, toothpastes and plastics (Figure 2). Beside these Ti and its alloys have several automotive applications and are used to fabricate aircrafts, naval ships, spacecrafts and missiles.

Figure 2. Titanium dioxide is the most commonly used compound of titanium (http://en.wikipedia.org/wiki/Titanium)

In 1947 J. Cotton introduced Ti and its alloys like implants with medical applications. Due to their favourable physical and chemical properties, Ti and Ti alloys are nowadays the metals most commonly applied as alloplastic (bio) materials, with an excellent biointegration and osseointegration perspective, ensuring a predictable and long lifetime for medical or dental implants.[3,4,5]

Ti is the sevenths most frequent metal in the earth's crust, it is a quite light material. Its density is 4.5 g/cm^3, considerably less than that of other metals used in dentistry, like gold (19.3 g/cm^3), CoCrMo (8.5 g/cm^3), or stainless steel (7.9 g/cm^3). In its unalloyed condition,

titanium is as strong as steel, but 45% lighter. Its melting point is between 1672-1727 °C, and the thermal properties are similar to dental tissues. Titanium's Vickers Hardness Number (VHN) is 210, similar to gold alloys type III, IV (hard): 135-250. Ti6Al4V alloy has a VHN of 320, which is close to the value of CoCr alloys: 350-390.[6,7] Commercially pure (CP) Ti is available in 4 grades, which vary according to the oxygen (0.18 to 0.40 weight percent) and iron (0.20 to 0.50 weight percent) contents (Table 1). Oxygen in particular has a great influence in the ductility and strength of the metal.[1,6]

Element	Grade 1	Grade 2	Grade 3	Grade 4	Ti6Al4V[a]
Nitrogen	0.03	0.03	0.05	0.05	0.05
Carbon	0.10	0.10	0.10	0.10	0.08
Hydrogen	0.015	0.015	0.015	0.015	0.0125
Iron	0.20	0.30	0.30	0.50	0.25
Oxygen	0.18	0.25	0.35	0.40	0.13
Titanium			Balance		

Table 1. Chemical compositions of titanium and its alloy.[8] Aluminium 6.00% (5.50 ~ 6.50), vanadium 4.00% (3.50 ~ 4.50), and other elements 0.1% maximum or 0.4% total. All are maximum allowable weight percent.

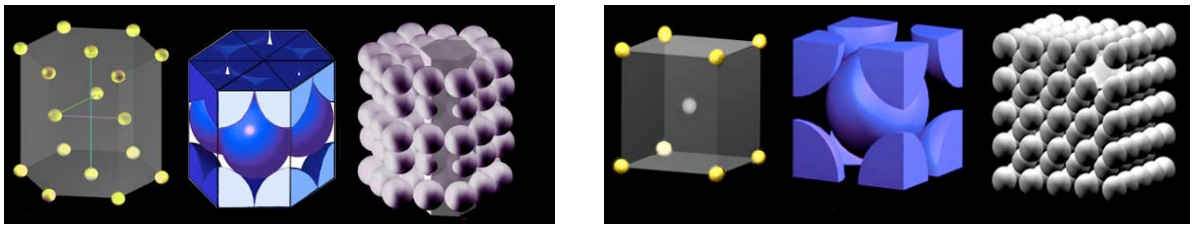
These slight concentration differences have a substantial effect on the physical and mechanical properties of the metal (Table 2).[1] Titanium can be alloyed with a wide variety of elements (see chapter 1.2) to alter its properties, mainly for the purpose of improving strength, high temperature performance, creep resistance, weldability, response to ageing heat treatments, and formability.

Properties	Grade 1	Grade 2	Grade 3	Grade 4	Ti6Al4V	Ti13Nb13Zr
Tensile strength (MPa)	240	345	450	550	860	1030
Yield strength (0.2% offset) (MPa)	170	275	380	485	795	900
Elongation (%)	24	20	18	15	10	15
Reduction of area (%)	30	30	30	25	25	45

Table 2. Mechanical properties of Ti and its alloys.[9]

Ti is a dimorphic allotrope: while at room temperature CP Ti has $\alpha-$phase (HCP-hexagonally closed packed), on heating in its crystalline structure an allotropic phase

transformation will occur. After 883 ºC, a body centered cubic (BCC), β−phase will form. β −form is stronger but more brittle than α−phase (Figure 3).[2,6]



α-Ti: HCP β-Ti: BCC

Figure 3. The crystalline structure of α-Ti is Hexagonal Close Packed (HCP) , in the case of β-Ti is Body Centered Cubic (BCC) (://www.ndt-ed.org/EducationResources/CommunityCollege/Materials/Structure/metallic_structures.htm)

The two most powerful/useful properties of Ti metal are the highest yield strength-to-density ratio of any metal,[1] and corrosion resistance.[2,6,7]

The strength of the material varies from a much lower value than that of 316 stainless steel or the CoCr alloys to a value about equal to that of annealed stainless steel of the cast CoCrMo alloy. But when compared by its specific strength (strength per density) the Ti alloys exceed any other implant materials (Figure 4). Ti, however, has poor shear strength making it less advantageous for bone screws, plates and similar applications.[1]

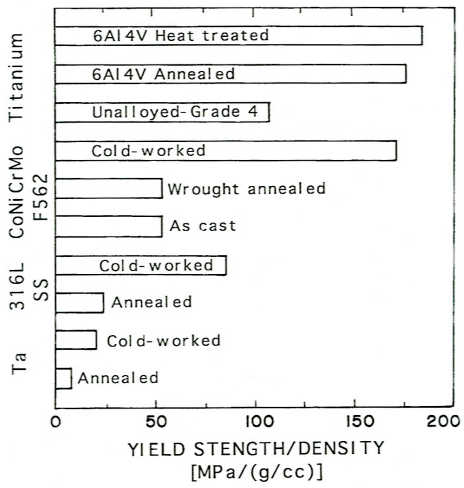


Figure 4. Yield strength to density ratio of commonly used implant materials.[1]

Ti and its alloys are resistant to corrosion because of the formation of an insoluble titanium oxide layer on the surface.[10] The reason is that, TiO_2 has one of the highest heats of reaction: ΔH = -912 kJ/mol. In air, the oxide, usually TiO_2, begins to form within nanoseconds (10^{-9} s) and reaches a thickness of 20–100 Å in 1 s (Figure 5). It is very adherent

to the parent Ti, protects the metal from other impurities and it is impenetrable to oxygen. TiO_2 may be catalytically active for a number of organic and inorganic chemical interactions influencing biological processes at the implant interface: the TiO_2 oxide film permits a compatible layer of molecules to attach.

Explanations for that are still largely unidentified, but the high dielectric constant of TiO_2 (ε = 50-170) versus 4-10 for alumina and dental porcelain can outcome in considerably stronger van der Waals bonds between molecules and TiO_2 than other oxides.[2]

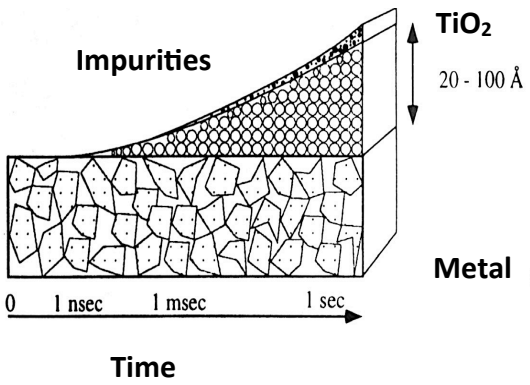


Figure 5. TiO_2 native/protective layer of metal Ti.

Both CP titanium and Ti6Al4V own exceptional corrosion resistance for a wide range of oxide states and pH levels. However, even in its passive condition, Ti is not totally inert. Its ions are released due to the chemical dissolution of titanium dioxide. Elevation of implant elements in blood can be observed (measured in the fibrous membrane encapsulating implants), but they are non toxic for Ti6Al4V: 21 ppm Ti, 10.5 ppm Al, 1 ppm V.[11]

Another important property is the elastic (or Young) modulus (E), which in case of load bearing implants integrated in bone preferably has to be similar to that of the bone elastic modulus. Young modulus of the nearby cortical bone is much smaller: E_{bone} = 17-24 GPa. Stainless steel, CoCr alloys are usually much stiffer than bone (E around 250 GPa), so they can shield the nearby bone from mechanical stress. Stress shielding results in a kind of disuse atrophy and the bone will resorb. For titanium and one of its widely used alloys, Ti6Al4V, E is about half of the other metals (110 GPa) therefore this property also makes Ti the material of choice in these applications (Figure 6).

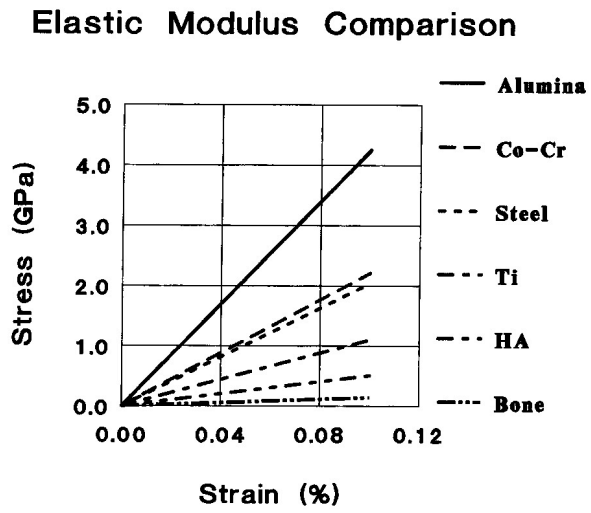


Figure 6. Elastic modulus of different biomaterials compared to bone.[12]

Titanium readily reacts with gaseous elements in air at high temperatures therefore prevention of metal from any contamination is required. Without a well controlled vacuum, titanium surfaces will be contaminated, reducing strength and ductility and promote cracking mechanism. Because of the low density of titanium it is difficult to cast in conventional, centrifugal-force casting machines. These make casting procedure more difficult and more expensive. Advanced casting techniques are nowadays applied, which combine centrifugal, vacuum, pressure and gravity in specially designed furnaces. Thus casting process has been developed and now it is available.[6]

1.2. Applications of titanium and its alloys in dentistry

Titanium and its alloys are widely used as medical or dental implants in consequence of their appropriate mechanical properties, good biocompatibility and excellent corrosion resistance.[13]

Their most important applications in dentistry are: endosseous dental implants, surgical implants for fixating or replacing hard tissue, crowns and multiple-unit fixed restorations, partial removable prosthesis used in prosthetic dentistry, Ti brackets and dental arch wires in orthodontic dentistry (Table 3), and appliances for endodontic treatment.

Metal or alloy	Application fields
Pure Ti	crowns, partial dentures, dental implants
Ti6Al4V alloys	structural elements for dental implants
TiNi alloys	orthodontic wires, endodontic instruments
Ti6Al7Nb	surgical implants
Ti13Nb13Zr, TiMo, Ti30Pd, Ti20Cu,	dental implant, orthodontic archwires,

Table 3. Applications of titanium and its alloys in dentistry (pure Ti: http://en.wikipedia.org/wiki/Titanium; Ti6Al4V:http://en.wikipedia.org/wiki/Titanium_alloy; TiNi alloys:http://en.wikipedia.org/wiki/Nickel_ titanium; Ti6Al7Nb, TiMo, Ti30Pd, Ti20Cu :http://en.wikipedia.org/wiki/Titanium_alloy, S. Génin, P. Laheurte, A. Eberhardt, MP. Filleul: Testing the mechanical properties of titanium molybdenum preformatted orthodontic archwires under tensile stress – A preliminary report *European Cells and Materials* Vol. 10. Suppl. 4, 2005 (page 11) ISSN 1473-2262; Ti13Nb13Zr: [14])

1.2.1. Prosthetic, endodontic and orthodontic appliances

Ti is used in prosthetic dentistry to manufacture titanium-ceramic crowns, multiple-unit fixed restorations, and denture base too (Figure 7).[6,7]

As shown by Boeckler et al. the clinical performances of the CAD/CAM (computer-aided design/computer-aided manufacturing) titanium ceramic crowns followed for 3 years were acceptable, with no biologic complications and a high cumulative survival rate.[15] Boeckler et al. found, that the CAD/CAM titanium-ceramic fixed partial dentures (FPDs) survived in the mouths of patients without major complications for 3 years, although the risk of porcelain fracture appeared to be relatively high.[16] Titanium and its alloys are not the best common used metals for porcelain-fused-to-metal (PFM) restorations, due to the arising difficulties in processing: high casting temperature (2000 ˚C), rapid oxidation, reactions with investments. Special low expansion porcelains (coefficient of expansion of 9×10^{-6}/°C) need to PFM restorations with titanium alloy (Ti6Al4V).[6]

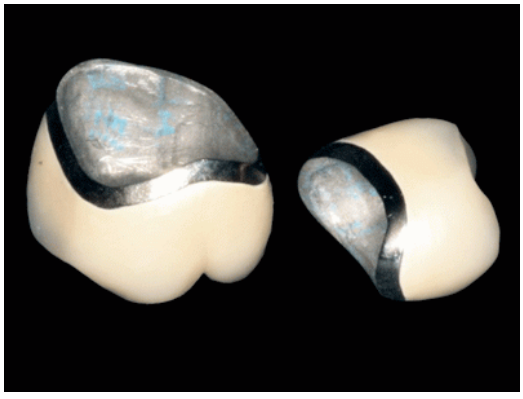


Figure 7. Gradia (aesthetic cover-up) on Pure Titanium Crown. (http://hktdental.com/html/2_gc_gradia.html)

Xie et al. examined the effect of different denture bases on the phonetic parameters.[17] It was found that titanium denture base provides a satisfying form of articulate position with thinner front palate thickness; therefore it is recommended as denture base. Titanium alloys can be used to manufacture wire clasps of removable partial denture too. The β Ti alloy wire may be applicable for abutment teeth with a large number of undercuts.[18] Clasps made from titanium and its alloy (Ti6Al4V) was compered with clasps made of cobalt-chromium. The results proved that there was more loss of retention for clasp made from cobalt-chromium than for titanium and its alloy; porosity in cobalt-chromium clasps was less than in titanium, and the porosity in titanium clasps did not cause fracture or permanent deformation.[6]

In conservative dentistry during root canal treatment Ni-Ti (nickel-titanium) or stainless steel hand instruments are applied frequently (Figure 8). For treating a tooth with a large curve of the root canal, the best solution is to choose very flexible instruments to avoid damages of the tooth. The NiTi files and reamers are five times more flexible than stainless steel.[6]

Figure 8. Nickel titanium (nitinol or NiTi) endodontic rotary files. (http://www.llu.edu/dentistry/news/index.page?story_id=1152)

Ti brackets are one of the most important applications of Ti in orthodontic dentistry.[19] Dental arch wires and orthopedic braces are usually made from the special titanium-nickel (TiNi) alloy.[1,20] The TiNi alloy show unusual properties, as after it is deformed the material can snap back to its previous shape following heating of the material (Figure 9).

This phenomenon is called *shape memory effect* and it was first observed by Buehler and Wiley at the US Naval Ordnance Laboratory. Since then, these alloys have several applications, as orthodontic archwire, intracranial aneurysm clip, vena cava filter, vascular stent, orthopedic staple. Ion-implanted surfaces of nickel-titanium archwires reduce the bracket friction.[6]

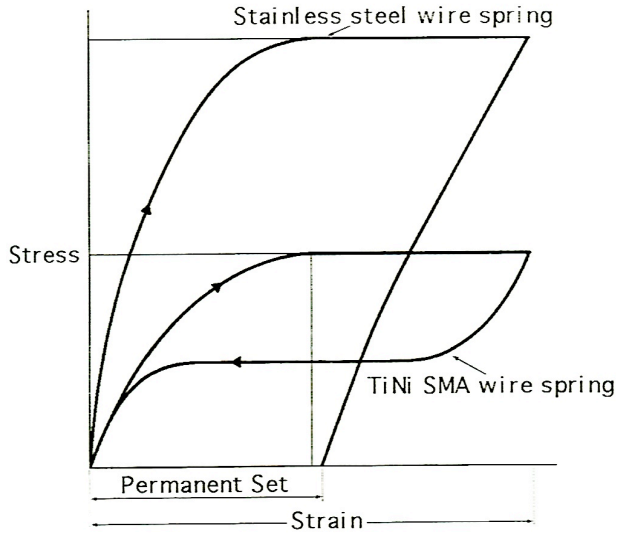


Figure 9. Stress and strain curve for the illustration of the *shape memory effect* of TiNi alloy compared with the stainless steel wire spring.[1]

β-titanium is another possibility to manufacture orthodontic wires (Figure 10**)**. The β-titanium orthodontic alloy (TMA) contains titanium and molybdenium. This alloy has outstanding formability, capability for permanent deformation and weldability.[6]

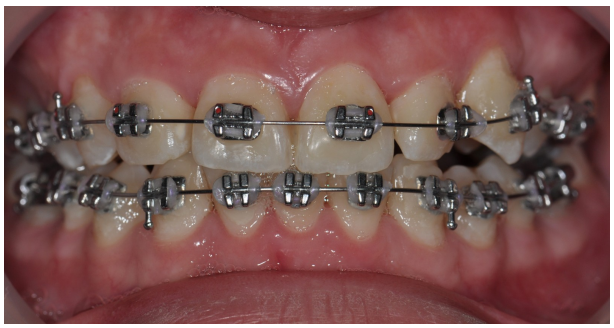


Figure 10. Orthodontic fix appliance (http://www.prestige-dental-care.com.my/blog/orthodontic-treatment/orthodontic-a-cases-with-fix-appliance-02.html)

Other titanium alloys (titanium-molybdenum alloy) are investigated to improve the features of archwires, because the new orthodontic techniques need special materials.

Doshi and Bhad-Patil investigated the static frictional resistance between three modern orthodontic brackets (made of ceramic with gold-palladium slot, ceramic, and stainless steel) and four archwires made of stainless steel, nickel-titanium, titanium-molybdenum alloy (TMA), and low-friction colored TMA.[21] According to their results the frictional values for colored TMA were comparable with those of the stainless steel wires. Ceramic with gold-palladium slot bracket and colored TMA archwire can be a good alternative to stainless steel in space closure with sliding mechanics.

1.2.2. Dental implants and factors influencing their biointegration

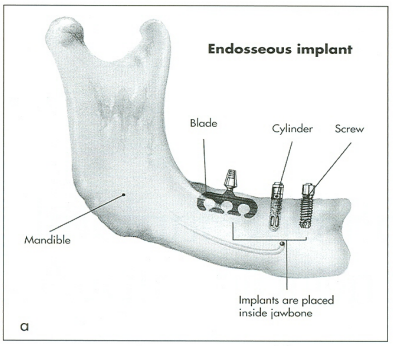
Ti was introduced in implant dentistry by chance in 1952 in the Laboratory of Vital Microscopy at the University of Lund, Sweden, by a team headed by Per Ingvar Brånemark, an orthopedic surgeon. Dr. Brånemark's team designed an optical chamber housed in a titanium cylinder, which was screwed into the rabbit's thighbone. After several months, they realized that the titanium cylinder had "fused" to the bone. He called this phenomena osseointegration. Many clinical trials were performed to test the success rate, the concept and the design of Ti implants. Finally, in 1982 the Toronto Conference on Osseointegration in Clinical Dentistry laid down the first rules on what is to be considered successful implant treatment and catalyzed also the acceptance and use of dental implants in North America.

Nowadays, endosseous dental implants, surgical implants for fixating or replacing hard tissue are mostly made from CP Ti and the most common Ti alloy, Ti6Al4V.[1,20]

Dental implants can be classified according to their location (Figure 11a, b, c):[6]

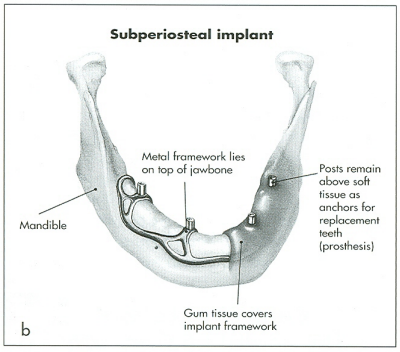
I. *Endosseus implants* (Figure 11a): are placed in mandibular or maxillar bone, through the oral mucosa and covers the edentulous ridge. There are three groups of endosseus implants:

- *root form:* inserted in maxillary and mandibular arch, in a case of completely or partially edentulous patients
- *blade (plate) form:* placed in maxillary and mandibular arch, for completely or partially edentulous patients
- *ramus frame:* inserted in a mandibular arch, for completely edentulous patients



II. *Subperiosteal implants* (Figure 11b)*:* It is placed on the surface of the bone, under the periosteum. There are three groups too:

- *complete:* it can be applied in the case of atrophized bone, but it needs adequate and stable bone to support the implant
- *unilateral:* placed on maxillary or mandibular arch
- *circumferential:* used for completely or partially edentulous patient



III. *Transosteal implants* (Figure 11c): It penetrates the inferior mandibular border, through the oral mucosa and covers the edentulous ridge. There are three groups too:

- *staple:* placed in the anterior bone
- *single pin:* placed in the anterior mandibular arch
- *multiple pin:* for completely or partially edentulous patient

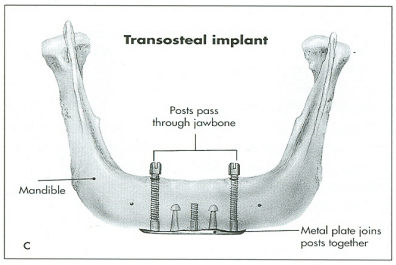


Figure 11. a, b, c Classification of dental implants based on their location.[6]

Nowadays, in clinical practice the root-form endosseus dental implants are the most frequently used. The success rate of mandibular implants is 96%, 94% and 86% at 5, 10, 15 years, respectively, while for maxillar implants this rate is lower 88%, 82% and 78%.[6]

The **success and long-term prognosis of endosseous implants** depend mainly on three factors: **osseointegration** (anchorage in the host bone), **gingival attachment** and the **appropriate transmission of masticatory force** (load transfer capacity).

Osseointegration is a complex process, during which a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant is provided.[6]

The healthy gingival attachment protects the bone-implant junction from oral bacteria. Damaging the soft tissues around the implant can lead also to damaging the hard tissues around the implant.

The gingiva surrounding the teeth is covered by three different stratified squamous epithelia surrounding the implant. These are gingival, sulcular and junctional epithelium. The junctional epithelium is the most apical portion, which is an undifferentiated epithelium with wide intercellular spaces, a likely portal of entry for bacterial antigens. The attachment of epithelial cells to metal constitutes the primary sealing mechanism of oral mucosa to dental implant. The soundness of this seal is one of the most important factors of the long-term success of the implant.[22]

The chemical characteristics of the implant surface are crucial to the adhesion and migration of cells because surface chemistry influences the adsorption of various serum proteins. The adsorption of certain proteins plays a key role in the subsequent cellular and molecular events that are critical to the attachment of cells to implant surfaces.[22]

For dental implants, likewise to biomaterials, bio- and osseointegration processes can be controlled at molecular and cellular level by modification of implant surface. There are several surface modification possibilities, usually classified as **physicochemical and biochemical methods**. The aim of the surface modification of biomaterials is to retain the key physical (bulk) properties of a biomaterial while modifying only the outermost surface to influence the biointeraction. Due to this the tissue-interface-related biocompatibility will be improved or changed.

The main research topics of our group are to investigate the biointegration of endosseous dental implants and how the chemical and surface microstructural modifications of these titanium implants influence their biointegration (or osseointegration).

The rationale for the surface modification of implants is therefore straightforward: to retain the key physical properties of an implant while modifying only the outermost surface to influence the bio-interaction. As a result, the implant manufacturing companies devote a lot of research work on developing an optimal Ti implant surface (e.g.: OsseoSpeed, ASTRATECH; SLActive, Straumann). This question is very complex, not only for the above mentioned problems, but also because a dental implant has several functional parts (root and neck), which are in contact with different biological tissues (alveolar bone, connective and epithelial tissue) (Figure 12). The optimal implant surface is different for any given purposes, thus, when the goal is to develop an implant surface, then the targeted functional part and the purpose of the modification has to be specified.

A -smooth surface for epithelial attachment and to prevent plaque formation (Polished surfaces: $R_a < 0.5$ μm).
B -machined, oblique part for proper connective tissue attachment
C -rough surface: developed for anchorage in the bone ($R_a > 0.5$ μm)

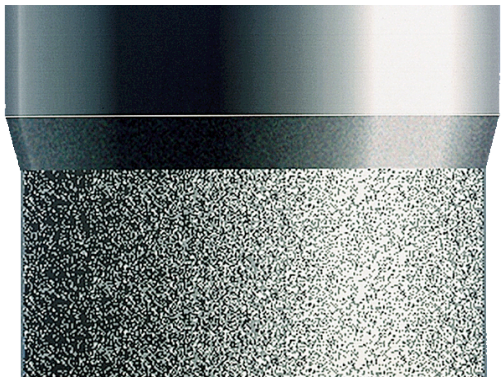


Figure 12. Three functional parts of implant.

In general, in case of a dental implant a smooth surface (A) is developed for epithelial attachment and to prevent plaque formation, a machined or rough oblique part (B) for proper connective tissue attachment, and a rough surface (C) for anchorage in the bone. The rough surface is commonly manufactured by one of the above mentioned physicochemical modifications, for e.g. oxidation, acid-etching and/or sand blasting.

Although my main research topic was not directly targeting these modified surfaces a lot of findings and research methods were useful when I investigated the chemical and surface microstructural alterations of titanium dental implants caused by different decontaminating and prophylactic agents. The *in vitro* (cell culturing) studies presented in this PhD work were performed with the intention to characterize the response of biological materials (living cells) to these altered surfaces as several experimental studies or clinical observations called the attention on these problems. It is my belief that these studies represent a huge positive contribution to clinical implant science, especially if we target the interaction of more and more used titanium with those chemical agents which are most commonly used in dentistry.

1.3. Prophylactic and chemical agents used in dentistry

1.3.1. Fluoride containing caries-preventive prophylactic agents used in dentistry

Patients regularly use different oral care products containing F^-, such as toothpastes, rinsing solutions, or prophylactic gels. The Ti alloys applied in the form of orthodontic wire or as the framework of a prosthesis, therefore come into contact with a wide range of preventive agents and these F^- containing materials can attack the surface of Ti.[23,24,25,26] SEM investigations have revealed that topical F^- solutions can cause stress corrosion cracking on

CP Ti.[27] Galvanic corrosion has been reported to occur between orthodontic wires and brackets (NiTi and CuNiTi) immersed in fluoride mouthwashes.[28] Such corrosion has two undesirable consequences: the mechanical performance of the wire-bracket system deteriorates, and the risk of local Ni^{2+} release is increased. As the pH of the rinses and gels used for caries prevention in dentistry ranges from 3.5 up to neutral, and the F^- concentration in these materials is between 1000 and 10,000 ppm,[29] it is essential for the dental practitioner to know whether a F^- -containing material can attack the Ti surface or can modify the corrosion resistance of the Ti surface of a dental implant, a prosthesis, or the wires of orthodontic braces. Besides 0.1–0.15% (1000–1500 ppm) F^-, toothpastes contain other constituents, such as rubbing, cleaning, foaming materials, and calcium complexes, which reduce the effectiveness of toothpastes by 25–50%.[30]

Oxidative agents are well known to exert a corrosive effect on the alloys used in dentistry, with the exceptions of Ti and other bioinert materials. Indeed, oxidative processes can thicken and condense the titanium oxide layer on the surface, improving the corrosion stability of the underlying Ti. On the other hand, reductive agents, such as fluoride (F^-), may have the opposite effect and attack this layer. Strietzel et al. demonstrated that Ti ion release was enhanced in the presence of F^-, and this effect was even further accelerated at low pH.[31] High F^- concentrations and an acidic pH are known to impair the corrosion resistance of Ti, and as a result crevice and pitting corrosion occur.[32,33,34] According to Velasco-Ortega this titanium alloy (Ti6Al4V) was neither cytotoxic nor genotoxic in any of the tests performed.[35]

Although all the above mentioned studies support the inconvenient effect of fluoride-containing prophylactic gels, there is a huge amount of data documenting that fluoride has a bone promoting activity when a controlled amount of fluoride is attached to the surface. Ellingsen et al. proved that, when fluoride ions are incorporated in the titanium oxide layer the retention of the implants was significantly increased, even when compared to rough surfaced implants.[36,37] The success of the TiO_2-blasted surface with a fluoride-modified titanium dioxide layer (OsseoSpeed implants, ASTRATECH) lies in the ability of the fluoride coating to stimulate the bone response leading to a connection between titanium and phosphate from tissue fluids. The free fluoride ions catalyze this reaction and induce the formation of fluoridated hydroxyapatite and fluorapatite in the surrounding bone.

The studies of Cooper et al. demonstrated that fluoride ion modification of TiO_2 grit-blasted CP titanium surfaces enhanced osteoblastic differentiation and interfacial bone formation.[38]

1.3.2. Chemical agents used for decontamination dental implant surfaces

Peri-implant infections involve peri-implant mucositis, defined as a reversible inflammatory change of the peri-implant soft tissues without bone loss, and peri-implantitis, an inflammatory process resulting in loss of supporting bone and associated with bleeding and suppuration.[39,40,41]

Several studies have evaluated peri-implant infections, but only a few were cross-sectional and provide information on the prevalence of peri-implant diseases among patients with implants functioning for ~10 years. The incidence of peri-implant mucositis has been reported to be in the range of 60% of implant recipients and in 48% of implants.[42,43] The prevalence of peri-implantitis was found to be around 15, 16, and 28% with respect to the recipients,[44] and 7 and 12% regarding implant sites.[45] The differences in the prevalence of peri-implantitis may be explained by differing criteria used for the diagnosis of peri-implantitis, as well as variations in maintenance procedures.[41,45]

The etiology of marginal peri-implantitis is based mainly on an infectious factor and a biomechanical factor.[46] Although the causes may differ in both cases, microbial colonization occurs on the surface of the implant.[40,47] If the conditions become pathogenic, bacteria start to proliferate, leading to inflammation around the implant. Peri-implant diseases have been primarily linked to Gram-negative anaerobic microflora.[48] The process is aggravated by microorganism colonization and their toxins, and extensive bone destruction will occur. The inflammation spreads apically thus, in very severe cases, therefore, the patient may lose the implant. Methods which remove the bacteria and the toxins from the surface of challenged implants would prevent or terminate the development of peri-implant bony defects.

The therapy of peri-implantitis in the surgical phase is a complex process, starting with surgical debridement of devitalized peri-implant tissue and continuing with decontamination of the exposed implant surface. The implant surface can be cleaned by mechanical (an air-powder abrasive) or chemical (citric acid, H_2O_2, chlorhexidine digluconate (CHX) or EDTA) procedures or with laser irradiation (CO_2, diode, Er:YAG or Nd:YAG).[49,50] To support

antimicrobial treatment, topical, and/or systemic antibiotics may be administered. After removal of damaged tissues from the peri-implant pocket, surgical treatment (guided tissue regeneration with or without the use of bone grafts and barrier membranes) promotes regeneration of any bone defect.[49,51]

For the chemical detoxification of implants, various cleaning solutions are used: CHX, H_2O_2, citric acid, phosphoric acid gel, delmopinol, Listerine^R, iodine, saline irrigation, beta-isodona, chloramine-T, and so forth. Besides these chemical agents, a number of systemic antibiotics can be applied to support the therapy: for example tetracycline, amoxicillin, augmentin, metronidazol, penicillin, and so forth.[41,49]

CHX is a commonly administered antimicrobial agent with a wide range of medical applications. It is used in dentistry as a mouthwash and topical antimicrobial. In the treatment of peri-implantitis it can serve as a rinsing solution,[52,53] or more often as an implant irrigation solution, in combination with systemic antibiotics.[49,51] Renvert et al. investigated the difference in effectiveness of minocycline microspheres and CHX gel, and concluded that the adjunctive use of these microspheres led to improved probing depths and bleeding scores, CHX alone resulting in only a limited reduction of the bleeding scores.[54,55] CHX is also effective in the surgical treatment of late peri-implant defects using guided tissue regeneration.[56,57] Recognizing the increasing interest in the functionalization of dental implant surfaces with antimicrobial agents prior to implantation, Barbour et al.[58] investigated the adsorption of CHX to TiO_2 crystals of anatase and rutile. Their results proved that CHX in 4-morpholinoethanesulfonic acid (MES) and phosphate-buffered saline (PBS) buffers adsorbed rapidly to anatase and rutile TiO_2, equilibrium being attained in less than 60s, with gradual desorption over a period of several days. More CHX adsorbed to anatase than to rutile, and the CHX desorbed more rapidly from anatase than from rutile, depending on the buffer used. The study by Burchard revealed that fibroblasts adhere more readily to surfaces exposed to CHX or saline than to those exposed to stannous fluoride.[59]

Saturated citric acid can also be applied for the decontamination of Ti surfaces in the surgical treatment of peri-implantitis with bone grafts and membranes.[60,61]

In a comparison of the effects of citric acid and 10% H_2O_2, Alhag et al. demonstrated that rough surfaces (with an enhanced TiO_2 layer and textured surface; Nobel Biocare AB^R , Gothenburg, Sweden) which were plaque-contaminated and cleaned with either solution, can re-osseointegrate.[62] H_2O_2 can be used successfully at a concentration of 3% in the surgical

treatment of peri-implantitis, employing bone substitutes with, or without, resorbable membranes.[63,64] Some authors, including Khoury have even used a combination of these three different cleaning solutions in the surgical therapy of peri-implantitis.[51] After removal of the granulomatous tissue, the surgical site was repeatedly rinsed with CHX, after which citric acid (pH = 1) was applied for 1 min to decontaminate the implant surface, this then being rinsed with H_2O_2 and 0.9% saline. Dennison et al. found that machined implants (without a surface coating) are decontaminated by a variety of methods (air-powder abrasive, citric acid solution, or CHX) more readily than hydroxyapatite-coated surfaces.[65]

The above-mentioned chemical agents are commonly applied in the therapy of peri-implantitis, but only investigations relating to the adsorption of CHX on different TiO_2 crystals (anatase and rutile) appear to have been conducted. When used for implant surface decontamination, these materials may alter the morphology and chemical structure of the surface.

2. Aims and questions to be answered

The aim of my PhD work was to investigate the effect of decontaminating and chemical solutions commonly used in dentistry on titanium surfaces. For that, I applied *in vitro* testing methods with human epithelial and osteoblast cells. These methods are regularly used for testing the biointegration of alloplastic materials, before performing the more expensive and time consuming *in vivo* or clinical studies. The examined problems presented in this work represent the first studies in this field. In the laboratory of the Faculty of Dentistry we can routinely separate epithelial and fibroblast cells from oral mucosa, allowing us to examine the response of the epidermal cells to different surfaces. Our experiments are very close to (mimic) the *in vivo* conditions, as we work with human oral mucosa.

In my first study, I investigated the effects of different F^--containing caries-preventive prophylactic rinses and gels on the surface structure and roughness of CP Ti, through the use of XPS and AFM. A further aim was to survey the attachment and proliferation of human epithelial cells after treatment of the Ti surface with an acidic NaF solution, a widely used F^--containing mouthwash or a gel. There has so far been no study of the behavior of epithelial cell growth on F^--treated Ti implant surfaces. The epithelial cell attachment and proliferation were examined by means of dimethylthiazol-diphenyl tetrazolium bromide (MTT) and protein content assays (the latter with bicinchoninic acid). For the visualization of cells, scanning electron microscopy (SEM) was applied. Overall we have examined:

- *The changes in the roughness of the F^--treated titanium surfaces*
- *The alterations in the composition of the titanium surfaces caused by different fluoride treatments*
- *The attachment ability of human epithelial cells to these modified surfaces*
- *The proliferation ability of human epithelial cells to these altered surfaces*

The aim of my second investigation, was to study the effects of three cleaning solutions in clinical use for peri-implantitis therapy. *In vitro* studies are essential in the development of such treatments, as these are the basic steps with which to reveal the action of cleaning solutions on the implant surface. Additionally, fewer animal experiments would be

required. In the present investigation, the effects of three different cleaning solutions (3% H_2O_2 solution, saturated citric acid (pH = 1) and CHX gel) on the chemical structure and surface roughness (R_a) of CP Ti were investigated, through the use of X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). A further aim was to survey the response of the biological environment to these changes, by examining the attachment and proliferation of human epithelial cells after treatment of the Ti surfaces with these solutions. The epithelial cell attachment and proliferation was again examined by means of MTT and protein-content assays.

Overall I examined:

- *The changes of the roughness of titanium surfaces after treatments with the different decontamination solutions*
- *The alterations in the composition of titanium surfaces due to the cleaning treatments*
- *The attachment ability of human epithelial cells to the cleaned surfaces*
- *The proliferation ability of human epithelial cells to the cleaned surfaces*

These studies represent a huge contribution to clinical implant science as the tested solutions are applied every day by the dental practitioner and more and more patients have titanium made appliances.

3. Materials and methods

3.1. Materials used for testing the effects of fluoride containing prophylactic agents (epithelial cell culturing experiments)

Ti discs (9 mm in diameter and 2 mm in thickness) were made from implant material (CP grade 4, CAMLOG Biotechnologies AG, Switzerland). The discs were mechanically polished to a surface roughness not exceeding 0.2 μm, the roughness needed for the neck of a dental implant.[66] The discs were cleaned in acetone and absolute ethanol in an ultrasonic bath for 15 min. Each sample was immersed for 1 h in one or other of (1) a caries-preventive prophylactic mouthwash (Elmex, GABA International AG, Switzerland) containing 250 ppm F^- (pH 4.4), which contains fluoride in form of Olaflur (bis-(hydroxyethyl)-aminopropyl-N-(hydroxyethyl)-octadecylamin dihydrofluoride) and potassium-fluoride; (2) an aqueous solution of 1% NaF (3800 ppm F^-, pH 4.5), where the pH of the NaF solution was set to 4.5 with lactic acid; or (3) a gel (Elmex, GABA GmbH, Germany) containing a total of 12,500 ppm (1.25%) F^- [pH 4.8 (10% in water); www.gaba. com], 2500 ppm (0.25%) in the form of the amine fluorides Olaflur and Dectaflur (hexadecylamine hydrofluoride), and the rest in the form of sodium fluoride (1%).

After 1 h, the samples were removed from the F^- containing medium, thoroughly washed with ultrapure water and dried. As the suggested use of the prophylactic rinse and the gel is once a day for 30 s and once a week for 2 min, respectively, our application time corresponds to the accumulated effect of regular usage of 4 months for the rinse and 7.5 months for the gel. The 1 h treatment time seems to be too long, but if we take in consideration that these prophylactic solutions are not rinsed after application, then we may think about shorter cumulative periods.

A PSIA XE-100 atomic force microscope (South Korea) was used to acquire information on the roughness of the sample surface., described in chapter: 3.3.

The chemical composition of the Ti surfaces was studied by XPS, described in chapter: 3.4.

The control and treated Ti discs were sterilized under UV-C radiation (20 s) for the epithelial cell culturing experiments. The cell cultures involved human gingival mucosa from healthy consenting adult (age 18–24) donors. All subjects enrolled in this research have

responded to an Informed Consent, and the scheme of the experiments has been approved by the Human Investigation Review Board of University of Szeged, as it complied with the ethical standards of the research, in accordance with the Helsinki Declaration. A quantity of 10^4 cells/ml/disc from the cell culture in the 3rd passage was plated on the Ti discs, in 48-hole cell culture plates. The cell adhesion was determined at 24 h, and the cell proliferation at 72 h. Three independent experiments were performed, and for each treatment five Ti samples were used. MTT measurements and protein content assays were used to investigate how the cells survived and proliferated on the surfaces treated with the materials containing different amounts of F^-.

Statistical analysis was done using Student's t-test for two samples, where $p = 0.05$ was considered as the level of significance.

3.2. Materials used for testing the effects of chemical agents in the treatment of peri-implantitis (epithelial cell culturing experiments)

Ti discs (9 mm in diameter and 1.5 mm in thickness) were made from CP grade 4 implant material (CAMLOG^R Biotechnologies AG, Switzerland). The surfaces of the discs were machined (through turning), with a roughness (R_a) < 0.2 μm, a typical roughness for the abutment of a dental implant.[66] The discs were cleaned in acetone and absolute ethanol in an ultrasonic bath for 15 min. After cleaning, the discs were treated with 3% H_2O_2, saturated citric acid (pH = 1) or CHX gel (Corsodyl^R dental gel; SmithKline Beecham Consumer Healthcare, UK). Corsodyl^R contains 1% w/w CHX. As in the usual clinical protocols, the durations of treatment were 5 min for H_2O_2 and CHX gel, and 1 min for citric acid. The control group was rinsed with ultrapure water for 5 min. After treatment, the samples were washed three times with ultrapure water and dried.

For AFM a PSIA XE-100 instrument (PSIA Inc., South Korea) was used, the chemical composition of the Ti surfaces was studied by XPS, described in the following chapters.

A quantity of 10^4 cells/0.5 ml medium/disc from the cell culture in the 3rd passage was plated on the Ti discs, in 48-hole cell culture plates. The cell adhesion was determined at 24 h, and the cell proliferation at 72 h. In all, four independent experiments were performed, and five Ti samples were used for each treatment.

3.3. Atomic force microscope (AFM)

A PSIA XE-100 atomic force microscope (South Korea) was used to acquire information on the roughness of the sample surface in our studies. AFM is a technique that measures forces between the AFM probe tip and the sample as it approaches and retracts from the investigated surface. The tips were contact silicon cantilevers (type: P/N 910M-NSC36) purchased from MikroMasch Eesti OU (Estonia). Cantilevers with spring constants of 0.95 and 1.75 N/m were used. The measurements were performed in contact mode, and the height, deflection, and 3D images with areas of 10 μm × 10 μm and 5 μm × 5 μm were captured. The surface roughness (R_a) was determined via the AFM software program (at least 10 independent measurements), and was defined as the arithmetic average of the surface height relative to the mean height. R_{pv} was also determined, as the difference between the highest (peak) and deepest (valley) values of the surface.

3.4. X-ray photoelectron spectroscopy (XPS)

The chemical composition of the Ti surfaces was studied by XPS. The photoelectrons were generated by Al Kα primary radiation (hν = 1486.6 eV) and analyzed with a hemispherical electron energy analyzer (PHOIBOS 150 MCD 9; manufactured by SPECS). The X-ray gun was operated at 150 W (12 kV, 12.5 mA). The binding energies were normalized with respect to the position of the C 1s peak of adventitious carbon, which was taken as 285.1 eV. The changes in the XPS spectra were measured after 30-60 min of He^+ bombardment, which was repeated several times. He^+ ions were generated with an ion gun energy of 5 kV, and the incident ion beam current was measured at 200 nA. The bombardment led to the removal of a thickness of ~10 nm from the surface material during the operation. Wide-range scans and high-resolution narrow scans of the Ti 2p, O 1s, and C 1s characteristic peaks were recorded.

3.5. *In vitro* cell culture testing

3.5.1. Epithelial cell separation from oral mucosa

Adult epidermal epithelial cells were isolated and cultured from inflammation-free oral mucosa of healthy donors (age 18-46) undergoing dento-alveolar surgery. The protocol of the experiments was approved by the Human Investigation Review Board at the University of Szeged: it complied with the ethical standards of research, in accordance with the Helsinki Declaration. All subjects enrolled in the research gave their signed informed consent.

Cell culturing techniques

After removing the mucosa specimens were washed in Salsol A solution (sterile isotonic salt solution: Human Rt, Gödöllő, Hungary) supplemented with 2% antibiotic, antimycotic solution (Sigma-Aldrich GmbH, Germany). Overnight incubation in dispase solution (Grade II, Roche Diagnostics, Mannheim, Germany) (Figure 13) was carried out at 4°C to separate the dermis from the epidermis.[67]

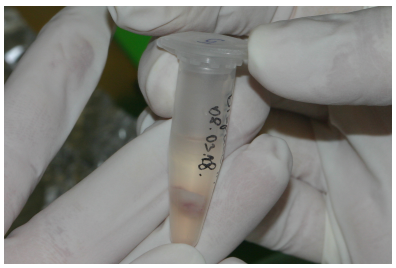
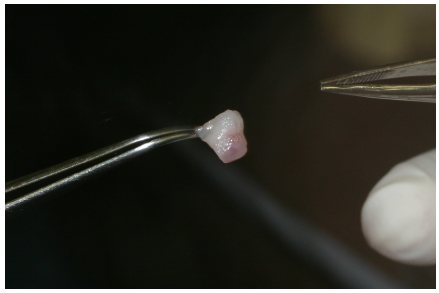


Figure 13. Mucosa in dispase after overnight incubation.

Next day, the epidermis was peeled off the dermis. (Figure 14 a, b)



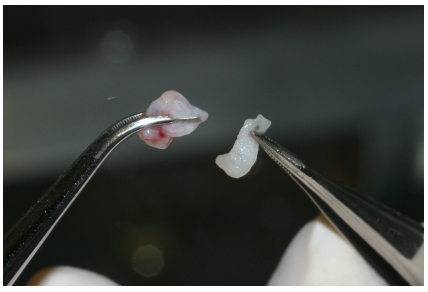


Figure 14. a: Mucosa before peeling. **b**: The epidermis was peeled off the dermis with forceps.

The epidermis was placed in 0.25% trypsin-EDTA solution (Sigma-Aldrich GmbH, Germany) for 5 min at 37°C. (Figure 15)

Figure 15. Epidermis in trypsin-EDTA solution.

Following trypsinization, the epidermis was torn apart mechanically and washed vigorously to release epidermal cells. The epidermal cell suspension was centrifuged at 200 g for 10 min at 4 °C. (Figure 16)

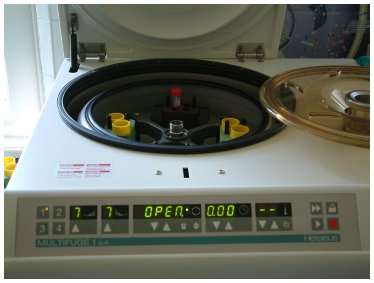


Figure 16. Centrifuge with cell mixture in centrifugal tube. The suspension was centrifuged at 200g for 10 minutes at 4 °C.

The supernatant was removed and the medium (KSFM) was added to the pellet, after this the pellet was suspended to cells (Figure 17.). The epidermal cells were then placed in 25 cm^2 tissue culture dishes (Orange Scientific, Belgium).[68,69]

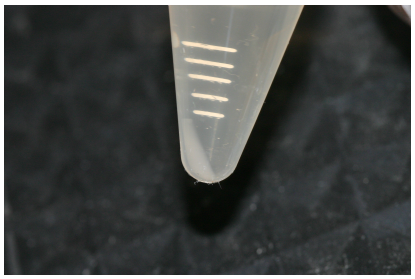


Figure 17. Pellet in centrifugal tube.

The oral epithelial cell culture medium consisted of keratinocyte serum-free medium (KSFM) with L-glutamine (Gibco BRL, Eggstein, Germany), supplemented with recombinant epidermal growth factor 2.5 µg/500 ml (Gibco BRL, Eggstein, Germany), bovine pituitary extract 25 mg/500 ml (Gibco), L-glutamine and antibiotic/antimycotic solution containing penicillin G sodium 1%, streptomycin sulfate 1% and amphotericin B 0.0025% (Sigma-Aldrich GmbH, Germany).

Fresh culture medium was added to the cells three times weekly. The primary epithelial cell cultures reached ~ 90% confluence in 8-16 days. (Figure 18 a, b)

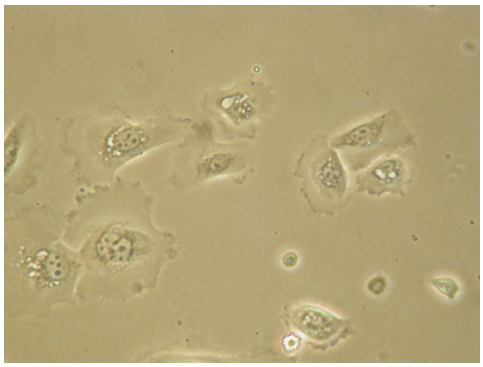
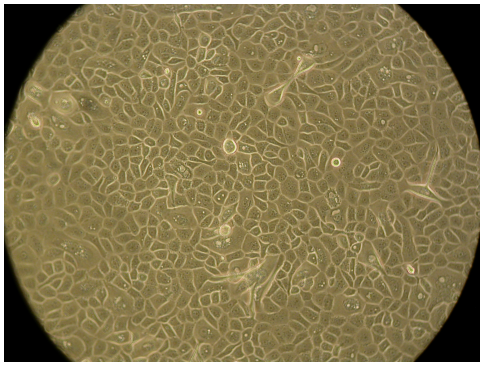


Figure 18. a) Few attached primary epithelial cells. (magnification: x 400)



b) Confluent epithelial cell culture. (magnification: x 200)

Confluent primary cultures were treated with phosphate-buffered saline (pH = 7.4, Gibco) and cells were harvested by a 2-4-min trypsinization with 0.25% trypsin-EDTA solution (Sigma-Aldrich GmbH, Germany). Harvested cells were divided into 2-4 equal parts at passages. Cultures were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

Counting of the cells was performed with "Bürker"-chamber in trypane blue (Figure 19).

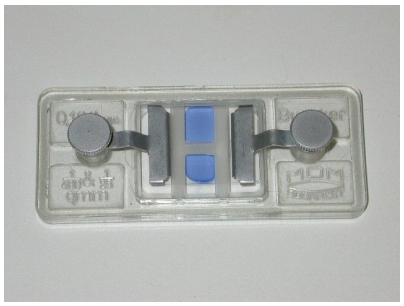


Figure 19. Bürker-chamber with cell suspension in trypane-blue.

3.5.2. Assays used for measurement of cell growth and proliferation

Protein content assay

The protein content of all the cells (both living and dead) was measured with a micro BCA™ protein assay kit. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline medium (the Biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing

bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 μg/ml). The BCA method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

A micro BCA™ protein assay kit (Figure 20) containing bicinchoninic acid was applied (Pierce, Rockford, IL, USA). An ascendant set of dilutions with an albumin standard (BSA) was made as control.

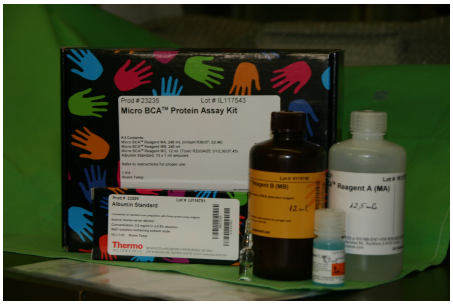


Figure 20. Micro BCA™ protein assay kit (Pierce, Rockford, IL, USA)

The cells were dissolved with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin), the reagent (green) was then applied, according to the manufacturer's instructions, and the solution was incubated for 2 h at 37 °C. Due to this reaction, the color changed to purple. E_{540} was determined with a Multiscan Ex spectrophotometer (Thermo Labsystems, Vantaa, Finland) and Ascent Software (Thermo Labsystems, Vantaa, Finland) at the Department of Dermatology and Allergology (University of Szeged, Faculty of Medicine).

MTT

The MTT is a rapid colorimetric assay widely used for cellular growth and survival study.[70] The growth of cultured cells was measured with a rapid colorimetric assay, which determines living cell numbers by the reduction of MTT. The MTT system is measuring the activity of living cells via mitochondrial dehydrogenases.

The MTT method is simple, accurate and yields reproducible results. The key component is (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTT. Solutions of MTT, dissolved in medium or balanced salt solutions without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals which are insoluble in aqueous solutions. The crystals are dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.

Cells were seeded into 48-well culture plates at a density of 3000 or 10^4 cells/well and grown on Ti discs in culture media for 24 or 72 h. The supernatant was removed and replaced with 0.5 mg/ml MTT solution (Sigma-Aldrich GmbH, Germany) in RPMI media without phenol red. After incubation for 4 h at 37 ^oC, the medium was gently removed from each well and the crystallized dye was solubilized with 2% sodium dodecyl sulfate (SDS) and 0.04 mM HCl in absolute isopropanol. The optical density of the color reaction at 540 nm (E_{540}) was determined with a Multiscan Ex spectrophotometer (Thermo Labsystems, Vantaa, Finland) and Ascent Software (Thermo Labsystems, Vantaa, Finland) at the Department of Dermatology and Allergology (University of Szeged, Faculty of Medicine).

Data presentation and statistical analysis

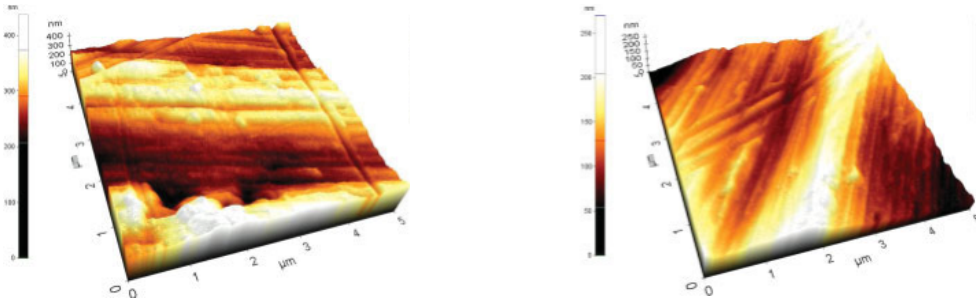
The means ± the standard errors of the mean (SEM) were calculated for the R_a (nm) values measured by AFM. The MTT and protein content assay data are presented as means ± SEM of OD_{540}. After normality testing, data were compared via one-way analysis of variance (ANOVA), followed by Tukey's, Scheffe, LSD, Benferroni and Sidak post hoc tests to determine statistical differences after multiple comparisons (SPSS 15.0, SPSS, Chicago, IL). A probability value < 0.05 was considered significant.

4. Results and Discussion

4.1. Evaluation of the effects of fluoride containing prophylactic gels on titanium implant surface

4.1.1. Surface characterization by AFM and XPS

Before treatment, the polished Ti samples were tested by AFM and XPS. Figures 21a and b reveal the almost parallel grooves on the control and mouthwash (250 ppm F^-) - treated samples; these grooves originate from the mechanical machining of the samples (the color becomes lighter on proceeding from the depths of the grooves toward the surface).



A B

Figure 21. 3D AFM pictures of typical (A) control (untreated) and (B) mouthwash-treated (250 ppm F^-, pH 4.4) Ti samples. The almost parallel grooves originate from the mechanical machining of the samples. The color becomes lighter on proceeding from the depths of the grooves toward the surface. Image size: 5 × 5 µm. Stájer et al.[71]

The AFM measurements gave R_a = 37.0 ± 2 nm for the control samples, and 51.3 ± 4 nm for the mouthwash-treated samples (Figure 22).[71]

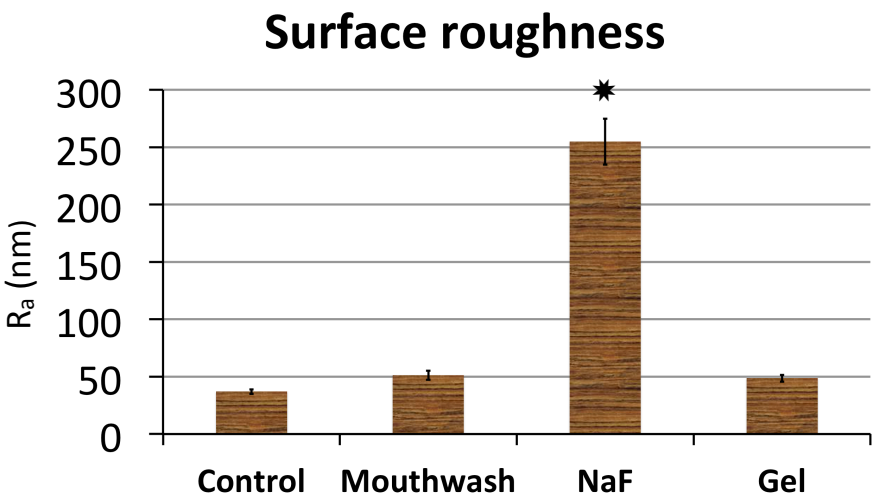
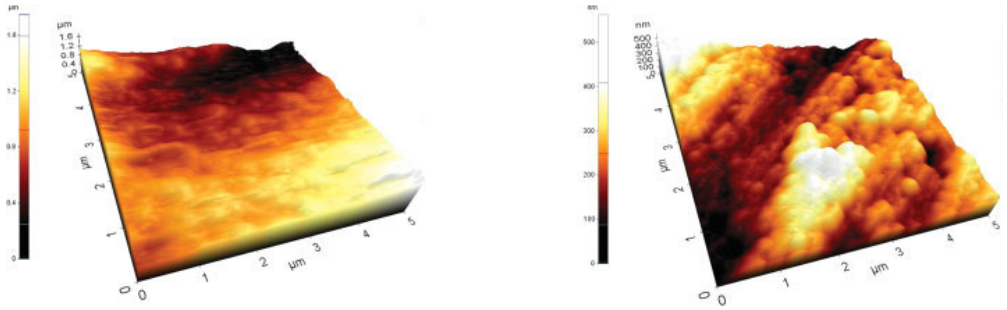


Figure 22. Overall bar-graph of the surface roughness (R_a) of the various samples. The AFM measurements gave R_a = 37.0 ± 2 nm for the control (untreated) samples, 51.3 ± 4 nm for the mouthwash-treated (250 ppm F^-, pH 4.4) samples, 254.8 ± 20 nm for the NaF solution (3800 ppm F^-, pH 4.5) and 48.6 ± 3 nm for the gel-treated (12,500 ppm F^-, pH 4.8) sample. Significant different (✷). Stájer et al.[71]

Although major differences can not be observed between the two samples, R_a was significantly ($p = 0.007$) higher than the control value. After treatment with 1% NaF solution (3800 ppm F^-, pH = 4.5), the Ti discs displayed the biggest increase in R_a (Figure 23A) the depth of the grooves was almost 7 times the control depth: $R_a = 254.8$ nm ± 20 nm ($p < 0.001$, Figure 21).



A B

Figure 23. 3D AFM pictures (A) of a Ti disc treated with 1% NaF (3,800 ppm F^-, pH 4.5), and (B) of a characteristic gel-treated (12,500 ppm F^-, pH 4.8) Ti sample. The Ti discs treated with NaF solution displayed an almost 7 times increase in R_a and for gel-treated samples the AFM picture revealed deep corrosive regions and granular forms. Image size: 5 X 5 µm. Stájer et al.[71]

On the discs immersed in the gel (12,500 ppm F^-) the AFM (Figure 23B) picture revealed deep corrosive regions and granular forms, and the average roughness of the gel-treated surface was significantly increased, $R_a = 48.6 \pm 3$ nm ($p = 0.005$), as compared with the control samples (Figure 22).

The results of the AFM and XPS measurements are presented in details in Stájer et al. and the thesis of A. Stájer (2012), therefore, are not considered as part of the results section of this thesis, and do not represent thesis points of this thesis. For a better understanding of the results of the epithelial cell culture testing we give a short description.[71] XPS revealed that the high F^- concentration and acidic pH of the gel and the 1% NaF solution resulted in strong corrosion and modification of the composition of the Ti surface. The complex Na_2TiF_6 was formed, bound strongly to the surface. Therefore it was important to test the response of epithelial cells the these modified surfaces.

4.1.2. MTT and protein content measurements

The results of MTT and protein concentration experiments relating to cell attachment (24-h observation) are illustrated in the bar-graphs of Figure 24A and B.

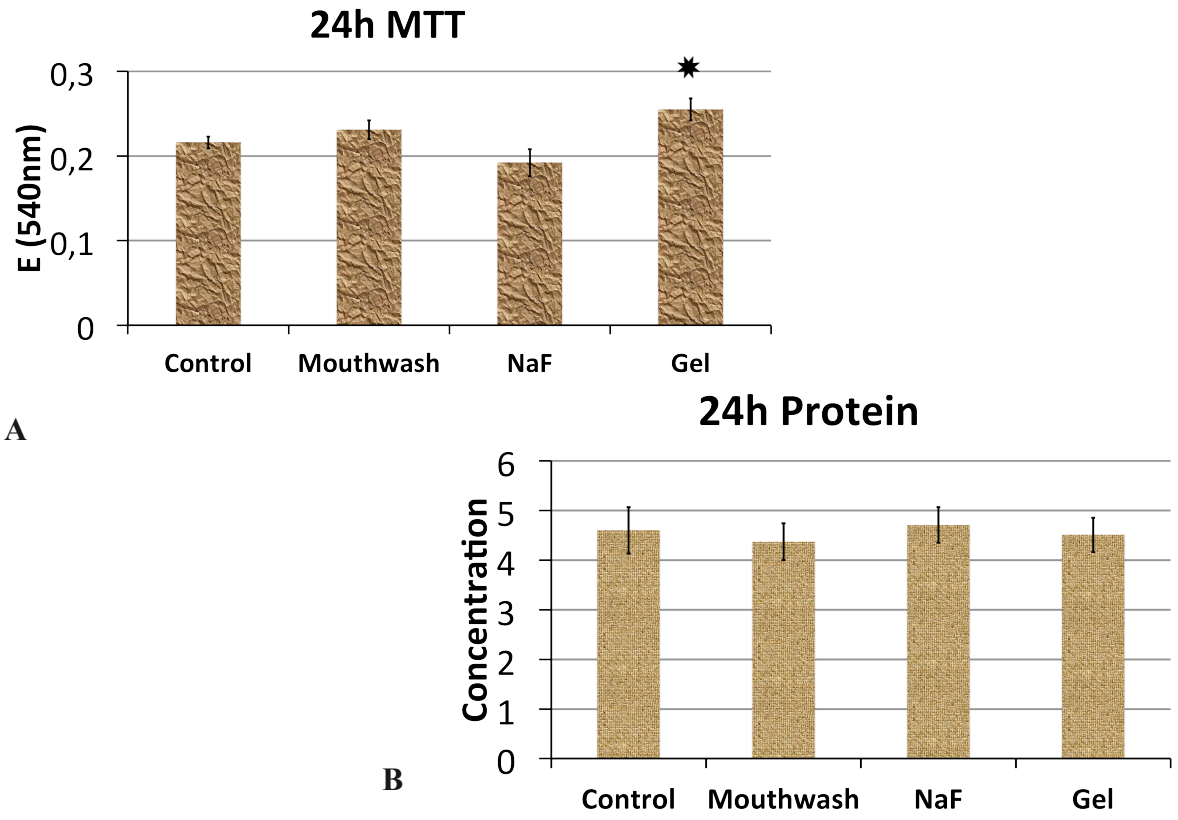


Figure 24. 24-h MTT (A) and protein concentration (B) results. The MTT results indicated that the epithelial cell attachment on the Ti surface was not disturbed significantly by immersion in the mouthwash or NaF, but after immersion in the gel, the attachment was significantly stronger. The protein concentration after 24 h was the same for all Ti samples, independently of the applied fluoride containing material. Significant different (✷).[71]

The MTT results indicated that the epithelial cell attachment on the Ti surface was not disturbed significantly by immersion in the mouthwash or NaF ($E_{540,Control}$ = 0.216 ± 0.007, $E_{540,Mouthwash}$ = 0.231 ± 0.011, $E_{540,NaF}$ = 0.192 ± 0.016). Following immersion in the gel, however, the attachment was significantly stronger ($E_{540,Gel}$ = 0.255 ± 0.013; p = 0.015). The protein concentration after 24 h was the same for all Ti samples, independently of the applied fluoride containing material ($c_{Control}$ = 4.60 ± 0.47).

The MTT and protein content assay results concerning cell proliferation (72-h observation) are presented in Figure 25A and B.

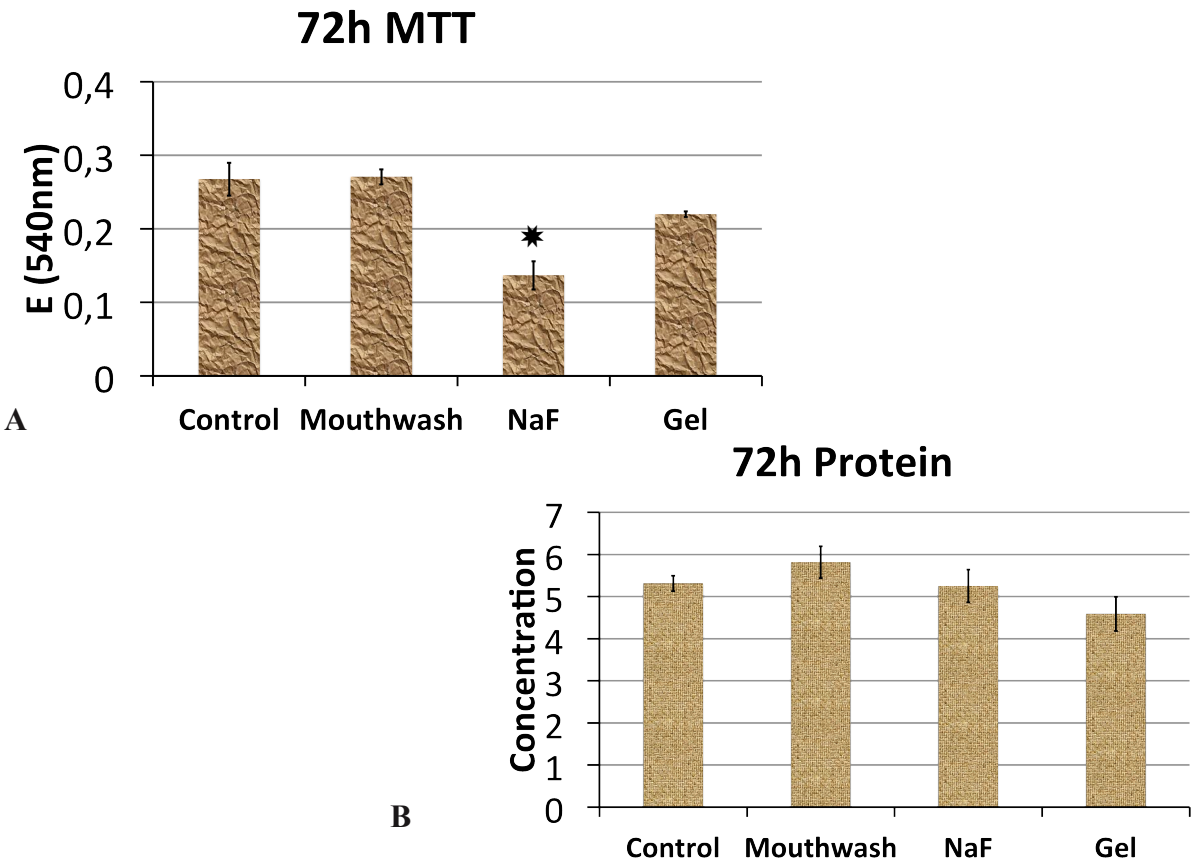


Figure 25. 72-h MTT (A) and protein content assay (B) results. The level of cell proliferation revealed by the MTT measurement was decreased significantly only in the case of the NaF-treated sample. The protein content assays demonstrated the same tendency as the MTT measurements for the gel-treated sample: a significant (but slight) decrease relative to the rinse treated sample. Significant different (✷).[71]

The level of cell proliferation revealed by the MTT measurement was decreased significantly ($p < 0.001$) only in the case of the NaF treated sample ($E_{540,Control} = 0.268 \pm 0.022$, $E_{540,NaF} = 0.137 \pm 0.004$, $E_{540,Mouthwash} = 0.271 \pm 0.01$, $E_{540,Gel} = 0.221 \pm 0.019$). The protein content assays demonstrated the same tendency as the MTT measurements for the gel-treated sample: a significant (but slight) decrease ($c_{Gel} = 4.59 \pm 0.41$ µg/ml; $p = 0.0312$) relative to the rinse-treated sample ($c_{Mouthwash} = 5.82 \pm 0.38$ µg/ml). A significant change was not detected for the NaF-treated sample ($c_{NaF} = 5.25 \pm 0.39$ µg/ ml; $c_{Control} = 5.31 \pm 0.18$ µg/ ml).

The influence of the surface roughness on epithelial cell growth has been studied by many authors and it has been proved that epithelial cells do not approach so closely to acid-etched and sand-blasted surfaces as to smooth (polished, $S_a < 0.5$ µm) surfaces.[72] Baharloo et al. observed that surfaces with smooth topography promote epithelial-cell growth, spreading, and the production of focal contacts on Ti surfaces.[73] Although our treatments were rather

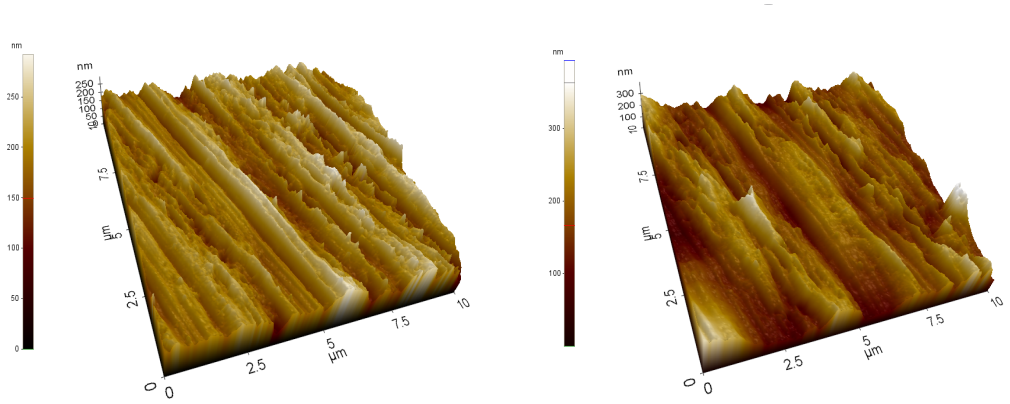
strong (for the gel and NaF, even the presence of Na_2TiF_6 was detected), the roughness never exceeded 0.5 μm, and the protein concentration was not decreased as compared with the control. This is in accordance with the findings of the above-mentioned authors.

The MTT method revealed a significant increase in cell attachment for the gel-treated sample, and a decrease in proliferation for the NaF-treated sample. The difference between the results obtained with these two methods is not yet understood, but may well be associated with the inherent differences between the methods. MTT assay measures the amount of living cells, while in the protein related measurements all the cells (viable and nonviable) are included.

4.2. Evaluation of the effects of decontaminating agents used for the treatment of peri-implantitis on titanium implant surface

4.2.1. AFM measurements

Before the cell culture experiments, the Ti samples were tested by AFM and XPS. Figures 26A and B reveal the almost parallel grooves on each sample, originating from the machining.



A B

Figure 26. 3D AFM pictures of (A) a typical control (untreated) sample and (B) a characteristic citric acid treated (saturated, pH = 1). The almost parallel grooves originate from the machining of the samples. The color becomes lighter on proceeding from the depths of the grooves toward the surface. Image size: 10× 10 μm.[74]

The AFM measurements gave $R_a = 22 \pm 3$ nm for the control samples (Figure 26A and 27), 25 ± 7 nm for the citric acid-treated samples (Figure 26B and 27), 30 ± 5 nm after treatment with 3% H_2O_2 solution (Figure 27), and 14 ± 4 nm for the CHX gel-treated discs

(Figure 27), probably a result of gel adsorption to the Ti surface.[57] The differences were not significant statistically.[74]

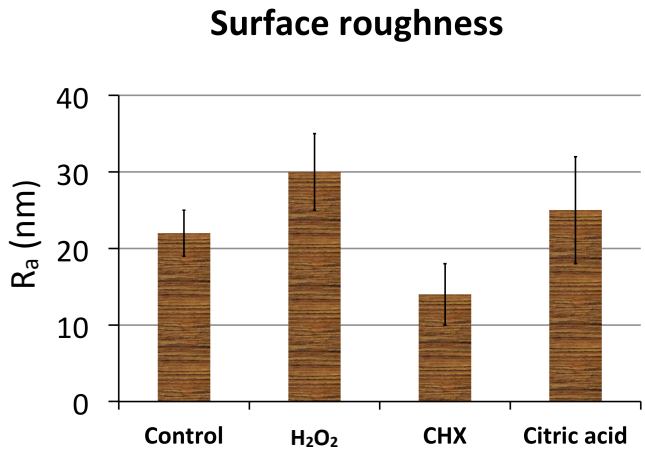


Figure 27. Overall bar-graph of the surface roughness (R_a) of the various samples. The AFM measurements gave R_a = 22 ± 3 nm (mean ± SEM) for the control (untreated) samples, 30 ± 5 nm for the H_2O_2 (3%)-treated samples, 14 ± 4 nm for the chlorhexidine gel-treated samples, and 25 ± 7 nm for the citric acid (pH = 1) - treated samples. Statistical analysis did not reveal any significant differences between the groups. [74]

4.2.2. XPS measurements

The XPS measurements revealed Ti, O, C and N in the topmost atomic layers of all samples (untreated and treated).

The binding energy of Ti 2p 3/2 electrons, which corresponds to Ti^{4+}, was measured at 458.6 ± 0.1 eV for each sample (Figure 28). The double Ti peaks (Ti 2p at 458.6 and 464 eV) and the O 1s signal (530 eV) demonstrate the presence of the TiO_2 layer.[75,76] The immersion in the different cleaning solutions did not change the Ti 2p signal of the surface (Figure 28).

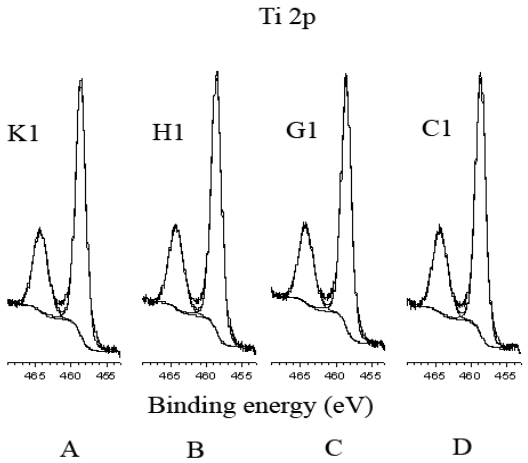


Figure 28. Ti 2p signals in XPS spectra of (A) control (K1), (B) H_2O_2- treated (H1), (C) chlorhexidine gel-treated (G1) and (D) citric acid treated (C1) Ti discs, confirming the presence of TiO_2 on all surfaces. [74]

Major changes were observed in the O 1s peak, which could be deconvoluted into three peaks (Figure 29). The most intense one, at ~ 530.1 eV, is that of lattice O in TiO_2, while

the peak at ~531.7 eV is due to surface OH groups. The third peak, at 532.9-533.0 eV, corresponds to the O in C-O and/or C=O bonds. The latter is most intense for the CHX gel-treated sample (Figure 29B), which can be explained in terms of the possible adsorption of CHX to the surface. [58]

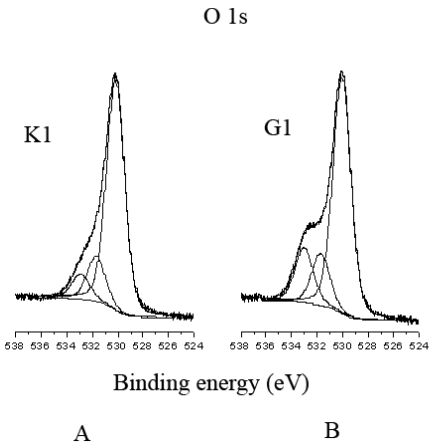


Figure 29. O 1s signals in XPS spectra of (A) control (K1) and (B) chlorhexidine gel-treated (G1) Ti discs. The signal was deconvoluted into three peaks: the most intense one (at 530.1 eV) is that of lattice O in TiO_2, while that at ~531.7 eV is due to surface OH groups. The third peak, at 532.9–533.0 eV, corresponds to the O in C-O and/or C=O bonds. The latter is most intense for the chlorhexidine gel-treated sample (Figure 30.B), which can be explained in terms of the possible binding of chlorhexidine digluconate to the surface. [74]

This is supported by the deconvolution of the C 1s signal (data not shown) which gave four peaks for all samples, the peak at 287 eV for the gel-treated sample proving more intense than those for the other samples.

The decrease in the C 1s signal (Figure 30) after a 30-60 min He^+ bombardment of the untreated sample indicates the presence of carbonaceous contamination, due to C-containing molecules remaining after cleaning or adsorbed later on the air-exposed surfaces. These elements are observed typically on Ti implant surfaces.[77,78]

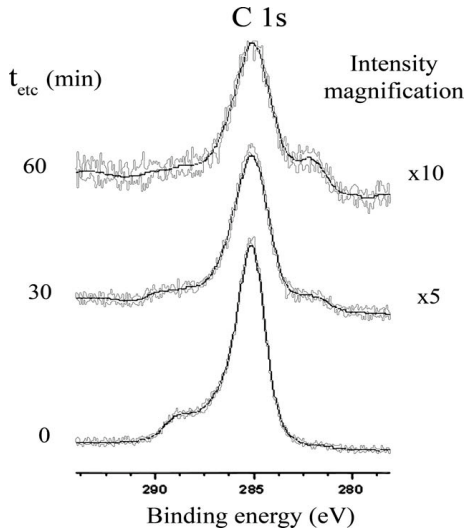


Figure 30. C 1s signals in the XPS spectra of the control Ti disc after 0 min (lowestmost curve), 30 min (middle) and 60 min (uppermost curve) of He^+ bombardment. The bombardment led to the removal of ~10 nm from the surface of the material during the operation. The decrease in the C 1s signal indicates the presence of carbonaceous contamination. [74]

4.2.3. Microscopic images

Optical microscopic images of epithelial cells are to be seen in Figure 31 at magnifications of 200 x. Figure 31A reveals, that there are only few attached cells as it is a primary epithelial cell culture, while Figure 31B shows a confluent epithelial cell culture.

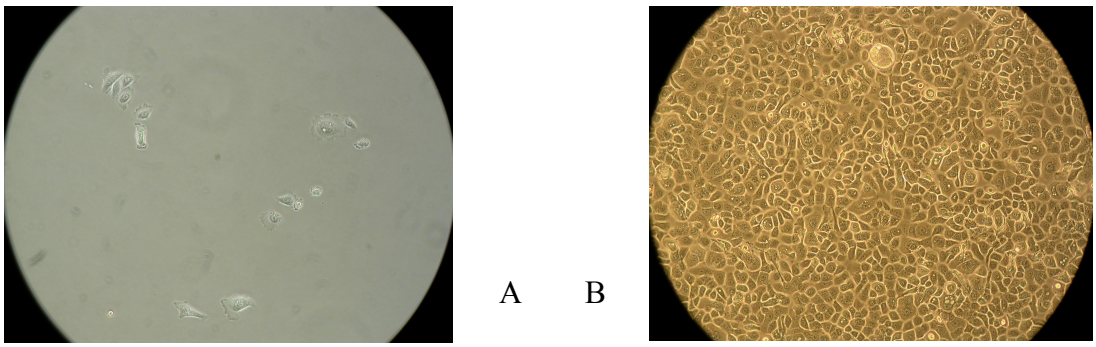
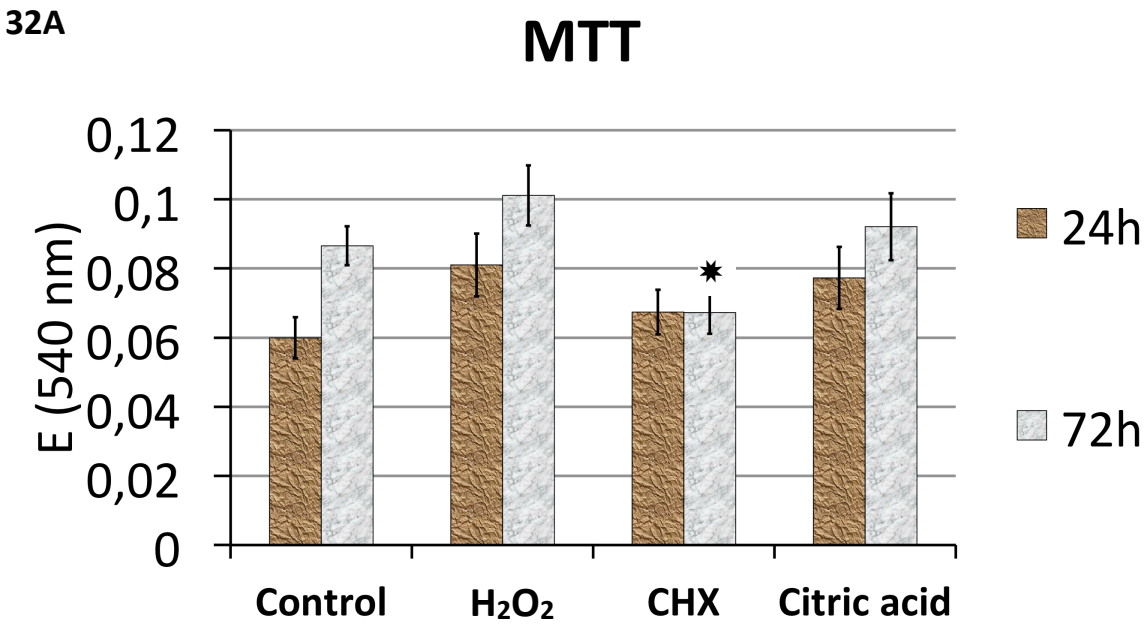


Figure 31. Optical microscopic images of A) epithelial cells of primary culture recorded with a Nikon TS 100 (Japan) microscope. There are few attached cells, magnification x 200. (B) Confluent epithelial cell culture (magnification: x 200).

4.2.4. MTT and protein content measurements

The results of MTT and protein concentration experiments relating to cell attachment (24-h observation) and cell proliferation are illustrated in the bar-graphs of Figure 32A and B.



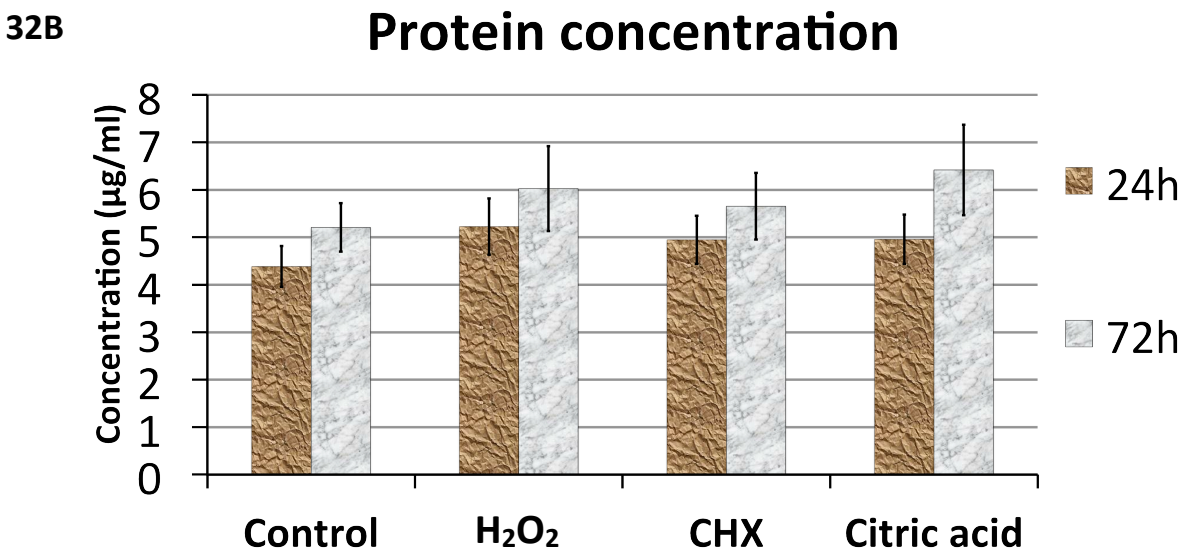


Figure 32. (A) 24-h and 72-h MTT and (B) 24-h and 72-h protein concentration results. The MTT data indicated that the epithelial cell attachment to the Ti surface was not disturbed significantly by the different cleaning solutions. H_2O_2 and citric acid treatment of the Ti discs induced slight increases in epithelial cell attachment and viability relative to the control and chlorhexidine gel-treated discs. The level of cell proliferation revealed by the MTT measurements was decreased significantly only in the case of the chlorhexidine gel-treated samples as compared with the H_2O_2-treated discs and the amount of cells was almost the same as at 24h. The protein concentration after 24 and 72h was the same for all Ti samples. Significant different (✷). [74]

The MTT results (Figure 32A) demonstrate that the epithelial cell attachment on the Ti surface was not disturbed significantly by the different cleaning solutions. More (but not significantly more) living cells were observed on the discs treated with H_2O_2 (E_{540,H_2O_2} = 0.081 ± 0.009) and citric acid ($E_{540,\text{Citric acid}}$ = 0.077 ± 0.008), than on the control ($E_{540,\text{Control}}$ = 0.059 ± 0.005) and CHX gel-treated discs ($E_{540,\text{CHX}}$ = 0.067 ± 0.006). The protein concentration after 24 h (Figure 32B) was similar for all samples: c_{Control} = 4.385 ± 0.429; $c_{H_2O_2}$ = 5.224 ± 0.592; c_{CHX} = 4.945 ± 0.508; $c_{\text{Citric acid}}$ = 4.957 ± 0.518. The level of cell proliferation revealed by the MTT measurements (Figure 32A) was increased slightly by the H_2O_2 (E_{540,H_2O_2} = 0.101 ± 0.008), and citric acid treatments ($E_{540,\text{Citric acid}}$ = 0.092 ± 0.009), and decreased by control ($E_{540,\text{Control}}$ = 0.086 ± 0.005) and gel treatments ($E_{540,\text{CHX}}$ = 0.067 ± 0.006). The H_2O_2-treated sample exhibited a significant ($p = 0.011$) increase as compared to the CHX gel-treated discs. No significant differences were observed among the other groups. The protein concentration after 72 h (Figure 32B) was similar for all Ti samples: c_{Control} = 5.207 ± 0.511; $c_{H_2O_2}$ = 6.025 ± 0.895; c_{CHX} = 5.654 ± 0.701; $c_{\text{Citric acid}}$ = 6.418 ± 0.953.[74]

5. Summary and Conclusions

5.1. Prophylactic agents used in dentistry

Our group demonstrated that the high F^- concentration and acidic pH of the gel and the 1% NaF solution resulted in strong corrosion and modification of the composition of the Ti surface.[71] As revealed by XPS a complex (Na_2TiF_6) was formed, bound strongly to the surface. R_a was increased significantly on all test samples as demonstrated by AFM. For the mouthwash-treated sample, $R_a = 51.3 \pm 4$ nm ($p = 0.007$), and for the gel treated sample, $R_a = 48.6 \pm 3$ nm ($p = 0.005$), as compared with $R_a = 37.0 \pm 2$ nm for the control surface. The 1% NaF solution-treated Ti discs displayed an almost 7-fold increase in roughness: $R_a = 254.8 \pm 19$ nm ($p < 0.001$), which is due to the fact that in aqueous solution in acidic pH hydrofluoric acid (HF) will form. We suppose that in case of gel, even if the F^- concentration is higher, the different agents like Olaflur and Dectaflur are fixing and chemically bounding (neutralizing) the F^-, impeding the formation of this acid. These results are presented in details in Stájer et al.[71] and the Ph.D thesis of Anette Stájer (2012).

My main task was to characterize the response of living cells (epithelial cell culturing) to these fluoride modified surfaces. The MTT measurements (24-h observation) demonstrated that the epithelial cell attachment on the Ti surface was not disturbed significantly by use of the mouthwash or NaF, but following gel treatment, the attachment was significantly stronger. The protein concentration was the same for all the Ti samples, independently of the applied F^- containing material. The cell proliferation (72-h observation) determined by MTT measurement was decreased significantly only for the NaF-treated samples. The protein content assays indicated almost the same tendency as the MTT measurements: for the gel-treated samples a significant decrease relative to the mouthwash-treated samples, but no significant change for the NaF-treated samples.

The main conclusions of this study are:

- It is advisable to take the adverse effects of a high F^- concentration and low pH into consideration when prophylactic gels are utilized by patients with implants or other dental appliances made of titanium.
- Epithelial cell culturing results can depend on the investigation method used.

5.2. Chemical agents used in the therapy of peri-implantitis

R_a was demonstrated by AFM to be similar for all groups. Only for the CHX gel-treated group was R_a lower (14 ± 4 nm), than that of controls (R_a = 22 ± 3 nm). These surfaces are therefore smooth, and equally suitable for epithelial cell attachment and proliferation. The influence of R_a on epithelial cell growth has been studied by many authors and it is known that epithelial cells do not attach so strongly to acid-etched or sand-blasted surfaces as to smooth (polished, S_a < 0.5 μm) surfaces.[72] Surfaces with a smooth topography promote epithelial cell growth, spreading, and the production of focal contacts on Ti surfaces.[73]

The XPS measurements proved the presence of an intact TiO_2 layer on both the untreated and the treated samples. A major change was observed for the CHX gel-treated sample, as the O 1s signal included an intense peak corresponding to the O in C-O and/or C=O bonds. This is assumed to be a result of the adsorption of CHX to the surface, as observed also by other authors.[58]

The MTT assays (24-h observation) showed that the epithelial cell attachment was not changed significantly on the Ti surfaces treated with the different cleaning solutions, and the protein-content assay supported this. The MTT method revealed differences in cell proliferation (72-h observation) between groups, with a significant increase in proliferation for the H_2O_2-treated sample relative to the CHX gel-treated one. The proliferation for the citric acid-treated samples was higher, but not significantly so, as compared to controls. The protein content assays indicated similar degrees of cell proliferation for the differently treated samples.[74]

The variations observed in the proliferation of epithelial cells cannot result from differences in R_a of the Ti samples, as the samples applied in this study had machined surfaces, with R_a between 0.014 and 0.030 μm, depending on the chemical agents used. As defined by Klinge et al. these surfaces are all smooth, and it appears improbable that epithelial cells are sensitive to such small changes in R_a.[72] The R_a values of the two surfaces do not differ significantly, therefore we presume the chemical composition of the CHX gel-treated surface is less favorable for the cells than the H_2O_2-treated surface.

The discrepancy between the results obtained with the two different methods (MTT and protein content assays) in cell proliferation is not yet understood, but may well be associated with the inherent differences between the methods. The MTT assay measures the number of living cells, whereas both viable and nonviable cells are included in the protein-related measurements. Thus, the latter method is effective, but not selective for viable cells.

The following conclusions can be drawn from this study:

- These findings also suggest that the results of epithelial cell culturing can depend on the investigation method applied.
- It is important to take into consideration the adsorption of CHX gel to the Ti surface when this material is used for dental implant decontamination.
- TiO_2 surface treatment with H_2O_2 or citric acid can result in the same or even better survival and proliferation of epithelial cells than in the untreated case. This is the most important finding of this study as these toxic decontamination solutions were not expected to improve cell attachment and proliferation at all.

In future we plan to perform further experiments and to add new sensitive methods (e.g. AlamarBlue) for a better determination of the amount of epithelial cells on differently treated Ti samples.

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I.

Corrosive effects of fluoride on titanium: Investigation by X-ray photoelectron spectroscopy, atomic force microscopy, and human epithelial cell culturing

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Abstract: High fluoride (F^-) concentrations and acidic pH impair the corrosion resistance of titanium (Ti). Effects of F^--containing caries-preventive prophylactic rinses, and gels on Ti were investigated by X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). Human epithelial cell attachment and proliferation were investigated by dimethylthiazol-diphenyl tetrazolium bromide (MTT) and protein content assays. Aqueous 1% NaF solution (3800 ppm F^-, pH 4.5) or high (12,500 ppm) F^- content gel (pH 4.8) strongly corroded the surface and modified its composition. XPS revealed formation of a strongly bound F^--containing complex (Na_2TiF_6). AFM indicated an increase in roughness (R_a) of the surfaces: 10-fold for the NaF solution and smaller for the gel or a mouthwash (250 ppm F^-, pH 4.4). MTT revealed that cell attachment was significantly increased by the gel, but was not disturbed by either the mouthwash or the NaF. Cell proliferation determined by MTT decreased significantly only for the NaF-treated samples; protein content assay experiments showed no such effect. This study indicates that epithelial cell culturing results can depend on the method used, and the adverse effects of a high F^- concentration and low pH should be considered when prophylactic gels are applied by patients with Ti implants or other dental devices. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 87A: 450–458, 2008

Key words: dental implant; fluoride; corrosion of titanium; epithelial cell culture; surface analysis

INTRODUCTION

Titanium (Ti) and its alloys are widely used as medical or dental implants in consequence of their good biocompatibility, excellent corrosion resistance, and appropriate mechanical properties.[1] Endosseous dental implants and surgical implants for fixating or replacing hard tissue are made from "commercially pure" Ti (CP Ti) and the most common Ti alloy, Ti-6Al-4V.[2,3] Ti is also used in prosthetic dentistry to manufacture crowns and multiple-unit fixed restorations,[4,5] and in orthodontic dentistry to produce Ti brackets.[6] Dental arch wires and orthopedic braces are usually made from the special TiNi shape memory alloy.[2,3]

Ti and its alloys are resistant to corrosion because of the formation of an insoluble titanium oxide layer on the surface.[7] In air, the oxide, usually TiO_2, begins to form within nanoseconds (10^{-9} s) and reaches a thickness of 20–100 Å in 1 s. It is very adherent to the parent Ti and impenetrable to oxygen.[8]

Oxidative agents are well known to exert a corrosive effect on the alloys used in dentistry, with the exceptions of Ti and other bioinert materials. Indeed, oxidative processes can thicken and condense the titanium oxide layer on the surface, improving the corrosion stability of the underlying Ti. On the other hand, reductive agents, such as fluoride (F^-), may have the opposite effect and attack this layer. Strietzel et al.[9]

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demonstrated that Ti ion release was enhanced in the presence of F^-, and this effect was even further accelerated at low pH. High F^- concentrations and an acidic pH are known to impair the corrosion resistance of Ti,[10] and as a result crevice and pitting corrosion occur.[11,12]

Patients regularly use different oral care products containing F^-, such as toothpastes, rinsing solutions, or prophylactic gels. The Ti alloys applied in the form of orthodontic wire,[13,14] or as the framework of a prosthesis, therefore come into contact with a wide range of preventive agents and these F^--containing materials can attack the surface of Ti.[15,16] SEM investigations have revealed that topical F^- solutions can cause stress corrosion cracking on CP Ti.[17] Galvanic corrosion has been reported to occur between orthodontic wires and brackets (NiTi and CuNiTi) immersed in fluoride mouthwashes.[18] Such corrosion has two undesirable consequences: the mechanical performance of the wire-bracket system deteriorates, and the risk of local Ni^{2+} release is increased.

Moreover, such F^--containing agents may come into contact with the neck part of dental Ti implants, which may extend into the oral cavity (Fig. 1). The long-term success of dental implants depends to a large extent on the gingival attachment to the neck of an implant. This mucosal seal ensures protection against bacterial attack and other injurious effects exerted by the oral environment. The epithelial attachment (junctional epithelium) may be anchored onto a rough or a smooth surface by hemidesmosomes through a preformed glycoprotein layer. A rough surface is more favorable for the plaque accumulation in the peri-implant crevices of the gingiva, which is an undesired effect in this very sensitive region of the implant. Accordingly, to avoid pathogenic plaque accumulation, the neck of an implant must be polished.[19,20] From this respect, it is easy to realize the great importance of the maintenance of the continuity of these surfaces.

In 1999, Nakagawa et al.[21] found a relation between the F^- concentration and the pH at which the corrosion of CP Ti occurred. The results of their anodic polarization and immersion tests indicated that the corrosion of Ti in a F^--containing solution depends on the concentration of hydrofluoric acid (HF). The passivation film on Ti was destroyed when the HF concentration in the solution was >30 ppm. In 1995, Boere[15] had demonstrated that the corrosion of Ti is enhanced in an acidic environment, because F^- in solution combines with H^+ to form HF, even if the NaF concentration is low.

Nakagawa et al.[22] investigated the corrosion behavior of Ti alloys: Ti-6Al-4V, Ti-6Al-7Nb, and the new alloy Ti-0.2Pd. Their experimental results demonstrated that even a low F^- concentration causes corrosion in an acidic environment. If Ti alloy contains at least 0.2% Pd, this process does not take place. The high corrosion resistance of this alloy is because of the surface enrichment of Pd promoting the repassivation of Ti.

The studies by Huang[23] indicated that, when the NaF concentration was >0.1%, the protectiveness of TiO_2 on Ti was destroyed by F^-, leading to the severe corrosion of Ti. In 2003, Huang[24] investigated the effects of F^- and albumin concentrations on the corrosion resistance of Ti-6Al-4V in acidic artificial saliva (pH 5). The X-ray photoelectron spectroscopy (XPS) results showed that when the NaF concentration was >0.1%, a hexafluorotitanate complex (Na_2TiF_6) was formed on the Ti surface, which destroyed the stable TiO_2 layer.

As the pH of the rinses and gels used for caries prevention in dentistry ranges from 3.5 up to neutral, and the F^- concentration in these materials is between 1000 and 10,000 ppm,[21] it is essential for the dental practitioner to know whether a F^--containing material can attack the Ti surface or can modify the corrosion resistance of the Ti surface of a dental implant, a prosthesis, or the wires of orthodontic braces. Besides 0.1–0.15% (1000–1500 ppm) F^-, toothpastes contain other constituents, such as rubbing, cleaning, foaming materials, and calcium complexes, which reduce the effectiveness of toothpastes by 25–50%.[25]

Although all the above-mentioned studies point to the deleterious effect of F^--containing prophylactic gels, there are a huge number of data documenting that F^- exerts a bone-promoting activity. Ellingsen et al. proved that, when F^- is incorporated in the titanium oxide layer, the retention of implants is significantly increased, even as compared with rough surface implants.[26,27] The success of a TiO_2-blasted surface with a F^--modified TiO_2 layer (OsseoSpeed implants, Astratech) is because of the ability of the F^- coating to stimulate the bone response, leading to binding between Ti and the phosphate from tissue fluids. The free F^- catalyzes this reaction and induces the formation of fluoridated hydroxyapatite and fluorapatite in the surrounding bone.[26]

The studies by Cooper et al., demonstrated that the F^- modification of TiO_2 grit-blasted CP Ti surfaces enhanced osteoblastic differentiation and interfacial bone formation.[28] As far as we are aware, there has so far been no study of the behavior of epithelial cell growth on F^--treated Ti implant surfaces.

In this work, the effects of different F^--containing caries-preventive prophylactic rinses and gels on the surface structure and roughness of CP Ti were investigated, through the use of XPS and atomic force microscopy (AFM). A further aim was to survey the attachment and proliferation of human epithelial cells after treatment of the Ti surface with an acidic NaF solution, a widely used F^--containing mouthwash or a gel. The epithelial cell attachment and proliferation

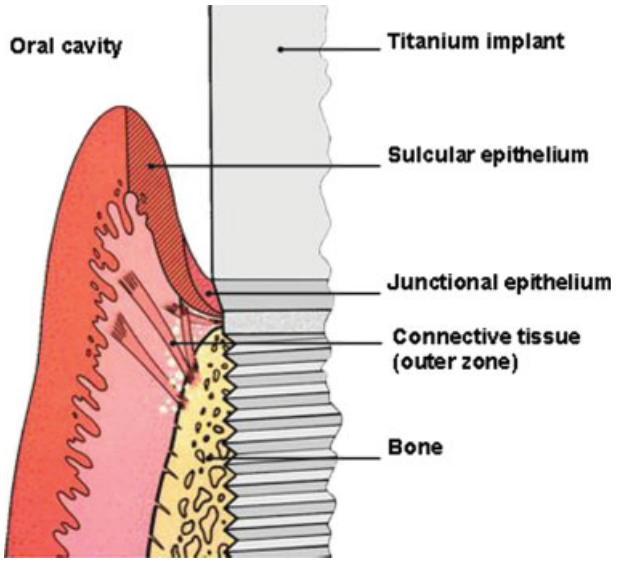

Figure 1. Epithelial attachment on a Ti implant surface, illustrating that F^--containing agents may come into contact with the neck part of dental Ti implants, which may extend into the oral cavity. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

were examined by means of dimethylthiazol-diphenyl tetrazolium bromide (MTT) and protein content assays. For the visualization of cells, scanning electron microscopy (SEM) was applied.

MATERIALS AND METHODS

Ti discs (9 mm in diameter and 2 mm in thickness) were made from implant material (CP grade 4, CAMLOG Biotechnologies AG, Switzerland). The discs were mechanically polished to a surface roughness not exceeding 0.2 μm, the roughness needed for the neck of a dental implant.[20] The discs were cleaned in acetone and absolute ethanol in an ultrasonic bath for 15 min. Each sample was immersed for 1 h in one or other of (1) a caries-preventive prophylactic mouthwash (Elmex, GABA International AG, Switzerland) containing 250 ppm F^- (pH 4.4), which contains fluoride in form of Olaflur (bis-(hydroxyethyl)-aminopropyl-*N*-(hydroxyethyl)-octadecylamin dihydrofluoride) and potassium-fluoride; (2) an aqueous solution of 1% NaF (3800 ppm F^-, pH 4.5), where the pH of the NaF solution was set to 4.5 with lactic acid; or (3) a gel (Elmex, GABA GmbH, Germany) containing a total of 12,500 ppm (1.25%) F^- [pH 4.8 (10% in water); www.gaba.com], 2500 ppm (0.25%) in the form of the amine fluorides Olaflur and Dectaflur (hexadecylamine hydrofluoride), and the rest in the form of sodium fluoride (1%).

After 1 h, the samples were removed from the F^--containing medium, thoroughly washed with ultrapure water and dried. As the suggested use of the prophylactic rinse and the gel is once a day for 30 s and once a week for 2 min, respectively, our application time corresponds to the accumulated effect of regular usage of 4 months for the rinse and 7.5 months for the gel. The 1-h treatment time seems to be too long, but if we take in consideration that these prophylactic solutions are not rinsed after application, then we may think about shorter cumulative periods.

The chemical composition of the Ti surfaces was studied by XPS. The photoelectrons were generated by Mg Kα primary radiation ($h\nu$ = 1253.6 eV) and were analyzed with a hemispherical electron energy analyzer (Kratos XSAM 800). The X-ray gun was operated at 210 W (14 kV, 15 mA). The binding energies were normalized with respect to the position of the C (1s) peak of adventitious carbon, which was taken as 285.1 eV. The changes in the XPS spectra were measured after 10 min of Ar^+ bombardment, which was repeated several times. Ar^+ was generated with an ion gun energy of 3 kV, and the incident ion beam current density was 4 $\mu A/cm^2$. The bombardment led to the removal of about 10 nm from the surface material in 10 min. Wide-range scans and higher-resolution narrow scans of the Ti 2p characteristic peaks were recorded.

A PSIA XE-100 atomic force microscope (South Korea) was used to acquire information on the roughness of the sample surface. AFM offers a new tool to study these surfaces on the micron to nanometer scale, using a technique that measures forces on the AFM probe tip as it approaches and retracts from the investigated surface. The tips were contact silicon cantilevers (type: P/N 910M-NSC36) purchased from

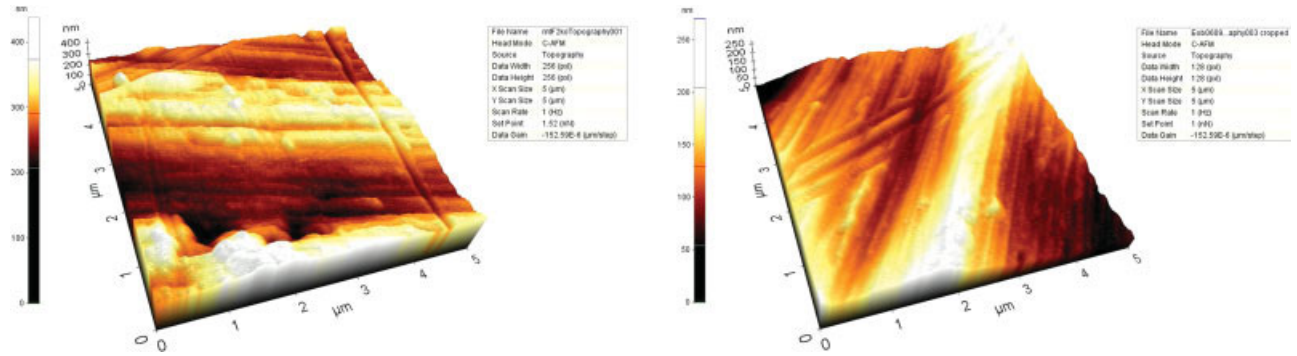

Figure 2. 3D AFM pictures of typical (A) control (untreated) and (B) mouthwash-treated (250 ppm F^-, pH 4.4) Ti samples. The almost parallel grooves originate from the mechanical machining of the samples. The color becomes lighter on proceeding from the depths of the grooves toward the surface. Image size: 5 × 5 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

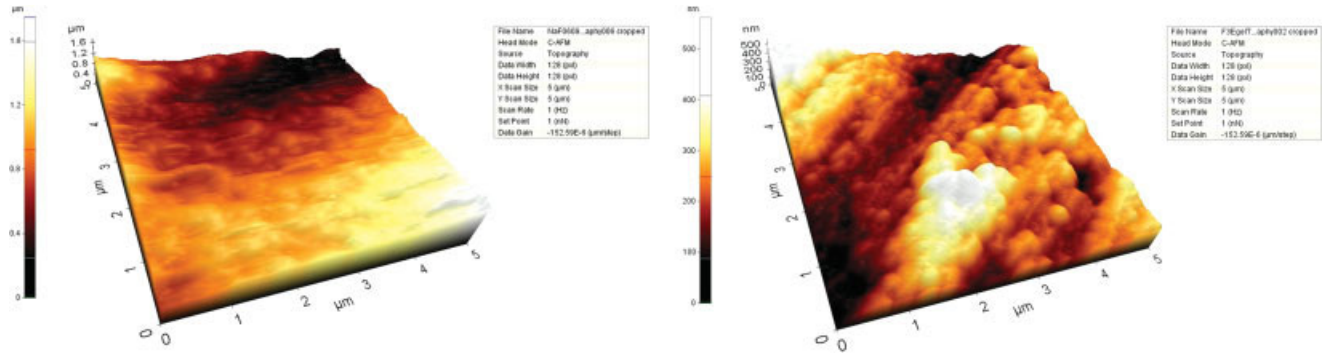

Figure 3. 3D AFM pictures (A) of a Ti disc treated with 1% NaF (3,800 ppm F^-, pH 4.5), and (B) of a characteristic gel-treated (12,500 ppm F^-, pH 4.8) Ti sample. The Ti discs treated with NaF solution displayed an almost 10 times increase in R_a and for gel-treated samples the AFM picture revealed deep corrosive regions and granular forms. Image size: 5 × 5 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MikroMasch Eesti OU (Estonia). Cantilevers with spring constants of 0.95 and 1.75 N/m were used. The measurements were performed in contact mode, and the height, deflection, and 3D images with areas of 10 μm × 10 μm and 5 μm × 5 μm were captured. The surface roughness (R_a) was determined via the AFM software program (at least 10 independent measurements), and was defined as the arithmetic average of the surface height relative to the mean height. R_{pv} was also determined, as the difference between the highest (peak) and deepest (valley) values of the surface. R_a was depicted graphically following section analysis.

The control and treated Ti discs were sterilized under UV-C radiation (20 s) for the epithelial cell culturing experiments. The cell cultures involved human gingival mucosa from healthy consenting adult (age 18–24) donors. All subjects enrolled in this research have responded to an Informed Consent, and the scheme of the experiments has been approved by the Human Investigation Review Board of University of Szeged, as it complied with the ethical standards of the research, in accordance with the Helsinki Declaration.

A quantity of 1×10^4 cells/mL/disc from the cell culture in the 3rd passage was plated on the Ti discs, in 48-hole cell culture plates. The cell adhesion was determined at 24 h, and the cell proliferation at 72 h. Three independent experiments were performed, and for each treatment five Ti samples were used.

MTT measurements and protein content assays were used to investigate how the cells survived and proliferated on the surfaces treated with the materials containing different amounts of F^-. The MTT is a rapid colorimetric assay widely used for cellular growth and survival study.[29] MTT gives a yellowish solution that is converted to the dark-blue water-insoluble MTT-formazan by mitochondrial dehydrogenases in living cells. The blue crystals are solubilized with acidified isopropanol and the intensity is measured colorimetrically at 570 nm. The adhering cells were removed by a lysis buffer (20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1 m*M* Na_2EDTA, 1 m*M* EGTA, 1% Triton X-100, 2.5 m*M* sodium pyrophosphate, 1 m*M* β-glycerophosphate, 1 m*M* Na_3VO_4, and 1 μg/mL leupeptine) and their protein content was determined with a micro Coomassie (Bradford) protein assay kit (Pierce, Rockford, IL) by following the instructions of the supplier, with bovine serum albumin as standard.

The samples were subsequently dehydrated in graded ethanol and acetone, and dried in critical point dryer (type SPI 1320). Mounted specimens were gold-coated by using an Edwards sputter coater, and viewed in a Hitachi S 2400 scanning electron microscope.

Statistical analysis was done using Student's *t*-test for two samples, where $p = 0.05$ was considered as the level of significance.

RESULTS AND DISCUSSION

AFM measurements

Before treatment, the polished Ti samples were tested by AFM and XPS. Figure 2(A,B) reveals almost the parallel grooves on the control and mouthwash (250 ppm F^-) -treated samples; these grooves origi-

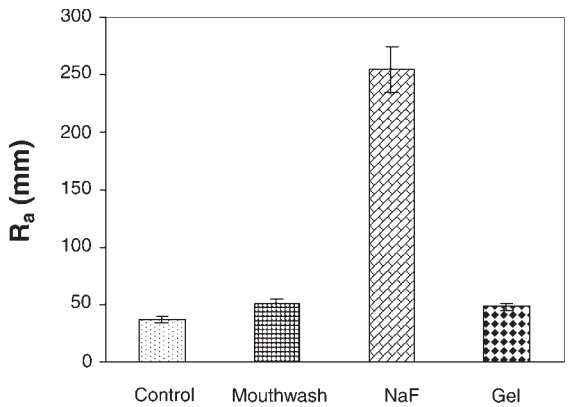

Figure 4. Overall bar-graph of the surface roughness (R_a) of the various samples. The AFM measurements gave R_a = 37.0 ± 2 nm for the control (untreated) samples, 51.3 ± 4 nm for the mouthwash-treated (250 ppm F^-, pH 4.4) samples, 254.8 ± 20 nm for the NaF solution (3800 ppm F^-, pH 4.5) and 48.6 ± 3 nm for the gel-treated (12,500 ppm F^-, pH 4.8) sample.

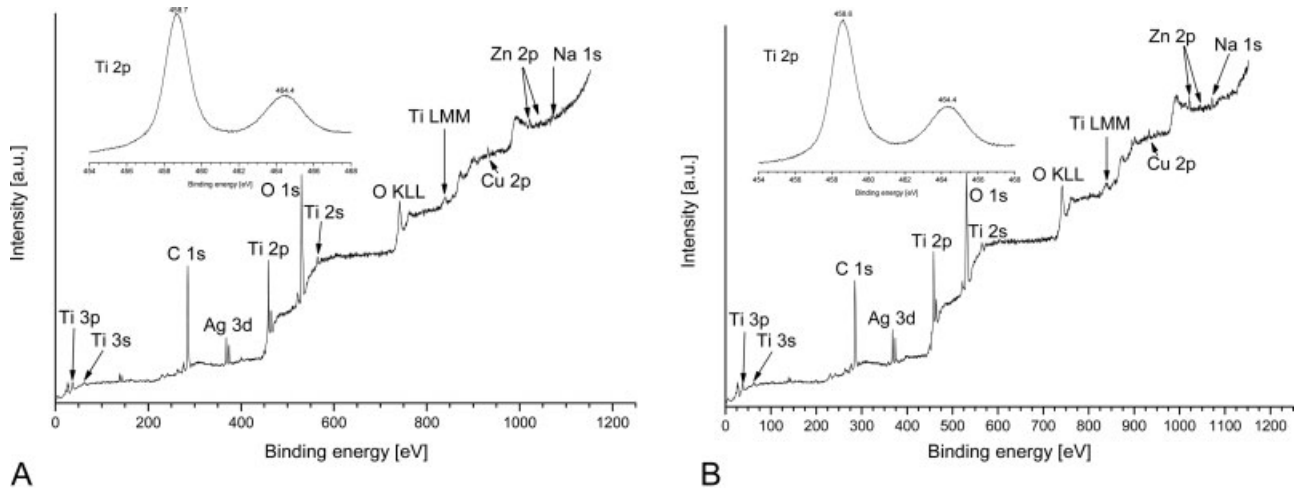

Figure 5. XPS spectra of (A) control and (B) mouthwash-treated (250 ppm F^-, pH 4.4) Ti discs. The XPS survey spectra confirmed the presence of O, C, and Ti. These elements are typically observed on Ti implant surfaces.

nate from the mechanical machining of the samples (the color becomes lighter on proceeding from the depths of the grooves toward the surface). The AFM measurements gave $R_a = 37.0 \pm 2$ nm for the control samples, and 51.3 ± 4 nm for the mouthwash-treated samples (Fig. 4). Although major differences can not be observed between the two samples, R_a was significantly ($p = 0.007$) higher than the control value.

After treatment with 1% NaF solution (3800 ppm F^-, pH = 4.5), the Ti discs displayed the biggest increase in R_a [Fig. 3(A)]: the depth of the grooves was almost 10 times the control depth: $R_a = 254.8$ nm $\pm$ 20 nm ($p < 0.001$, Fig. 4).

On the discs immersed in the gel (12,500 ppm F^-) the AFM [Fig. 3(B)] picture revealed deep corrosive regions and granular forms, and the average roughness of the gel-treated surface was significantly increased, $R_a = 48.6 \pm 3$ nm ($p = 0.005$), as compared with the control samples (Fig. 4).

XPS measurements

The XPS survey spectra of control and rinse-treated samples in Figure 5(A,B) confirmed the presence of O, C, and Ti. The C 1s signal indicates the presence of carbonaceous contamination, because of C-containing molecules remaining after chemical cleaning or adsorbed later on the air-exposed surfaces. These elements are typically observed on Ti implant surfaces.[30] Trace amounts of Ag, Cu, Zn, and Na could also be detected[31] originating from external contamination.

The double peaks of Ti (Ti 2p at 458 and 464 eV binding energy) and the O 1s signal (530 eV) demon-

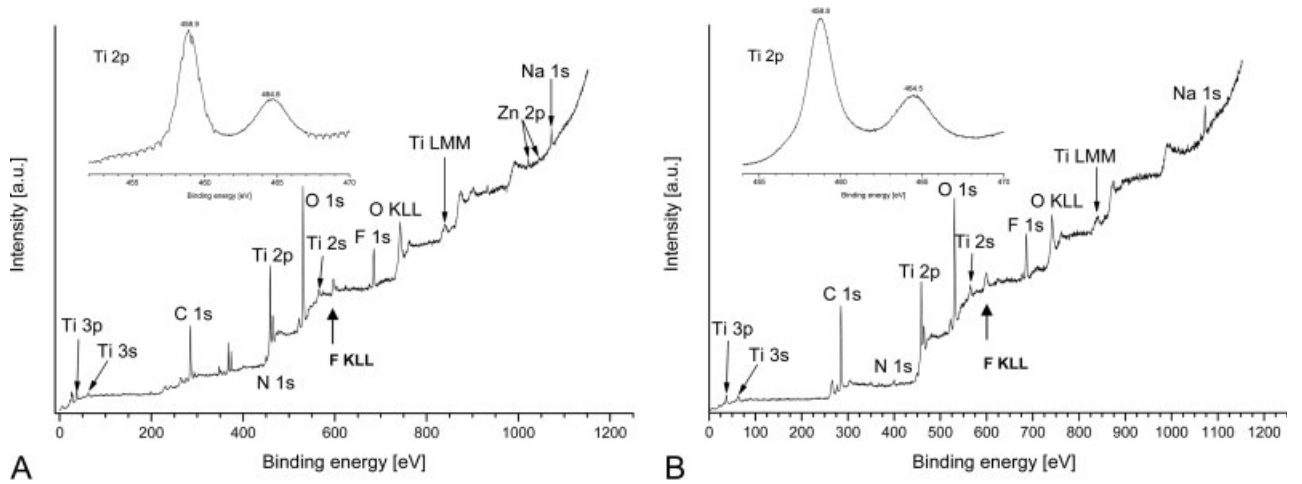

Figure 6. XPS spectra of (A) NaF (3800 ppm F^-, pH 4.5) and (B) gel-treated (12,500 ppm F^-, pH 4.8) Ti discs. Three new peaks can be observed on the spectra originating from Na_2TiF_6, which modifies the TiO_2 layer of the surface.

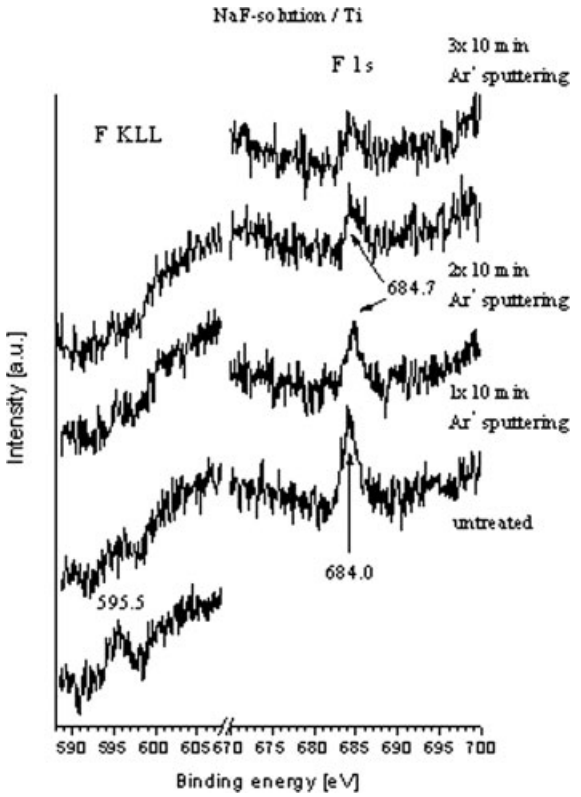

Figure 7. High-resolution XPS spectra of the NaF-treated (3800 ppm F^-, pH 4.5) Ti disc after 10, 20, and 30 min of Ar^+ bombardment. After 10 min of Ar^+ bombardment, repeated XPS investigation revealed that about 10 nm was removed from the surface of the material, but the F 1s peak at 684.7 eV persisted, proving that the binding between the Ti and F^- was very strong.

strate the presence of the TiO_2 layer.[32,33] The immersion in the mouthwash containing 250 ppm F^- did not change the XPS spectrum of the surface [Fig. 5(B)].

After the NaF and gel treatments, three new peaks were to be seen on the spectra [Fig. 6(A,B)]. One of them, at a binding energy of 1080 eV, is the characteristic line of Na 1s, which resulted from NaF. Two other peaks appeared in the 600–700 eV binding energy region. The line at 600 eV originates from F KLL,[23]and the F 1s peak at 685.3 eV from Na_2TiF_6, which modifies the TiO_2 layer of the surface.[23,24]

After 10 min of Ar^+ bombardment, repeated XPS investigation revealed that about 10 nm was removed from the surface of the material, but the F 1s peak at 684.7 eV persisted (Fig. 7), proving that the binding between the Ti and F^- was very strong.

MTT and protein content measurements

The results of MTT and protein concentration experiments relating to cell attachment (24-h observation) are illustrated in the bar-graphs of Figure 8(A,B). The MTT results indicated that the epithelial cell attachment on the Ti surface was not disturbed significantly by immersion in the mouthwash or NaF ($E_{540,control} = 0.216 \pm 0.007$, $E_{540,mouthwash} = 0.231 \pm 0.011$, $E_{540,NaF} = 0.192 \pm 0.016$). Following immersion in the gel, however, the attachment was significantly stronger ($E_{540,gel} = 0.255 \pm 0.013$; $p = 0.015$). The protein concentration after 24 h was the same for all Ti samples, independently of the F^- material applied ($c_{control} = 4.60 \pm 0.47$).

The MTT and protein content assay results concerning cell proliferation (72-h observation) are presented in Figure 9(A,B). The level of cell proliferation revealed by the MTT measurement was decreased significantly ($p < 0.001$) only in the case of the NaF-treated sample ($E_{540,control} = 0.268 \pm 0.022$, $E_{540,NaF} = 0.137 \pm 0.004$, $E_{540,mouthwash} = 0.271 \pm 0.01$, $E_{540,gel} = 0.221 \pm 0.019$). The protein content assays demonstrated the same tendency as the MTT measurements for the gel-treated sample: a significant (but slight) decrease ($c_{gel} = 4.59 \pm 0.41$ μg/mL; $p = 0.0312$) relative to the rinse-treated sample ($c_{mouthwash} = 5.82 \pm 0.38$ μg/mL). A significant change was not detected for the NaF-treated sample ($c_{NaF} = 5.25 \pm 0.39$ μg/mL; $c_{control} = 5.31 \pm 0.18$ μg/mL).

The influence of the surface roughness on epithelial cell growth has been studied by many authors and it has been proved that epithelial cells do not approach so closely to acid-etched and sand-blasted surfaces as to smooth (polished, $S_a < 0.5$ μm) surfaces.[34] Baharloo et al.[35] observed that surfaces with smooth topography promote epithelial-cell growth, spreading, and the production of focal contacts on Ti surfaces.

Although our treatments were rather strong (for the gel and NaF, even the presence of Na_2TiF_6 was detected), the roughness never exceeded 0.5 μm, and the protein concentration was not decreased as compared with the control. This is in accordance with the findings of the above-mentioned authors.

The MTT method revealed a significant increase in cell attachment for the gel-treated sample, and a decrease in proliferation for the NaF-treated sample. The difference between the results obtained with these two methods is not yet understood, but may well be associated with the inherent differences between the methods. MTT assay measures the amount of living cells, while in the protein related measurements all the cells (viable and nonviable) are included.

SEM observations

The SEM micrographs showed the same pictures of the surface structure of the differently treated Ti samples as those seen on AFM: a comparatively smooth surface for the mouthwash-treated discs [Fig. 10(A)] and a rougher surface with granules because of the corrosive effect of the F^--containing gel [Fig. 10(B)].

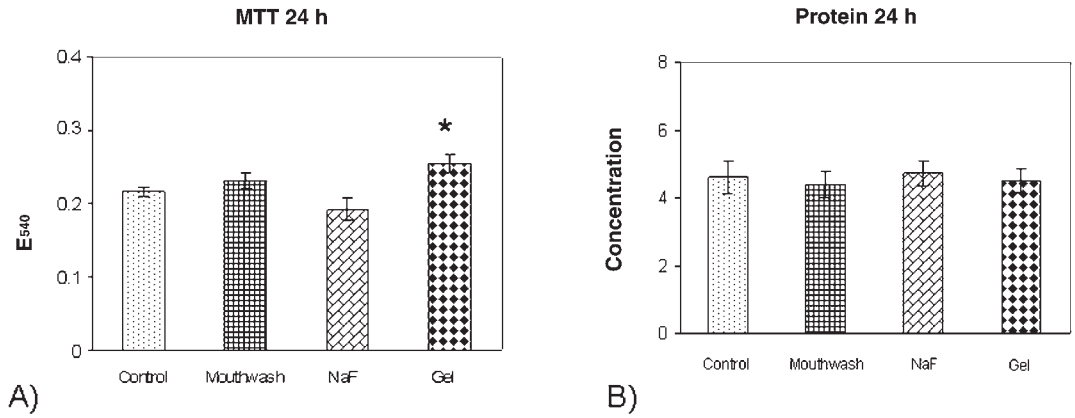

Figure 8. 24-h MTT (A) and protein concentration (B) results. The MTT results indicated that the epithelial cell attachment on the Ti surface was not disturbed significantly by immersion in the mouthwash or NaF, but after immersion in the gel, the attachment was significantly stronger. The protein concentration after 24 h was the same for all Ti samples, independently of the F^- material applied.

SEM of the Ti discs after 24-h human epithelial cell binding revealed human epithelial cells bound independently to selected sites on the prepared titanium surface and they exhibited a spherical morphology (not shown). Figure 10 illustrates the SEM pictures of the adhesion of the human epithelial cells to titanium surface after 72-h culturing. Number of the attached and proliferated cells is visible with spreading behaviors. However, we could not see any morphological differences on the adhesion and the growing of cells either on control or treated titanium surfaces [Fig. 10(A,B)].

CONCLUSIONS

R_a was demonstrated by AFM to be increased significantly on all test samples. For the mouthwash-treated sample, $R_a = 51.3 \pm 4$ nm ($p = 0.007$), and for the gel-treated sample, $R_a = 48.6 \pm 3$ nm ($p = 0.005$), as compared with $R_a = 37.0 \pm 2$ nm for the control surface. The 1% NaF solution-treated Ti discs displayed an almost 10-fold increase in roughness: $R_a = 254.8 \pm 19$ nm ($p < 0.001$), which is due to the fact that in aqueous solution in acidic pH hydrofluoric acid (HF) will form. We suppose that in case of gel, even if the F^- concentration is higher, the different agents like Olaflur and Dectaflur are fixing and chemically bounding (neutralizing) the F^-, impeding the formation of this acid.

XPS revealed that the high F^- concentration and acidic pH of the gel and the 1% NaF solution resulted in strong corrosion and modification of the composition of the Ti surface. The complex Na_2TiF_6 was formed, bound strongly to the surface.

The MTT results (24-h observation) showed that the epithelial cell attachment on the Ti surface was not

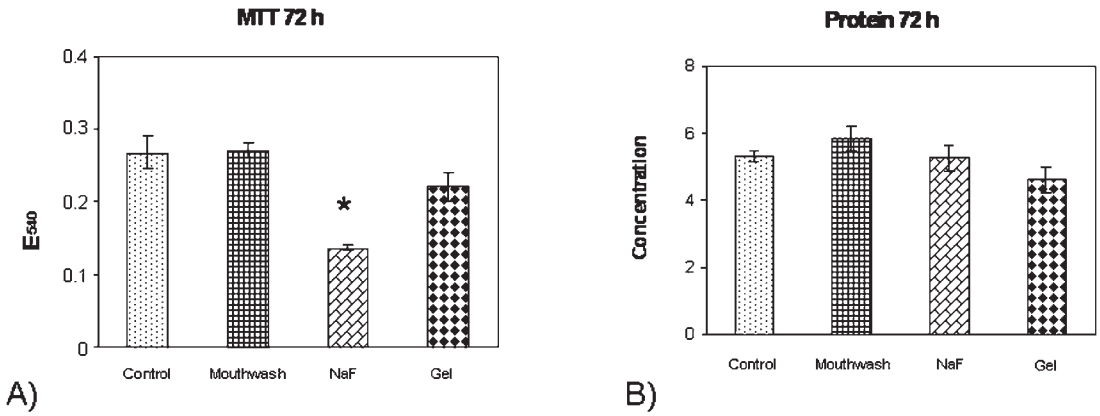

Figure 9. 72-h MTT (A) and protein content assay (B) results. The level of cell proliferation revealed by the MTT measurement was decreased significantly only in the case of the NaF-treated sample. The protein content assays demonstrated the same tendency as the MTT measurements for the gel-treated sample: a significant (but slight) decrease relative to the rinse-treated sample.

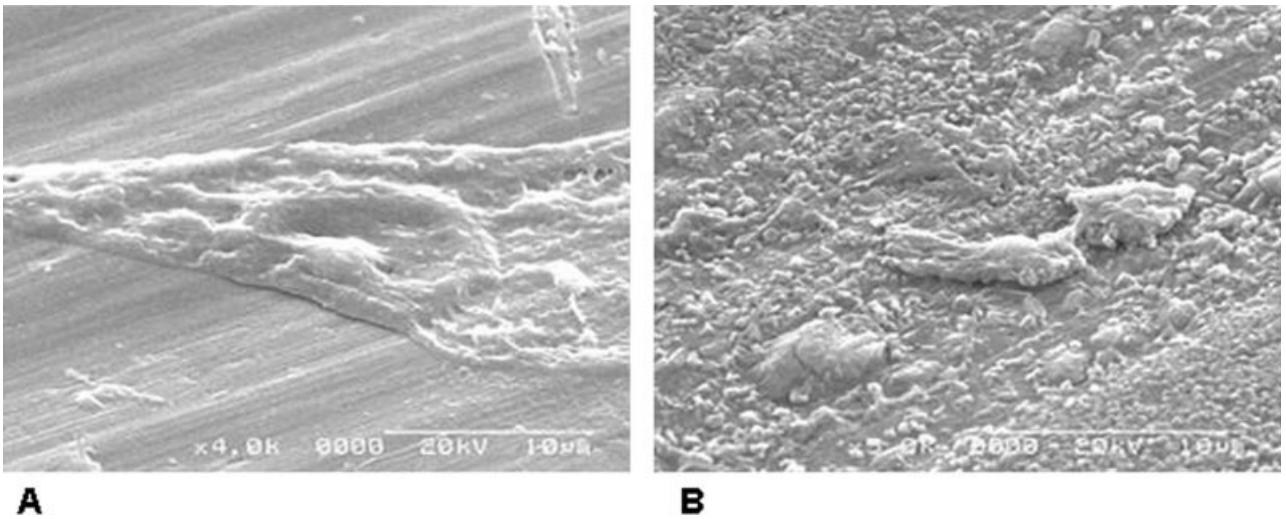

Figure 10. SEM images of epithelial cells on (A) mouthwash- (250 ppm F^-, pH 4.4) and (B) gel-treated (12,500 ppm F^-, pH 4.8) Ti samples (72-h observation). Magnification: ×4000 and ×5000, respectively. Number of the attached and proliferated cells are visible with spreading behaviors, however, we could not see any morphological differences on the adhesion and the growing of cells either on control or treated titanium surfaces.

disturbed significantly by use of the mouthwash or NaF, but following gel treatment, the attachment was significantly stronger. The protein concentration was the same for all the Ti samples, independently of the F^- material applied.

The cell proliferation (72-h observation) determined by MTT measurement was decreased significantly only for the NaF-treated samples. The protein content assays indicated almost the same tendency as the MTT measurements: for the gel-treated samples a significant decrease relative to the mouthwash-treated samples, but no significant change for the NaF-treated samples.

These results suggest that epithelial cell culturing results can depend on the investigation method used, and it is advisable to take the adverse effects of a high F^- concentration and low pH into consideration when prophylactic gels are utilized by patients with implants or other dental appliances made of titanium.

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II.

Effects on titanium implant surfaces of chemical agents used for the treatment of peri-implantitis

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Abstract: The treatment of peri-implantitis, which causes tissue deterioration surrounding osseointegrated implants, involves surface decontamination and cleaning. However, chemical cleaning agents may alter the structure of implant surfaces. We investigated three such cleaning solutions. Commercially pure (grade 4) machined titanium discs (CAMLOG Biotechnologies AG, Switzerland) were treated with 3% H_2O_2 (5 min), saturated citric acid (pH = 1) (1 min) or chlorhexidine gel (5 min), and their surface properties were examined by atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS). Human epithelial cell attachment (24-h observation) and proliferation (72-h observation) were investigated via dimethylthiazolyl-diphenyltetrazolium bromide (MTT) and bicinchoninic acid (BCA) protein content assays. AFM revealed no significant difference in roughness of the three treated surfaces. XPS confirmed the constant presence of typical surface elements and an intact TiO_2 layer on each surface. The XPS peaks after chlorhexidine gel treatment demonstrated C—O and/or C=O bond formation, due to chlorhexidine digluconate infiltrating the surface. MTT and BCA assays indicated similar epithelial cell attachments in the three groups; epithelial cell proliferation being significantly higher after H_2O_2 than after chlorhexidine gel treatment (not shown by BCA assays). These agents do not harm the Ti surface. Cleaning with H_2O_2 slightly enhances human epithelial cell growth, in contrast to chlorhexidine gel. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 94B:222–229, 2010.

Key Words: peri-implantitis, chemical agent, H_2O_2, citric acid, chlorhexidine gel, atomic force microscopy, X-ray photoelectron spectroscopy, epithelial cell culture

INTRODUCTION

Achievement of the biointegration of alloplastic materials is one of the most important targets of research in the medical, dental, and biological sciences. The most frequently used medical implants are dental implants that serve to replace human teeth. As the average human lifespan is currently increasing, ever more people have missing teeth or need tooth replacement.

Titanium (Ti) and Ti alloy prostheses are widely employed as they possess the best osseointegration properties with a predictably long lifetime.[1,2] The long-term benefits of such implants rely on the responses of the various surrounding tissues (the alveolar bone, or the conjunctive and epithelial parts of the mucosa). The failure of a dental implant is caused mainly by the inflammatory processes affecting the soft and hard tissues.[3]

Peri-implant infections involve peri-implant mucositis, defined as a reversible inflammatory change of the peri-implant soft tissues without bone loss, and peri-implantitis, an inflammatory process resulting in loss of supporting bone and associated with bleeding and suppuration.[3–5] Several studies have evaluated peri-implant infections, but only a few were cross-sectional and provide information on the prevalence of peri-implant diseases among patients with implants functioning for ~10 years. The incidence of peri-implant mucositis has been reported to be in the range of 60% of implant recipients and in 48% of implants.[6,7] The prevalence of peri-implantitis was found to be around 15, 16, and 28% with respect to the recipients,[6–8] and 7 and 12% regarding implant sites.[7,8] The differences in the prevalence of peri-implantitis may be explained by differing criteria used for the diagnosis of peri-implantitis, as well as variations in maintenance procedures.[5,9]

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The etiology of marginal peri-implantitis is based mainly on an infectious factor and a biomechanical factor.[10] Although the causes may differ in both cases, microbial colonization occurs on the surface of the implant.[4,11] If the conditions become pathogenic, bacteria start to proliferate, leading to inflammation around the implant. Peri-implant diseases have been primarily linked to Gram-negative anaerobic microflora.[12] The process is aggravated by microorganism colonization and their toxins, and extensive bone destruction will occur. The inflammation spreads apically thus, in very severe cases, therefore, the patient may lose the implant. Methods which remove the bacteria and the toxins from the surface of challenged implants would prevent or terminate the development of peri-implant bony defects.

The therapy of peri-implantitis in the surgical phase is a complex process, starting with surgical debridement of devitalized peri-implant tissue and continuing with decontamination of the exposed implant surface. The implant surface can be cleaned by mechanical (an air-powder abrasive) or chemical (citric acid, H_2O_2, chlorhexidine digluconate (CHX) or EDTA) procedures or with laser irradiation (CO_2, diode, Er:YAG or Nd:YAG).[13,14] To support antimicrobial treatment, topical, and/or systemic antibiotics may be administered.[13] After removal of damaged tissues from the peri-implant pocket, surgical treatment (guided tissue regeneration with or without the use of bone grafts and barrier membranes) promotes regeneration of any bone defect.[13,15]

For the chemical detoxification of implants, various cleaning solutions are used: CHX, H_2O_2, citric acid, phosphoric acid gel, delmopinol, Listerine^R, iodine, saline irrigation, beta-isodona, chloramine-T, and so forth. Besides these chemical agents, a number of systemic antibiotics can be applied to support the therapy: for example tetracycline, amoxicillin, augmentin, metronidazol, penicillin, and so forth.[5,13]

CHX is a commonly administered antimicrobial agent with a wide range of medical applications. It is used in dentistry as a mouthwash and topical antimicrobial. In the treatment of peri-implantitis it can serve as a rinsing solution,[16,17] or more often as an implant irrigation solution, in combination with systemic antibiotics.[13,15] Renvert et al.[18,19] investigated the difference in effectiveness of minocycline microspheres and CHX gel, and concluded that the adjunctive use of these microspheres led to improved probing depths and bleeding scores, CHX alone resulting in only a limited reduction of the bleeding scores. CHX is also effective in the surgical treatment of late peri-implant defects using guided tissue regeneration.[20,21]

Recognizing the increasing interest in the functionalization of dental implant surfaces with antimicrobial agents prior to implantation, Barbour et al.[22] investigated the adsorption of CHX to TiO_2 crystals of anatase and rutile. Their results proved that CHX in 4-morpholinoethanesulfonic acid (MES) and phosphate-buffered saline (PBS) buffers adsorbed rapidly to anatase and rutile TiO_2, equilibrium being attained in less than 60 s, with gradual desorption over a period of several days. More CHX adsorbed to anatase than to rutile, and the CHX desorbed more rapidly from anatase than from rutile, depending on the buffer used.

The study by Burchard[23] revealed that fibroblasts adhere more readily to surfaces exposed to CHX or saline than to those exposed to stannous fluoride.

Saturated citric acid can also be applied for the decontamination of Ti surfaces in the surgical treatment of peri-implantitis with bone grafts and membranes.[24,25] In a comparison of the effects of citric acid and 10% H_2O_2, Alhag et al.[26] demonstrated that rough surfaces (with an enhanced TiO_2 layer and textured surface; Nobel Biocare AB®, Gothenburg, Sweden) which were plaque-contaminated and cleaned with either solution, can re-osseointegrate. H_2O_2 can be used successfully at a concentration of 3% in the surgical treatment of peri-implantitis, employing bone substitutes with, or without, resorbable membranes.[27,28]

Some authors, including Khoury,[15] have even used a combination of these three different cleaning solutions in the surgical therapy of peri-implantitis. After removal of the granulomatous tissue, the surgical site was repeatedly rinsed with CHX, after which citric acid (pH = 1) was applied for 1 min to decontaminate the implant surface, this then being rinsed with H_2O_2 and 0.9% saline.

Dennison et al.[29] found that machined implants (without a surface coating) are decontaminated by a variety of methods (air-powder abrasive, citric acid solution, or CHX) more readily than hydroxyapatite-coated surfaces.

The above-mentioned chemical agents are commonly applied in the therapy of peri-implantitis, but only investigations relating to the adsorption of CHX on different TiO_2 crystals (anatase and rutile) appear to have been conducted. When used for implant surface decontamination, these materials may alter the morphology and chemical structure of the surface. The aim of our investigation, therefore, was to study the effects of three cleaning solutions in clinical use for peri-implantitis therapy.

In vitro studies are essential in the development of such treatments, as these are the basic steps with which to reveal the action of cleaning solutions on the implant surface. Additionally, fewer animal experiments would be required.

In the present investigation, the effects of three different cleaning solutions (3% H_2O_2 solution, saturated citric acid (pH = 1) and CHX gel) on the chemical structure and surface roughness (R_a) of CP Ti were investigated, through the use of X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). A further aim was to survey the response of the biological environment to these changes, by examining the attachment and proliferation of human epithelial cells after treatment of the Ti surfaces with these solutions. The epithelial cell attachment and proliferation was examined by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and protein-content assays (the latter with bicinchoninic acid).

MATERIALS AND METHODS

Ti discs (9 mm in diameter and 1.5 mm in thickness) were made from CP grade 4 implant material (CAMLOG^R Biotechnologies AG, Switzerland). The surfaces of the discs were machined (through turning), with a roughness (R_a) < 0.2 μm, a typical roughness for the abutment of a dental

implant.[30] The discs were cleaned in acetone and absolute ethanol in an ultrasonic bath for 15 min. After cleaning, the discs were treated with 3% H_2O_2, saturated citric acid (pH = 1) or CHX gel (Corsodyl^R dental gel; SmithKline Beecham Consumer Healthcare, UK). Corsodyl^R contains 1% w/w CHX. As in the usual clinical protocols, the durations of treatment were 5 min for H_2O_2 and CHX gel, and 1 min for citric acid. The control group was rinsed with ultrapure water for 5 min. After treatment, the samples were washed three times with ultrapure water and dried.

For AFM a PSIA XE-100 instrument (PSIA Inc., South Korea) was used to acquire information on R_a. AFM is a high resolution imaging technique to study such surfaces on the micron to nanometer scale, via a technique that measures forces on the AFM probe-tip as it approaches and retracts from the investigated surface. The tips were contact silicon cantilevers (type: P/N 910M-NSC36) purchased from Mikro-Masch Eesti OU (Estonia). Cantilevers with spring constants of 0.95 and 1.75 N/m were used. The measurements were performed in contact mode, and the height, deflection, and 3D images with areas of 10 × 10 μm and 5 × 5 μm were captured. R_a was determined via the AFM software program (at least six independent measurements) as the arithmetic average of the surface height relative to the mean height.

The chemical composition of the Ti surfaces was studied by XPS. The photoelectrons were generated by Al Kα primary radiation (hν = 1486.6 eV) and analyzed with a hemispherical electron energy analyzer (PHOIBOS 150 MCD 9; manufactured by SPECS). The X-ray gun was operated at 150 W (12 kV, 12.5 mA). The binding energies were normalized with respect to the position of the C 1s peak of adventitious carbon, which was taken as 285.1 eV. The changes in the XPS spectra were measured after 30-60 min of He^+ bombardment, which was repeated several times. He^+ ions were generated with an ion gun energy of 5 kV, and the incident ion beam current was measured at 200 nA. The bombardment led to the removal of a thickness of ~10 nm from the surface material during the operation. Wide-range scans and high-resolution narrow scans of the Ti 2p, O 1s, and C 1s characteristic peaks were recorded.

Cell-culturing techniques

Adult epidermal epithelial cells were isolated and cultured from inflammation-free oral mucosa of healthy donors (age, 18–46) undergoing dento-alveolar surgery. The protocol of the experiments was approved by the Human Investigation Review Board at the University of Szeged: it complied with the ethical standards of research, in accordance with the Helsinki Declaration. All subjects enrolled in the research gave their signed informed consent.

Mucous membrane specimens were first washed in Salsol A solution (Human Rt, Gödöllö, Hungary) supplemented with 2% antibiotic, antimycotic solution (Sigma-Aldrich GmbH, Germany). Overnight incubation in dispase solution (Grade II, Roche Diagnostics, Mannheim, Germany) was carried out at 4°C to separate the dermis from the epidermis.[31] Next day, the epidermis was peeled off the dermis. The epidermis was placed in 0.25% trypsin-EDTA solution (Sigma-Aldrich GmbH, Germany) for 5 min at 37°C. Following trypsinization, the epidermis was torn apart mechanically and washed vigorously to release epidermal cells. The epidermal cell suspension was centrifuged at 200*g* for 10 min at 4°C. The epidermal cells were then placed in 25 cm^2 tissue culture dishes (Orange Scientific, Belgium).

The oral epithelial cell culture medium consisted of keratinocyte serum-free medium with L-glutamine (Gibco BRL, Eggstein, Germany), supplemented with recombinant epidermal growth factor 2.5 μg/500 mL (Gibco BRL, Eggstein, Germany), bovine pituitary extract 25 mg/500 mL (Gibco), L-glutamine and antibiotic/antimycotic solution containing penicillin G sodium 1%, streptomycin sulfate 1%, and amphotericin B 0.0025% (Sigma-Aldrich GmbH, Germany).

Fresh culture medium was added to the cells three times per week. The primary epithelial cell cultures reached ~90% confluence in 8–16 days. Confluent primary cultures were treated with phosphate-buffered saline (pH = 7.4, Gibco) and cells were harvested by a 2–4 min trypsinization with 0.25% trypsin-EDTA solution (Sigma-Aldrich GmbH, Germany). Harvested cells were divided into two to four equal parts at passages. Cultures were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Optical microscopic images of epithelial cells were recorded with a Nikon TS 100 (Japan) microscope at magnifications of 200×.

Measurement of cell growth and proliferation

The control and treated Ti discs were sterilized on both surfaces under UV-C radiation (20 min) before the epithelial cell culturing experiments.

The growth of cultured epithelial cells was measured with a rapid colorimetric assay, which determines living cell numbers by the reduction of MTT.[32] Cells were seeded into 48-well culture plates at a density of 10^4 cells/well and grown on Ti discs in culture media for 24 or 72 h. The supernatant was removed and replaced with 0.5 mg/mL MTT solution (Sigma-Aldrich GmbH, Germany) in RPMI media without phenol red. After incubation for 4 h at 37°C, the medium was removed gently from each well and the crystallized dye was solubilized with 2% sodium dodecyl sulfate (SDS) and 0.04 m*M* HCl in absolute isopropanol. The optical density of the color reaction at 540 nm (OD_{540}) was determined with a Multiscan Ex spectrophotometer (Thermo Labsystems, Vantaa, Finland) and Ascent Software (Thermo Labsystems, Vantaa, Finland) at the Department of Dermatology and Allergology (University of Szeged).

The protein content of the cells (both living and dead) was also measured. A micro BCA™ protein assay kit containing bicinchoninic acid was applied (Pierce, Rockford, IL). An ascendant set of dilutions with an albumin standard (bovine serum albumin (BSA); Pierce) was made as control. The cells were dissolved with lysis buffer (20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1 m*M* Na_2EDTA, 1 m*M* EGTA, 1% Triton X-100, 2.5 m*M* sodium pyrophosphate, 1 m*M* β-glycerophosphate, 1 m*M* Na_3VO_4 and 1 μg/mL leupeptin), the reagent (green) was then applied, according to the manufacturer's instructions, and the solution was incubated for 2 h at 37°C.

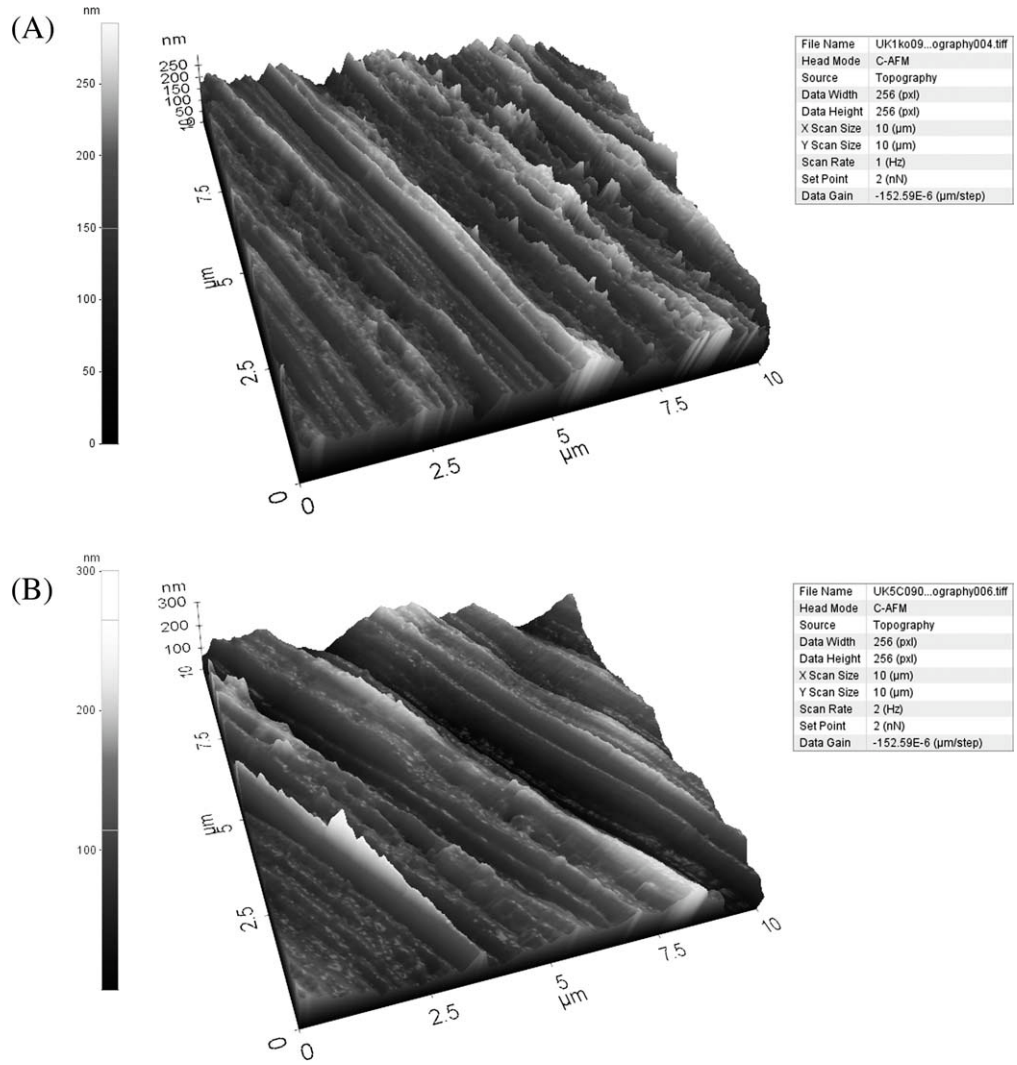

FIGURE 1. 3D AFM pictures of (A) a typical control (untreated) sample and (B) a characteristic citric acid-treated (saturated, pH = 1). The almost parallel grooves originate from the machining of the samples. The color becomes lighter on proceeding from the depths of the grooves toward the surface. Image size: 10 × 10 μm.

Because of this reaction, the color changed to purple. OD_{540} was determined with a Multiscan Ex spectrophotometer (Thermo Labsystems, Vantaa, Finland) and Ascent Software (Thermo Labsystems, Vantaa, Finland) at the Department of Dermatology and Allergology (University of Szeged).

A quantity of 10^4 cells/0.5 mL medium/disc from the cell culture in the third passage was plated on the Ti discs, in 48-hole cell culture plates. The cell adhesion was determined at 24 h, and the cell proliferation at 72 h. In all, four independent experiments were performed, and five Ti samples were used for each treatment.

Data presentation and statistical analysis

The means ± the standard errors of the mean (SEM) were calculated for the R_a (nm) values measured by AFM. The MTT and protein content assay data are presented as means ± SEM of OD_{540}. After normality testing, data were compared via one-way analysis of variance (ANOVA), followed by Tukey's and Scheffe *post hoc* tests to determine statistical differences after multiple comparisons (SPSS 15.0, SPSS, Chicago, IL). A probability value < 0.05 was considered significant.

RESULTS

AFM measurements

Before the cell culture experiments, the Ti samples were tested by AFM and XPS. Figure 1(A,B) reveal the almost parallel grooves on each sample, originating from the machining (the color becomes lighter on proceeding from the depths of the grooves toward the surface). The AFM measurements gave $R_a = 22 \pm 3$ nm for the control samples [Fig. 1(A) and 2], 25 ± 7 nm for the citric acid-treated samples (Fig. 1(B) and 2), 30 ± 5 nm after treatment with 3% H_2O_2 solution (Fig. 2), and 14 ± 4 nm for the CHX gel-treated discs (Fig. 2), probably a result of gel adsorption to the Ti surface.[22] The differences were not significant statistically.

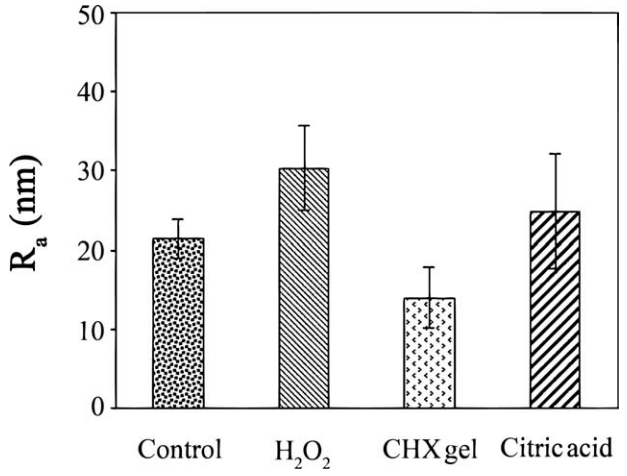

FIGURE 2. Overall bar-graph of the surface roughness (R_a) of the various samples. The AFM measurements gave $R_a = 22 \pm 3$ nm (mean ± SEM) for the control (untreated) samples, 30 ± 5 nm for the H_2O_2 (3%)-treated samples, 14 ± 4 nm for the chlorhexidine gel-treated samples, and 25 ± 7 nm for the citric acid (pH = 1)-treated samples. Statistical analysis did not reveal any significant differences between the groups.

XPS measurements

The XPS measurements revealed Ti, O, C, and N in the topmost atomic layers of all samples (untreated and treated).

The binding energy of Ti 2p 3/2 electrons, which corresponds to Ti^{4+}, was measured at 458.6 ± 0.1 eV for each sample (Fig. 3). The double Ti peaks (Ti 2p at 458.6 and 464 eV) and the O 1s signal (530 eV) demonstrate the presence of the TiO_2 layer.[33,34] The immersion in the different cleaning solutions did not change the Ti 2p signal of the surface (Fig. 3).

Major changes were observed in the O 1s peak, which could be deconvoluted into three peaks (Fig. 4). The most intense one, at ~530.1 eV, is that of lattice O in TiO_2, while the peak at ~531.7 eV is due to surface OH groups. The third peak, at 532.9–533.0 eV, corresponds to the O in C—O and/or C=O bonds. The latter is most intense for the CHX gel-treated sample [Fig. 4(B)], which can be explained in terms of the possible adsorption of CHX to the surface.[22] This is supported by the deconvolution of the C 1s signal (data not shown) which gave four peaks for all samples, the peak at 287 eV for the gel-treated sample proving more intense than those for the other samples.

The decrease in the C 1s signal (Fig. 5) after a 30–60 min He^+ bombardment of the untreated sample indicates the presence of carbonaceous contamination, due to C-containing molecules remaining after cleaning or adsorbed later on the air-exposed surfaces. These elements are observed typically on Ti implant surfaces.[35]

Microscopic images

Optical microscopic images of epithelial cells are to be seen in Figure 6 at magnifications of 200×. Figure 6(A) reveals there are only few attached cells as it is a primary epithelial cell culture, while Figure 6(B) shows a confluent epithelial cell culture.

MTT and protein content measurements

The results of MTT and protein concentration experiments relating to cell attachment (24-h observation) and cell proliferation are illustrated in the bar-graphs of Figure 7. The MTT results [Fig. 7(A)] demonstrate that the epithelial cell attachment on the Ti surface was not disturbed significantly by the different cleaning solutions. More (but not

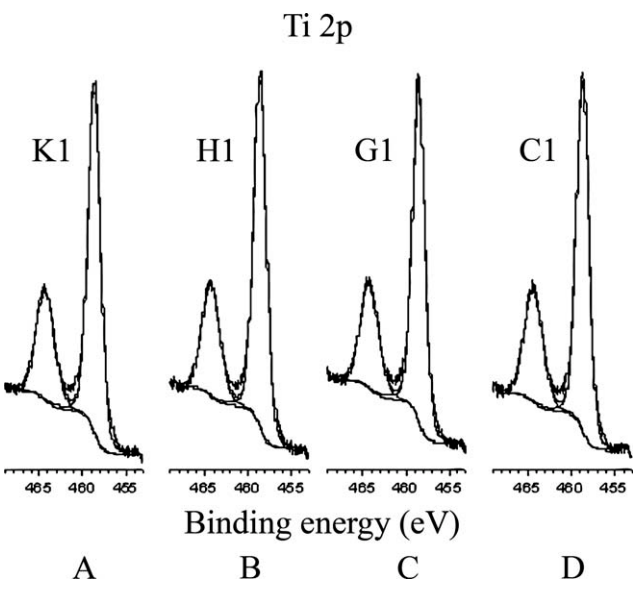

FIGURE 3. Ti 2p signals in XPS spectra of (A) control (K1), (B) H_2O_2-treated (H1), (C) chlorhexidine gel-treated (G1) and (D) citric acid-treated (C1) Ti discs, confirming the presence of TiO_2 on all surfaces.

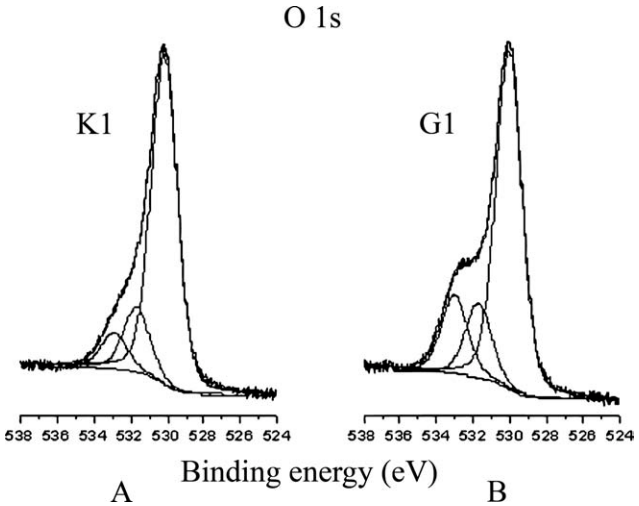

FIGURE 4. O 1s signals in XPS spectra of (A) control (K1) and (B) chlorhexidine gel-treated (G1) Ti discs. The signal was deconvoluted into three peaks: the most intense one (at 530.1 eV) is that of lattice O in TiO_2, while that at ~531.7 eV is due to surface OH groups. The third peak, at 532.9–533.0 eV, corresponds to the O in C—O and/or C=O bonds. The latter is most intense for the chlorhexidine gel-treated sample [Fig. 4(B)], which can be explained in terms of the possible binding of chlorhexidine digluconate to the surface.

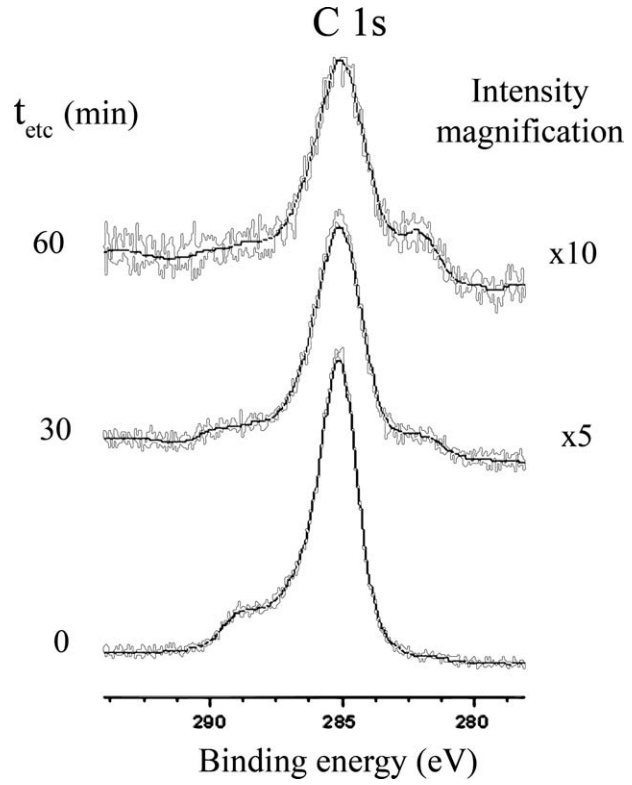

FIGURE 5. C 1s signals in the XPS spectra of the control Ti disc after 0 min (lowestmost curve), 30 min (middle) and 60 min (uppermost curve) of He^+ bombardment. The bombardment led to the removal of ~10 nm from the surface of the material during the operation. The decrease in the C 1s signal indicates the presence of carbonaceous contamination.

significantly more) living cells were observed on the discs treated with H_2O_2 and citric acid than on the control and CHX gel-treated discs. The protein concentration after 24 h [Fig. 7(B)] was similar for all samples. The level of cell proliferation revealed by the MTT measurements [Fig. 7(A)] was increased slightly by the H_2O_2 and citric acid treatments. The H_2O_2-treated sample exhibited a significant ($p = 0.011$) increase as compared to the CHX gel-treated discs. No significant differences were observed among the other groups. The protein concentration after 72 h [Fig. 7(B)] was similar for all Ti samples.

DISCUSSION

R_a was demonstrated by AFM to be similar for all groups. Only for the CHX gel-treated group was R_a lower (14 ± 4 nm), than that of controls. These surfaces are therefore smooth, and equally suitable for epithelial cell attachment and proliferation. The influence of R_a on epithelial cell growth has been studied by many authors and it is known that epithelial cells do not attach so strongly to acid-etched or sand-blasted surfaces as to smooth (polished, $S_a < 0.5$ μm) surfaces.[36] Surfaces with a smooth topography promote epithelial cell growth, spreading, and the production of focal contacts on Ti surfaces.[37]

The XPS measurements proved the presence of an intact TiO_2 layer on both the untreated and the treated samples. A major change was observed for the CHX gel-treated sample, as the O 1s signal included an intense peak corresponding to the O in C—O and/or C=O bonds. This is assumed to be a result of the adsorption of CHX to the surface, as observed by other authors.[22]

The MTT assays (24-h observation) showed that the epithelial cell attachment was not changed significantly on the Ti surfaces treated with the different cleaning solutions, and the protein-content assay supported this. The MTT method revealed differences in cell proliferation (72-h observation) between groups, with a significant increase in proliferation for the H_2O_2-treated sample relative to the CHX gel-treated one. The proliferation for the citric acid-treated samples was higher, but not significantly so, as compared to controls. The protein content assays indicated similar degrees of cell proliferation for the differently treated samples. The variations observed in the proliferation of epithelial cells cannot result from differences in R_a of the Ti samples, as the samples applied in this study had machined surfaces, with R_a between 0.014 and 0.030 μm, depending on the chemical

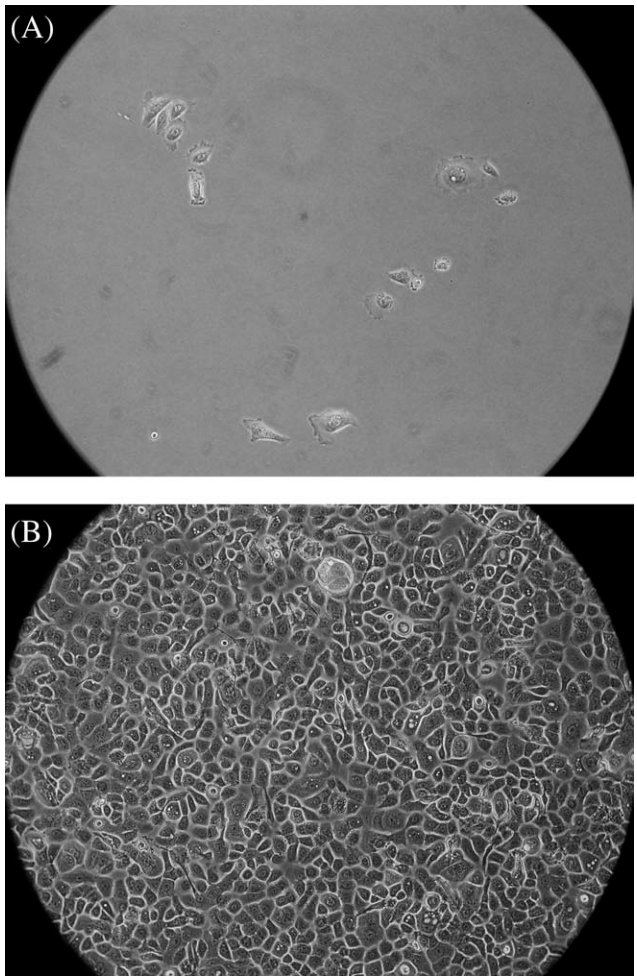

FIGURE 6. Optical microscopic images of A) epithelial cells of primary culture recorded with a Nikon TS 100 (Japan) microscope. There are few attached cells, magnification ×200. (B) Confluent epithelial cell culture (magnification ×200).

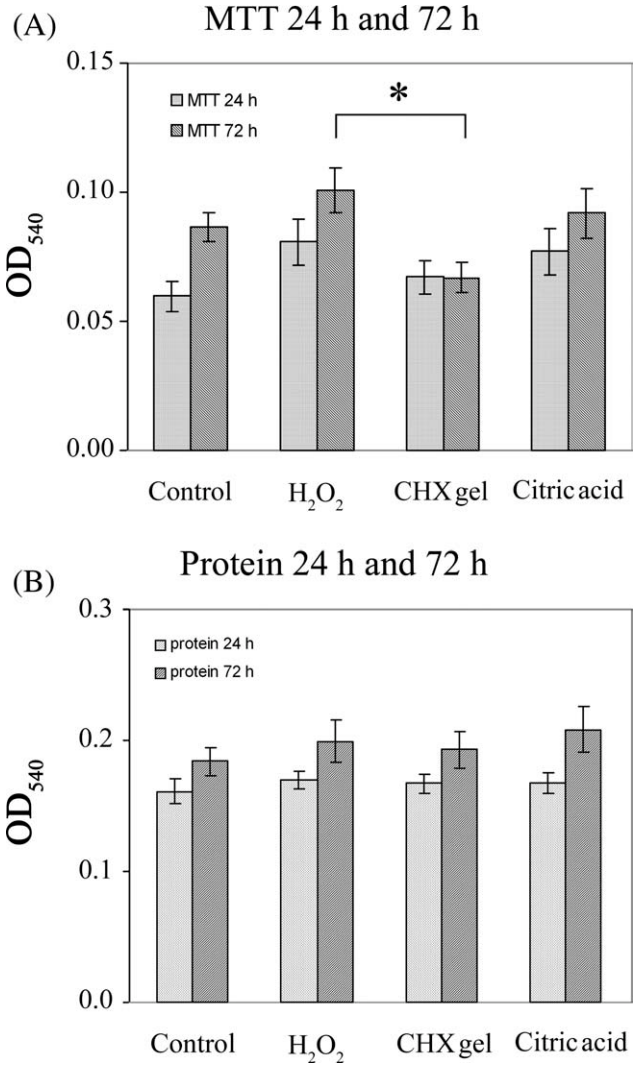

FIGURE 7. (A) 24-h and 72-h MTT and (B) 24-h and 72-h protein concentration results. The MTT data indicated that the epithelial cell attachment to the Ti surface was not disturbed significantly by the different cleaning solutions. H_2O_2 and citric acid treatment of the Ti discs induced slight increases in epithelial cell attachment and viability relative to the control and chlorhexidine gel-treated discs. The level of cell proliferation revealed by the MTT measurements was decreased significantly only in the case of the chlorhexidine gel-treated samples as compared with the H_2O_2-treated discs and the amount of cells was almost the same as at 24 h. The protein concentration after 24 and 72 h was the same for all Ti samples.

agent used. As defined by Klinge et al.[36] these surfaces are all smooth, and it appears improbable that epithelial cells are sensitive to such small changes in R_a. As the R_a values of the two surfaces do not differ significantly, we presume the chemical composition of the CHX gel-treated surface is less favorable for the cells than the H_2O_2-treated surface.

The discrepancy between the results obtained with the two different methods (MTT and protein content assays) in cell proliferation is not yet understood, but may well be associated with the inherent differences between the methods. The MTT assay measures the number of living cells, whereas both viable and nonviable cells are included in the protein-related measurements. Thus, the latter method is effective, but not selective for viable cells.

These findings suggest that the results of epithelial cell-culturing can depend on the investigation method applied, and that it is advisable to take into consideration the adsorption of CHX gel to the Ti surface when this material is used for dental implant decontamination. TiO_2 surface treatment with H_2O_2 or citric acid can result in the same or even better survival and proliferation of epithelial cells than in the untreated case. This is an important finding as these toxic decontamination solutions were not expected to improve cell attachment and proliferation at all.

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III.

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Dekontaminálóanyagok hatása a titánfelszín biointegrációs tulajdonságaira: *in vitro* humán epithel sejtkultúra vizsgálatok

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A peri-implantitisz terápiájában a kontaminálódott fogászati implantátum-felszín fertőtlenítése és kémiai tisztítása alapvető fontosságú. Fontos azonban az is, hogy a tisztítás ne eredményezzen a titánfelszínen olyan változást, ami az implantátum biointegrációra való alkalmasságát hátrányosan befolyásolná. A szerzők Grade 4-es tisztaságú, esztergált felszínű CP titán korongokat (CAMLOG^{TM} Biotechnologies AG, Svájc) kezeltek 3% H_2O_2-dal (5 perc), túltelített citromsavval (pH = 1; 1 perc) vagy klórhexidin géllel (5 perc). A korongok felszínét kezelés előtt és után atomi erő mikroszkóppal (AFM) és röntgen-fotoelektron spektroszkóppal (XPS) vizsgálták. A biointegrációra való alkalmasság minősítésére humán orális epithel sejtek 24 óra eltelte utáni kitapadását és 72 óra alatt bekövetkező sejtosztódását értékelték a szerzők dimetiltiazolil-difeniltetrazólium bromid (MTT) teszttel és bicinkoninil sav (BCA) fehérje-meghatározó módszerrel. Az AFM mérések nem mutattak szignifikáns különbséget a felületek kezelés előtti és utáni érdessége között. Az XPS eredmények alapján a TiO_2 réteg (1-25 nm) szerkezete a kezeléseket követően egyik mintán sem változott. Az MTT és BCA vizsgálatok hasonló mértékű sejtletapadást mutattak mindegyik vizsgálati csoportban, a sejt-proliferáció MTT vizsgálatának eredménye viszont szignifikánsan magasabb értékű volt a H_2O_2-dal kezelt korongok esetében, mint a klórhexidin géllel kezelt korongokon. Következtetések A vizsgált korongok felszínén a dekontaminálóanyagokkal való kezelés nem járt a biointegrációra való alkalmasságot károsan befolyásoló hatással. A H_2O_2-dal történő tisztítás után némileg növekedett a sejtosztódás mértékének a mutatója, a klórhexidin gélhez viszonyítva.

Kulcsszavak: peri-implantitisz, implantátumfelszín, dekontamináció, epithel sejtkultúra

Bevezetés

A titánnak és ötvözeteinek kedvező tulajdonságaik miatt, széles körű orvosi és fogorvosi alkalmazásai ismertek [21, 24]. Erősen reaktív fém, nanoszekundumok (10^{-9} s) alatt 20–100 Å vastagságú oxidréteg keletkezik a felszínén. Ez a réteg átjárhatatlan az oxigén és más szennyeződések számára, ezáltal korrózióresisztenssé válik [19]. Kis sűrűsége (4,43 g/cm^3; az acélnál 45%-kal könnyebb, de ugyanolyan erős) és kiemelkedő biokompatibilitása teszi a fogászati implantológia illetve az arc-, állcsont- és szájsebészet ideális anyagává.

Az implantátumok élettartamát az anyaguk, szerkezetük és az adott igénybevétel mellett nagymértékben befolyásolja a környező lágy- és keményszövetek (az alveoláris csont, a kötőszövet és a hámréteg) állapota. A fogászati műgyökerek behelyezését követő esetlegesen kialakuló szövődményekért általában eme szövetekben kialakuló, gyorsan terjedő gyulladás a felelős.

A peri-implantális gyulladások közül a peri-implantális mucositis olyan reverzíbilis gyulladás, amely az implantátum körüli lágyszöveteket érinti, csontpusztulás nélkül. Ezzel szemben a peri-implantitisz esetén lágy- és keményszövetekre terjedő gyulladás figyelhető meg, mely visszafordíthatatlan, és csontlebontódással jár [2, 39]. Három, klinikailag releváns tanulmányban Brånemark-implantátumok behelyezése után követéses vizsgálatokat végeztek, hogy a peri-implantitisz előfordulási gyakoriságát meghatározzák [11, 28, 29]. *Fransson és mtsai* [11] minimum 5 éves követés során 662 páciens esetében, 3413 behelyezett implantátumot vizsgáltak. A páciensek 28%-ában progresszív csontpusztulást (egyéves és több mint ötéves vizsgálat között kialakult csontpusztulás) tapasztaltak. Az implantátumok számára vonatkoztatva, ez a százalék 12,4% volt. *Renvert és mtsai* [28] átlagosan 10,8 éves követéses vizsgálatukban (213 páciens, 976 Brånemark-implantátummal) azt tapasztalták, hogy a behelyezett implantátumok 14,9%-ánál volt több mint három csavar-

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menetnyi csontpusztulás. Ez minimum 1,8 mm-nyi csontveszteségnek felel meg, és ezt tekintették peri-implantitisznek. *Roos-Jansåker és mtsai* [29] 9–14 éves követéses vizsgálatot végeztek 218 páciens 1057 implantátumának behelyezését követően. A klinikai és radiológiai értékelés szerint, kimutatták, hogy a páciensek 16%-ánál (kortól, nemtől, behelyezés helyétől, dohányzási szokásoktól függetlenül) alakult ki peri-implantitisz, míg az implantátumokra vonatkoztatva 6,6%-nál. A peri-implantitisz meghatározásánál szintén a 3 csavarmenetnél nagyobb csontpusztulást vették figyelembe. Az előfordulás gyakoriságában tapasztalható különbségek többféle okra vezethetők vissza. Többek között a peri-implantitisz diagnosztikai kritériumainak meghatározása, az implantátum behelyezésének konkrét jellemzői és a páciensek különböző gyógyulási hajlama is hozzájárulhat a különbség kialakulásához. A fertőzés és a mechanikai faktor (túlterhelés a szuprastruktúra elkészítését követően) döntő fontosságú a peri-implantális gyulladások etiológiájában [38]. Habár az okok különbözők, mindkét faktor esetében bakteriális kolonizáció figyelhető meg az implantátum felszínén [18, 27]. A dentális implantátum körüli gyulladás kialakulásáért és fennmaradásáért leginkább a Gram-negatív anaerob mikroflóra a felelős [20]. Ha a környezeti tényezők a kórokozók számára optimálisak, akkor a baktériumok és toxinjaik nagymértékben felhalmozódnak, és gyorsan progrediáló, apikálisan terjedő csontpusztulás alakul ki, amely súlyos esetben az implantátum elvesztéséhez vezet.

A peri-implantitisz terápiájában az elhalt szövet maradéktalan eltávolítása és a kontaminálódott felszín tisztítása alapvető fontosságú, amelyet sebészi technikákkal egészíthetünk ki. Az implantátum felszínének tisztítása történhet mechanikai úton (homokfúvás), kémiai anyagokkal (citromsav, foszforsav, H_2O_2, klórhexidin-diglükonát [CHX], delmopinol, jód, klóramin-T, etiléndiamin-tetraecetsav [EDTA]) vagy különböző lézerek segítségével (CO_2, dióda, Er:YAG, Nd:YAG). Sebészeti beavatkozásként alkalmazható az irányított szöveti regeneráció, a csontgraftok és -membránok különböző típusai [18, 33]. Súlyos esetben, szisztémás és lokális antibiotikum kezeléssel egészíthetjük ki a terápiát [14, 27, 33, 36].

A CHX általánosan alkalmazott szer a fogorvosi kezelések során, szájöblögetőként és helyi antimikrobiális szerként is javasolják. A peri-implantitisz terápiájában a CHX oldatát öblögetőként, gyakran átöblítő oldatként használják, kiegészítve szisztémás antibiotikum adással [1, 14, 30, 33]. *Renvert és mtsai* [25, 26] a CHX és a minociklin hatásosságát vizsgálták. Tapasztalataik alapján a minociklin a periimplantális tasak szondázási mélységére és a vérzési index alakulására is pozitív hatással volt, míg a CHX csak enyhén csökkentette a vérzési index értékét. A CHX alkalmazása hatékony kiegészítésnek bizonyult sebészi terápia esetében is, irányított szöveti regeneráció során [12, 35].

Barbour és mtsai [6] vizsgálták a CHX kötődését anatáz és rutil TiO_2 kristályokhoz. Kísérleteik során a CHX-et foszfát- és 4-morfolinoetánszulfonil sav (MES) pufferben vitték fel a TiO_2 kristályokra. Több CHX kötődött az anatáz TiO_2 kristályokhoz mint a rutilhoz, és gyorsabban vált le (deszorbeálódott) az anatázról, mint a rutilról, a puffertípustól függően. Burchard [8] tanulmánya alapján a fibroblasztok szívesebben tapadnak ki a CHX-el kezelt felületre, mint az ón-fluoriddal (SnF_2) kezeltre.

A peri-implantitisz sebészi kezelése során túltelített citromsav-oldatot is gyakran használnak a kontaminálódott implantátum tisztítására [10, 34]. Érdes Ti implantátumok felszínének (Nobel Biocare™, Göteborg, Svédország) plakkal történő kontaminálódása után, a citromsavas és a 10%-os H_2O_2-os kezelés hatására, azt tapasztalták, hogy mindkét anyag esetén újból összeointegrálódtak az implantátumok [3]. A 3%-os H_2O_2 hatásosnak bizonyult a peri-implantitisz sebészi terápiájában is, membrán alkalmazása esetén [31, 32].

Khoury [14] vizsgálataiban ennek a három anyagnak (citromsav, CHX és H_2O_2) a kombinációját alkalmazta a peri-implantitisz sebészi terápiájában. Az elhalt szövetek eltávolítása után a kontaminálódott implantátumfelszínt többször CHX-el mosta, majd citromsavval kezelte 1 percig, amelyet H_2O_2-dal és fiziológiás sóoldattal öblített le.

Dennison és mtsai [9] azt tapasztalták, hogy könnyebb a bevonat nélküli esztergált felszínű implantátumokat dekontaminálni citromsavval vagy CHX-vel, mintha hidroxi-apatittal lenne borítva a felszín.

A CHX, a citromsav és a H_2O_2 gyakran alkalmazott kémiai ágensek a peri-implantitisz terápiájában. A szakirodalomban nem találtunk olyan tanulmányokat, amelyek azt vizsgálták volna, hogy ezek az anyagok megváltoztatják-e a titán (Ti) felszín összetételét, felületi érdességét, és ezáltal befolyásolják-e a biológiai környezet válaszát. Kísérleteink tervezésekor azt tűztük ki célul, hogy nyomon kövessük az egyes anyagokkal történő kezelést követően a Ti felület összetételében, érdességében bekövetkezett esetleges változásokat. Vizsgáljuk továbbá a humán epithél sejtek tapadási és proliferációs készségének változását a próbatestek felületén, és értékeljük a biológiai környezet válaszát a különböző dekontamináló anyagokra.

Vizsgálati anyag és módszer

CP grade 4-es tisztaságú esztergált felszínű Ti korongokat használtunk (átmérő: 9 mm, vastagság: 1,5 mm, CAMLOG™ Biotechnologies AG, Svájc) a fogászati implantátumok nyaki részére jellemző érdességgel (R_a < 0,2 μm) [7]. A korongokat acetonnal és abszolút etanollal mostuk ultrahangos fürdőben, 15 percig. Tisztítás után a próbatesteket 3%-os H_2O_2-vel, túltelített citromsavval (pH=1) vagy CHX géllel (Corsodyl dental gel; SmithKline Beecham Consumer Healthcare, Nagy-Britannia) kezeltük. A Corsodyl 1% w/w CHX-t tartal-

maz. A kezelés időtartamát 5 percben határoztuk meg a H_2O_2 és a CHX gél esetében, míg 1 percben a citromsavnál. A kezelések után a próbatesteket háromszor mostuk ultratiszta vízzel, majd levegőn szárítottuk. A kontroll-csoportot ultratiszta vízzel mostuk 5 percig.

Az AFM vizsgálatot a PSIA XE-100 készülékkel (Dél-Korea) végeztük. Az AFM módszer lehetőséget nyújt a felszínek érdességének mikronos-nanométeres nagyságrendű vizsgálatára, miközben a szilikon tartókarra rögzített AFM tű (típusa: P/N 910M-NSC36 (MikroMasch Eesti OU, Észtország) megközelíti és eltávolodik a vizsgált felszíntől. A vizsgálatokat kontakt módban végeztük, a magassági, deflekciós és a 3D képeket rögzítettük. 10x10 μm és 5x5 μm-es felvételeket készítettünk. Az érdesség (R_a) meghatározását az AFM software program segítségével végeztük (legalább 6 független mérés alapján).

A Ti felszín kémiai összetételét XPS készülék segítségével értékeltük. A fotoelektronok Al Kα primer sugárzásból származtak (hν = 1486,6 eV), melyeket hemiszférikus elektronenergia-analizátor segítségével értékeltünk (PHOIBOS 150 MCD 9; SPECS). A röntgenágyút 150 W-on működtettük (12 kV, 12,5 mA). A kötési energiát normalizáltuk a bekötött szén C 1s csúcsához viszonyítva (285,1 eV). Az XPS spektrumban mutatkozó változásokat 30–60 perc He^+ bombázást követően detektáltuk. A He^+ ionokat ionágyúval (5 kV) generáltuk, és a beeső ionsugarat 200 nA-nél mértük. A bombázás kb. 10 nm felszíni anyagot távolított el. Széles illetve nagy felbontású, keskeny spektrumokat vettünk fel, és a Ti 2p, O 1s és C 1s karakterisztikus vonalakat vizsgáltuk.

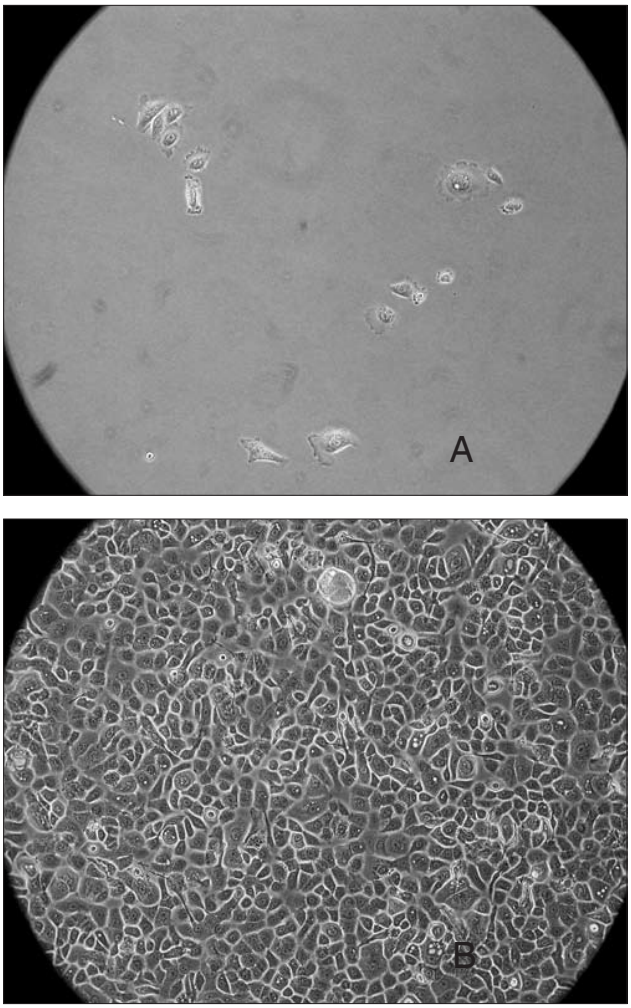
Sejtkultúra-vizsgálatok

Egészséges páciensekből – egyébként is szükséges szájsebészeti beavatkozás során – eltávolított gyulladásmentes nyálkahártyából izoláltunk orális epithel sejteket. A donorok életkora 18 és 46 év között volt. A vizsgálati protokollt a Szegedi Tudományegyetem Humán Orvosbiológiai Etikai Bizottsága jóváhagyta, a kutatásetikai mérték mindenben megfelelt a Helsinki Egyezménynek.

A nyálkahártya-darabokat először 2% antibiotikum-antimikotikum oldattal (Sigma-Aldrich GmbH, Németország) kiegészített Salsol A oldatban mostuk (Human Rt., Gödöllő, Magyarország). Ezután a mintákat dispase enzimoldatban (Grade II, Roche Diagnostics, Mannheim, Németország) inkubáltuk egy éjszakán át, 4 °C-on. Másnap elválasztottuk egymástól a dermiszt és az epidermiszt [16]. Az izolált epidermiszt 0,25%-os trypszin-EDTA oldatban inkubáltuk (Sigma-Aldrich GmbH, Németország) 5 percig, 37 °C-on, így a szövetből sejteket nyertünk. A sejtszuszpenziót 200 g-n 10 percig 4 °C-on centrifugáltuk, majd a továbbiakban az epidermális sejteket 25 cm^2-es flaskákban tenyésztettük (Orange Scientific, Belgium).

Az orális epithel sejteket keratinocita szérummentes mediumban (Gibco BRL, Eggstein, Németország) tenyésztettük. A tápfolyadékot 5 μg/ml rekombináns epidermális növekedési faktorral (Gibco BRL, Eggstein, Németország), 50 mg/ml borjú agyalapi mirigy-kivonattal (Gibco), 1% L-glutaminnal és 1% antibiotikum/antimikotikum oldattal egészítettük ki (1% penicillin G, 1% streptomycin szulfát és 0,0025% amphotericin B; Sigma-Aldrich GmbH, Németország).

A tápfolyadékot hetente háromszor cseréltük le a sejttenyészeteken. A primer epithel sejtkultúra 8–16 nap alatt vált konfluenssé. A konfluens primer kultúrákat PBS-el mostuk (phosphate-buffered saline, pH = 7,4, Gibco) és 2-4-percig kezeltük 0,25%-os trypsin-EDTA oldattal (Sigma-Aldrich GmbH, Németország). A sejteket 2–4 egyenlő részbe passzáltuk. A kultúrákat 37 °C-on, párás környezetben, 5%-os CO_2 tartalom mellett tenyésztettük.



1. ábra
Humán epithél sejtkultúra fénymikroszkópos felvételei. Az *(A)* felvételen néhány letapadt epithél sejt látható, míg a *(B)* felvételen konfluens tenyészet. 200×-os nagyítás.

Inverz optikai mikroszkóppal (Nikon TS 100, Japán) felvételeket készítettünk a tenyésztőoldatban lévő letapadt sejtekről 200x nagyításban. A primer *(1a. ábra)* és konfluens *(1b. ábra)* tenyészetben láthatóak az

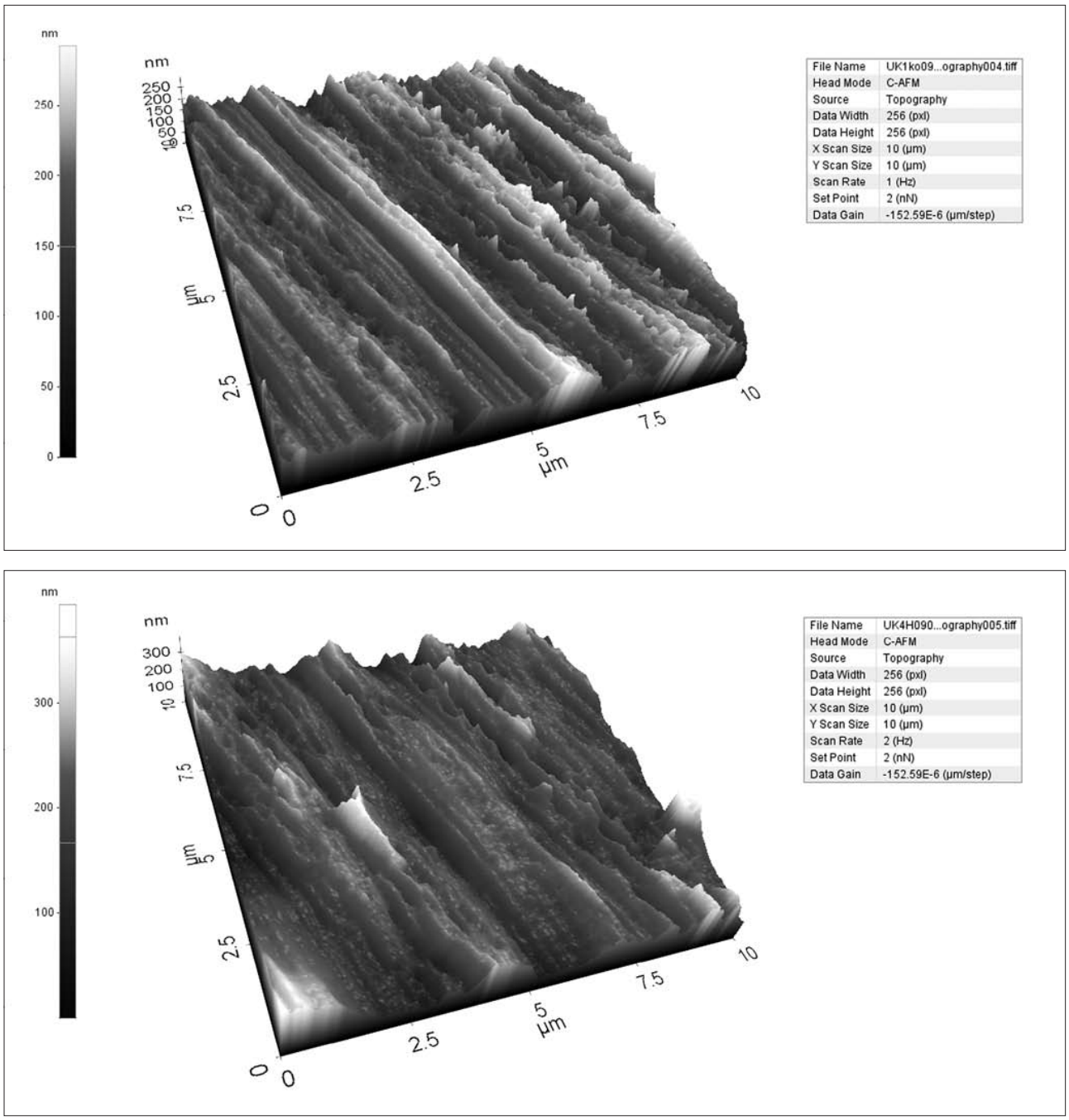
egészséges sejtek, a tápban fertőzésre utaló jel nem látható.

Sejtletapadás és proliferáció vizsgálata

A sejttenyésztés előtt a kontroll és kezelt Ti-korongok mindkét felszínét UV-C alatt 20 percig sterilizáltuk.

A vizsgálatainkhoz harmadik passzázsban lévő orális epithel sejtkultúrát használtunk. A sejt letapadást 24 h, a proliferációt 72 h elteltével vizsgáltuk. Négy független kísérletet végeztünk, minden csoportban 5 korongszámmal.

A dimetiltiazolil-difeniltetrazólium bromid (MTT) vizsgálat során az élő sejtek mitokondriális enzimjeik segítségével redukáltuk a sárga színű MTT-t, amely során kék színű formazán kristályok keletkeznek. A kristályok feloldása után kapott oldat színintenzitása arányos a mintában lévő sejtek számával [22]. Ezt a módszert alkalmaztuk először a sejtek letapadásának és túlélésének vizsgálatánál. A Ti korongokat 48-lyukú sejttenyésztő edénybe tettük, majd mindegyikre 10^4 sejtet szélesztettünk. A sejteket 24 vagy 72 h időtartamig tenyésztettük a Ti próbatesteken. Ezután a felülúszót el-

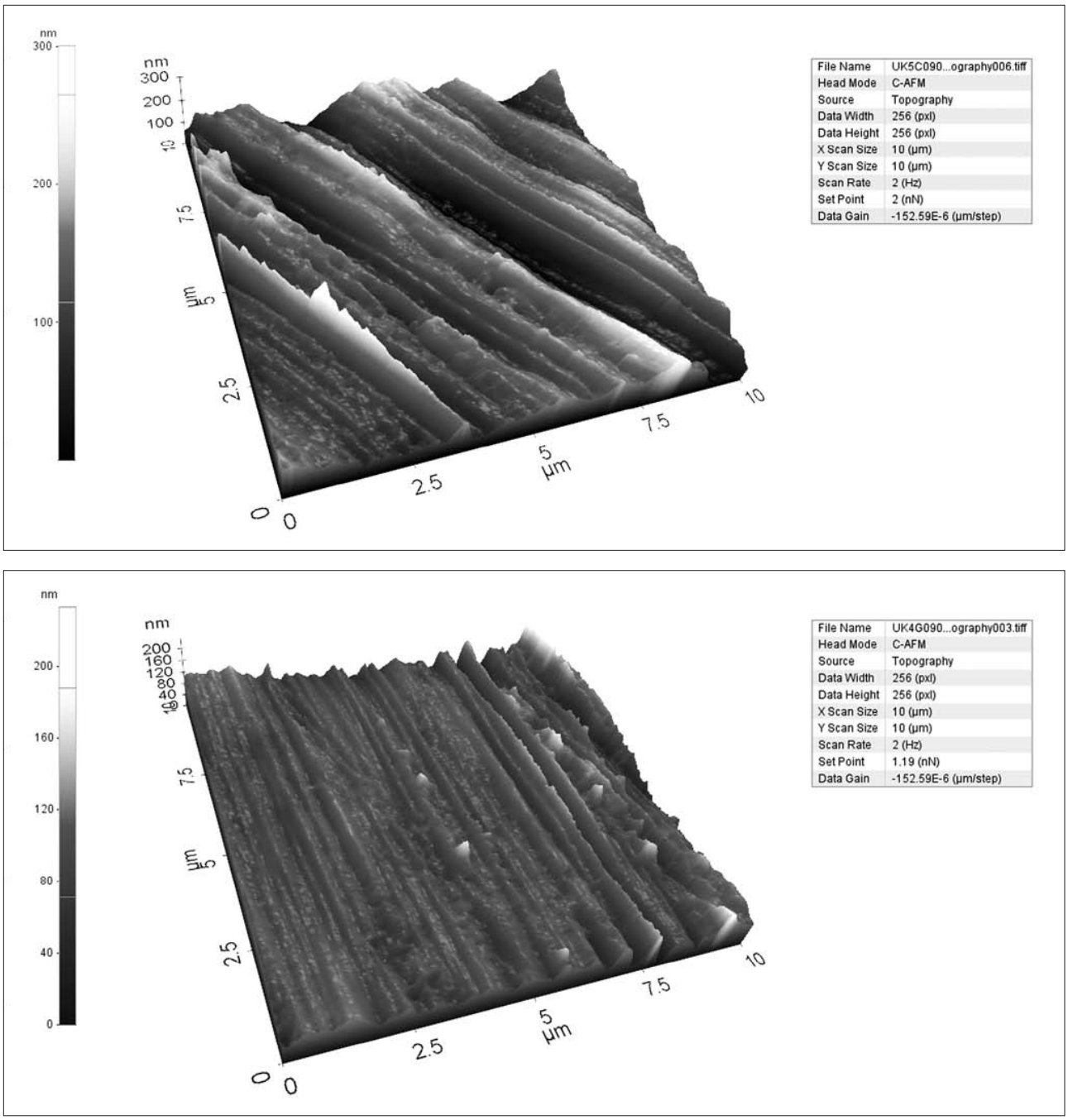


2. ábra

Kontroll (kezeletlen) minta *(A)* és 3%-os H_2O_2-vel kezelt korong *(B)* felszíni érdességének 3 dimenziós (3D) AFM felvétele. Az esztergált felszínre jellemző párhuzamosan futó barázdák színe egyre világosabb a barázda mélyétől felfelé. Méret: 10x10 µm

távolítottuk, majd a sejtekre RPMI tápfolyadékban oldott, 0,5 mg/ml koncentrációjú MTT (Sigma-Aldrich GmbH, Németország) festéket mértünk, amellyel a sejteket 4 órán át inkubáltuk, 37 °C-on. Ezután a felülúszót óvatosan eltávolítottuk, majd a kristályokat 2%-os sodium dodecyl szulfát oldatban (SDS) és 0,04 mM sósavas isopropanolban feloldottuk. Az optikai denzitást (OD) 540 nm-nél mértük Multiscan Ex spektrofotométer (Thermo Labsystems, Vantaa, Finnország) és Ascent Software (Thermo Labsystems, Vantaa, Finnország) segítségével.

A fehérjemennyiség meghatározását (élő és elhalt sejtekből) „micro BCA™ protein assay kit"-tel (Pierce, Rockford, IL, USA) végeztük. A fehérjemérés standardjaként borjúszérum-albumint (Pierce, USA) használtunk. A sejteket lízis pufferrel feltártuk (20 mM Tris-HCl, pH 7,5; 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2,5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 és 1 µg/ml leupeptin), majd rámértük a zöld színű reagenst. Az oldatot 2 órán át inkubáltuk 37 °C-on. Az oldat lila színűvé vált a benne lévő fehérje mennyiségével arányosan. Az op-



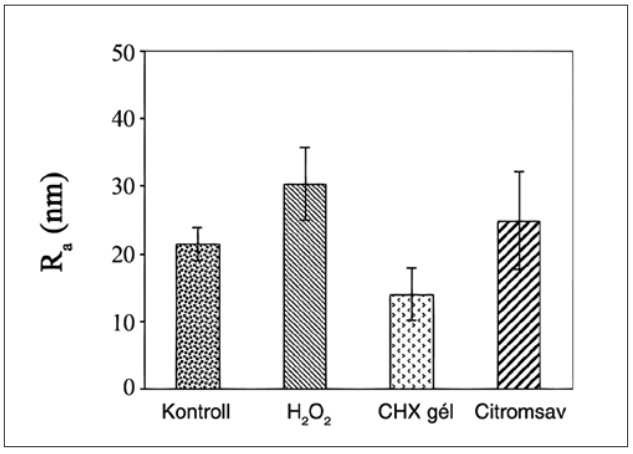
3 a-b. ábra
Túltelített citromsavval (pH = 1) *(A)* és klórhexidin (CHX) géllel kezelt korongok *(B)* felülete látható a háromdimenziós AFM felvételeken. Méret: 10x10 µm

tikai denzitást (OD) 540 nm-en mértük Multiscan Ex spektrofotométer (Thermo Labsystems, Vantaa, Finnország) és Ascent Software (Thermo Labsystems, Vantaa, Finnország) segítségével.

Adatok feldolgozása, statisztika
Átlag ± átlag szórása (standard error of the mean – SEM) értékeket számoltuk ki az AFM, az MTT és a fehérjetartalom vizsgálat esetében is. Normalitás vizsgálat után, egytényezős varianciaanalízist végeztünk (ANOVA), majd Tukey és Scheffé *post hoc* teszteket alkalmaztunk az értékek páronkénti összehasonlítására (SPSS 15.0, SPSS, Chicago, Illinois, USA). A szignifikancia-szintet 0,05-nek vettük ($p < 0,05$).

Eredmények

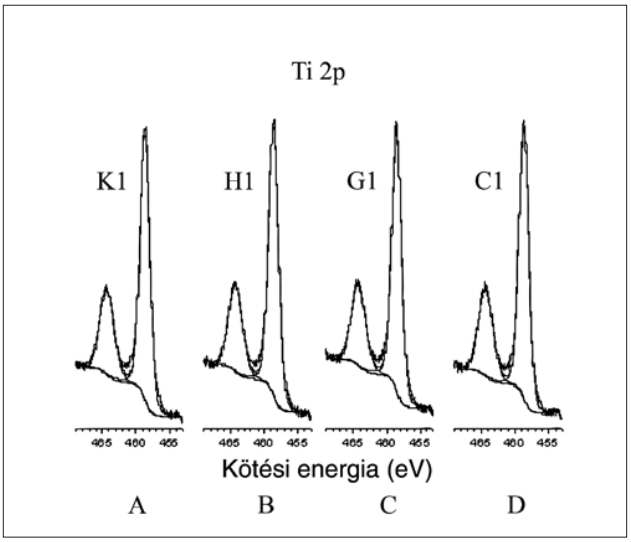
AFM vizsgálat
Az *in vitro* vizsgálatok előtt a Ti-korongok felszínét AFM és XPS segítségével vizsgáltuk. A *2a és 2b ábrák*on jól látható, hogy párhuzamosan futó barázdák vannak az esztergált felszínen, a szín egyre világosabb a barázda mélyétől felfelé. Az AFM mérések a kontroll-csoportban $R_a = 22 \pm 3$ nm felületi érdességet adtak *(2a. ábra)*. A citromsavval kezelt mintákon 25 ± 7 nm *(3a és 4 ábra)*, míg a 3% H_2O_2 csoportba tartozó próbatesteken 30 ± 5 nm volt az érdesség *(2b és 4 ábra)*. A CHX géllel kezelt csoportban 14 ± 4 nm volt *(3b és 4 ábra)*. A csökkenést – amely nem volt szignifikáns – valószínűleg a gél Ti-felszínhez történő adszorpciója okozta [6].



4. ábra
A kontroll, a H_2O_2-vel, a klórhexidin (CHX) géllel és a citromsavval kezelt Ti-korongok felületi érdességének (R_a) ábrázolása oszlopdiagramon, az átlag értékek és az átlag szórásának jelölésével. Az AFM vizsgálat a kontroll (kezeletlen) csoportban $R_a = 22 \pm 3$ nm (átlag ± SEM) felületi érdesség értéket adott. Ez az érték 30 ± 5 nm a H_2O_2 (3%) –dal kezelt csoportban, 14 ± 4 nm a CHX géllel történt kezelés esetén, míg 25 ± 7 nm a citromsavval (pH = 1) kezelt próbatesteken. A statisztikai analízis nem mutatott szignifikáns különbséget a csoportok között.

XPS vizsgálat
Az XPS vizsgálat során a kezelt és kezeletlen minták felszínén is jelen voltak az általában megfigyelhető elemek: a Ti, O, C és N.

A Ti^{4+}-nak megfelelő Ti 2p 3/2 elektronok kötési energiája 458,6 ± 0,1 eV-nál volt mérhető minden mintán *(5. ábra)*. A kettős Ti csúcsok (Ti 2p, 458,6 és 464 eV-nál) és az O 1s jel (530 eV) bizonyítja a TiO_2 réteg jelenlétét [4,15]. A különféle oldatokkal történő kezelés nem változtatta meg a korongok felszínein a Ti 2p jelet (5. ábra).

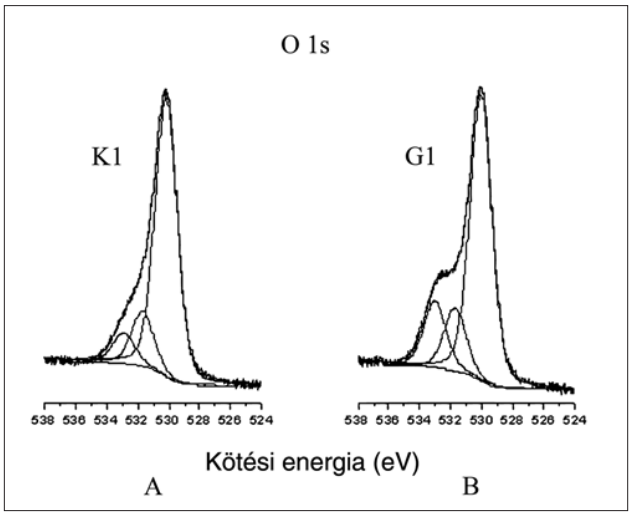


5. ábra
A kontroll (*A*; K1), a H_2O_2-vel (*B*; H1), a klórhexidin géllel (*C*; G1) és a citromsavval (*D*; C1) kezelt Ti korongok Ti 2p jele az XPS spektrumban. A TiO_2 minden felszínen jelen van

Változást tapasztaltunk azonban az O 1s csúcsnál, amelyet három csúcsra lehet felbontani *(6a. és 6b. ábrák)*. A legintenzívebb, ~ 530,1 eV-nál mérhető, amely a TiO_2-ban lévő O-t jelzi, míg a ~ 531,7 eV-nál mért csúcs a felszíni OH csoportoknak köszönhető. Az 532,9-533,0 eV között mérhető harmadik csúcs a C-O és/vagy C=O kötésekben jelenlévő O-tól származik. Ez utóbbi jel a CHX géllel kezelt minták esetében volt a legintenzívebb *(6b. ábra)*, amely valószínűleg a CHX felszínbe történő adszorpciójából származik [6]. Ezt a C 1s jel felbontása is alátámasztja (nem közölt ábra), melyet minden mintánál 4 csúcsra lehetett bontani. A géllel kezelt mintáknál a 287 eV –nál mért csúcs intenzívebb volt, mint a többi csoport esetében.

A kezeletlen mintákon a C 1s jel gyengülése tapasztalható *(7. ábra)*, 30–60 perc He^+ bombázás után. Ez azoknak a szénszennyeződéseknek köszönhető, amelyek a tisztítás után maradtak a felszínen, vagy a levegőből adszorbeálódtak a tárolás során. Ezek az elemek általában jelen vannak a Ti implantátum felszínén [23].

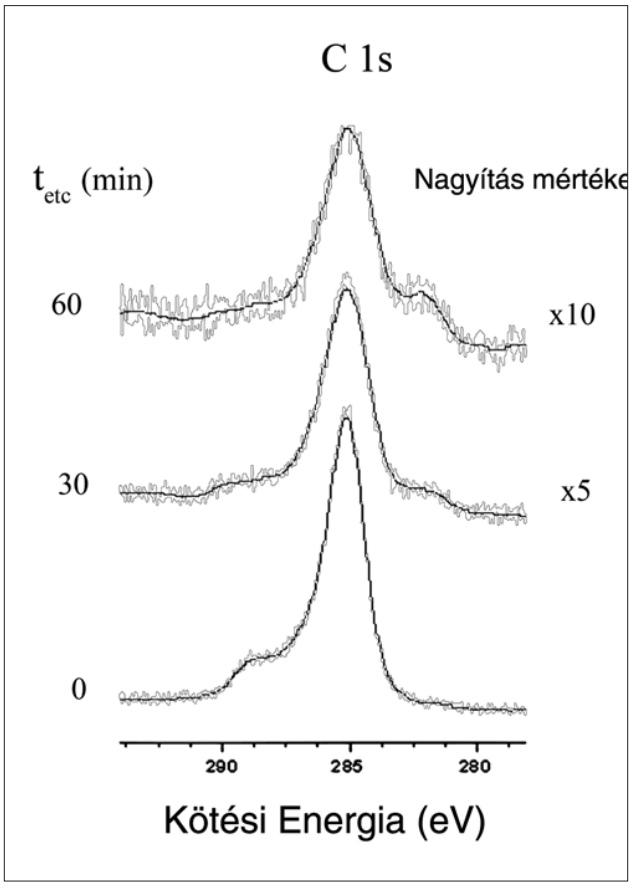
MTT- és fehérjemennyiség-meghatározás
A titánkorongokon 24 és 72 órán át növesztett sejtekkel végzett MTT mérés eredményeit a *8. ábrá*n látható diagrammon ábrázoltuk. A 24 óra után végzett MTT mérés nem mutatott szignifikáns különbséget az egyes



6. ábra
Az XPS spektrum O 1s jelei a kontroll *(A)*
és a klórhexidin (CHX) géllel kezelt *(B)* korongok esetében.
A jel három csúcsra oszlik: a legintenzívebb (530.1 eV)
a TiO_2-ban lévő O-t jelzi, míg az ~ 531,7 eV-nál mért csúcs
a felszíni OH csoportoknak köszönhető.
A harmadik, 532.9–533.0 eV közötti csúcs a C-O és/vagy C=O
kötésekben jelenlévő O-től származik.
Az utóbbi jel a CHX géllel kezelt korongok esetében
a legintenzívebb *(B),* ami a CHX felszínbe történő
kötődésével magyarázható

csoportokban mért abszorbanciák között. ($OD_{540,kontroll}$ = 0,059 ± 0,006, $OD_{540,H2O2}$ = 0,081 ± 0,009, $OD_{540,CHX\ gél}$ = 0,067 ± 0,006, $OD_{540,citromsav}$ = 0,077 ± 0,009). Nagyobb értéket kaptunk a H_2O_2-dal és a citromsavval kezelt korongok esetében, mint a kontroll és a CHX géllel kezelt próbatesteken, azonban a különbség nem volt szignifikáns. A 72 óra után végzett MTT teszt enyhe sejtmennyiség növekedést mutatott a H_2O_2-dal és a citromsavval kezelt korongokon. A H_2O_2-dal kezelt korongokon lévő sejt proliferáció mértéke szignifikánsan magasabbnak (p = 0,011) bizonyult a CHX géllel kezelt csoporthoz viszonyítva ($OD_{540,kontroll}$ = 0,087 ± 0,006, $OD_{540,H2O2}$ = 0,101 ± 0,009, $OD_{540,CHX\ gél}$ = 0,067 ± 0,006, $OD_{540,citromsav}$ = 0,092 ± 0,009). A többi csoport között nem találtunk szignifikáns eltérést.

A titánkorongokon 24 és 72 órán át növesztett sejtekkel végzett fehérjevizsgálat eredményeit a *9. ábrá*n lévő diagrammon láthatjuk. A 24 órát követő fehérjetartalom-mérés hasonló értékeket adott mind a 4 csoportban: $OD_{540,kontroll}$ = 0,162 ± 0,009, $OD_{540,H2O2}$ = 0,170 ± 0,007, $OD_{540,CHX\ gél}$ = 0,168 ± 0,007, $OD_{540,citromsav}$ = 0,168 ± 0,008. A fehérjekoncentráció-mérés eredménye 72 óra után hasonló volt mind a négy csoportban: $OD_{540,kontroll}$ = 0,185 ± 0,011, $OD_{540,H2O2}$ = 0,199 ± 0,016, $OD_{540,CHX\ gél}$ = 0,194 ± 0,014, $OD_{540,citromsav}$ = 0,209 ± 0,017.

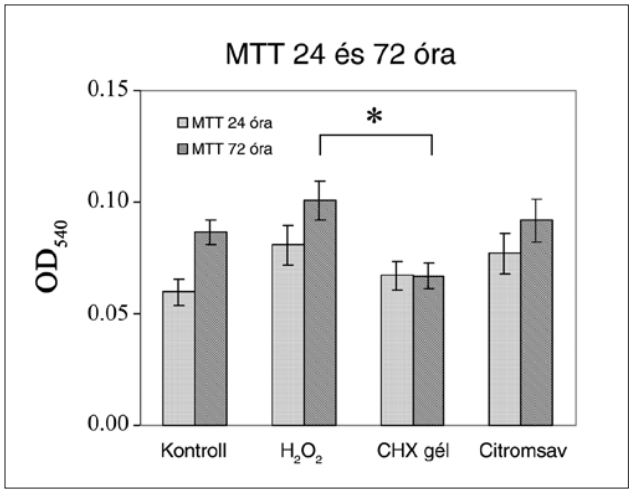


7. ábra
Kontroll-minták XPS spektrumának C 1s jele,
növekvő időtartamú He^+ bombázást követően.
A legalsó görbe 0 perc bombázást, a középső görbe 30 perc,
míg a legfelső görbe 60 perc He^+ bombázást jelent.
A bombázás ~ 10 nm anyagot távolít el a felszínről.
A C 1s jel fokozatos gyengülése igazolja a szén szennyeződések
jelenlétét.

Megbeszélés

Az epithel sejtek különböző érdességű felszíneken történő letapadását, túlélését több tanulmányban vizsgálták már. Kimutatták, hogy az epithel sejtek nem tapadnak olyan erősen a savmaratott vagy homokfúvott felszínhez, mint a simához (polírozott, R_a < 0,5 μm) [17]. A sima felszínek elősegítik az epithel sejtek növekedését, osztódását és kapcsolódását a Ti-felszínhez [5]. Mivel az AFM vizsgálat során kapott R_a érdesség értékek hasonlóak voltak mind a négy csoportban és egyedül csak a CHX géllel kezelt csoport esetében tapasztaltunk enyhe csökkenést a kontroll korongokhoz képest, megállapítható, hogy a felszínek simák

voltak, és egyformán alkalmasak a sejtek letapadására, proliferációjára. A sejt-proliferáció vizsgálatánál kapott különbségeket nem eredményezhette a felszínek érdességei között mutatkozó különbség, mert vizsgálatunkban az esztergált felszínű korongok érdessége mind 0,014 és 0,030 μm között változott, az alkalmazott szertől függően. *Klinge* szerint az ilyen felszínek simának tekinthetők, és nem valószínű, hogy az epithel sejtek érzékelik az ilyen kis érdességbeli változásokat [17]. *Stájer* vizsgálatában azt tapasztalta, hogy savas pH és nagy fluoridkoncentráció jelenléte együttesen okozhatja a titán felszín korrózióját, mely negatívan befolyásolja az epithel sejtek letapadását, proliferációját [37]. A hazai irodalomban többek között *Joób-Fancsaly és mtsai* [13] vizsgálták már korábban fibroblaszt és oszteoblaszt sejtek proliferációs készségét különböző fizikai beavatkozásokkal módosított titán



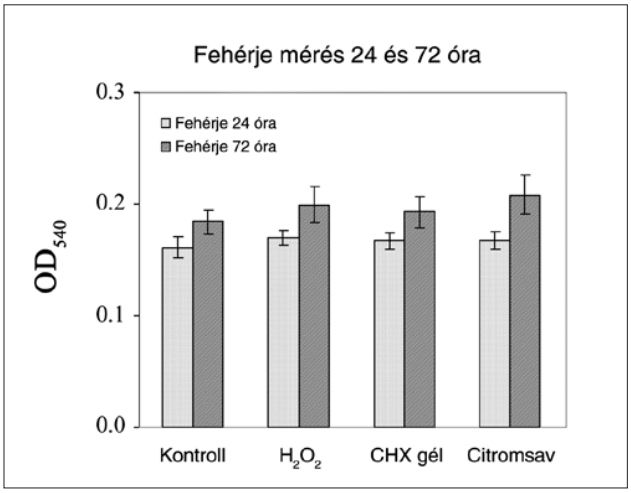
8. ábra
MTT vizsgálat eredményei 24 (letapadás) és 72 óra (proliferáció) elteltével. Élő sejtmennyiségek átlagai és az átlagok szórása látható a kontroll, a H_2O_2-vel, a klórhexidin (CHX) géllel és a citromsavval kezelt Ti korongok esetében. A H_2O_2-vel kezelt korongokon mért élő sejt mennyiség 72 óra után szignifikánsan nagyobb volt, mint a CHX géllel kezelt korongokon. A többi csoport esetében nem tapasztaltunk statisztikailag szignifikáns különbséget

felszíneken, így ez a kutatási terület Magyarországon is eredményes múltra tekint vissza.

Az XPS vizsgálat a kezeletlen és kezelt korongok esetében is intakt TiO_2 réteget mutatott ki. Változást a CHX géllel kezelt korongok esetében tapasztaltunk, mivel az O 1s jelben intenzív csúcs jelent meg a C-O és/vagy C=O kötésben lévő O-nek köszönhetően. Ez az eredmény a CHX gél felszínbe történő adszorpcióját bizonyítja, melyet más szerzők munkái is alátámasztanak [6].

A korongokon 24 órán át tenyésztett sejteken végzett MTT vizsgálat nem mutatott szignifikáns eltérést a különböző kémiai anyagokkal kezelt csoportok között. A fehérjekoncentráció mérése sem mutatott szignifikáns eltérést az egyes csoportok között.

A 72 órát követő MTT vizsgálat különbségeket mutatott ki a csoportok között a sejt proliferációban. Szignifikánsan magasabb értéket kaptunk a H_2O_2-dal kezelt csoportban a CHX géllel kezelt mintákhoz képest, míg a protein koncentráció mérésénél nem kaptunk statisztikailag értékelhető különbséget. Az eredmények közötti eltérést a módszerek különbözőségével magyarázhatjuk: míg az MTT vizsgálat csak az élő sejteket méri, addíg a fehérje koncentráció vizsgálatánál az élő és elhalt sejtekből származó fehérjét is lemérjük. Mivel az érdességek nem voltak szignifikánsan különbözőek, ezért a CHX géllel kezelt korongok esetében mért kisebb sejtmennyiséget a felszíni összetételben bekövetkezett változásnak tulajdonítjuk. A citromsavval kezelt minták esetében is magasabb volt a proliferáció mértéke a kontroll mintákhoz viszonyítva, azonban ez a különbség nem bizonyult szignifikánsnak.



9. ábra
A fehérjemérés eredményei 24 és 72 óra elteltével. A fehérjemennyiség átlagai és átlagainak szórásai láthatóak a kontroll, a H_2O_2-vel, a CHX géllel és a citromsavval kezelt Ti korongok esetében. A statisztikai analízis során nem találtunk szignifikáns különbséget a különböző csoportok között

Eredményeink alapján elmondhatjuk, hogy a CHX gél alkalmazása során a felszínen anyagbeépülés (adszorpció) történhet a titánfelszínbe. A TiO_2 felszín tisztítása H_2O_2-val vagy citromsavval hasonló vagy jobb hatással volt a sejtek túlélésére és szaporodására, a kontrollhoz képest. *In vitro* kísérleteink bizonyították, hogy az előbb említett két dekontaminálló anyag hatékonyan alkalmazható a peri-implantitisz terápiájában, mivel nemhogy nem csökkentette, hanem még növelte is a sejtek proliferációját. Ez igazán figyelemre méltó, hiszen valójában toxikus anyagokról van szó.

Hosszú távú terveink között szerepel, oszteoblaszt

sejtekkel is elvégezni ezeket a kísérleteket, hogy a keményszöveti sejtek reakcióját is értékelhessük ezen kémiai tisztító anyagokra. A jövőben szeretnénk a titán felszínt is változtatni, hogy ne csak esztergált, hanem más, például polírozott vagy homokfújt-savmaratott felszínen is vizsgálhassuk a sejtek letapadását. Végül, de nem utolsósorban a felületre vitt anyagokat is változtatjuk majd, hogy minél szélesebb körű információt gyűjthessünk és adhassunk át a gyakorló fogorvosoknak (implantológusoknak) a különböző tisztító anyagok és a titán kölcsönhatására vonatkozóan.

Köszönetnyilvánítás

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Dr. Ungvári K, Dr. Pelsőczi KI, Kormos B, Oszkó A, Prof. Dr. Rakonczay Z, Dr. Radnai M,
Prof. Dr. Kemény L, Prof. Dr. Nagy K, Prof. Dr. Fazekas A, Dr. Turzó K:

Impact of decontaminating solutions on titanium surface: an epithelial cell culture study

Introduction The effects of three different decontaminating solutions in clinical use for peri-implantitis therapy on the chemical structure and surface roughness of commercially pure (CP) Ti were investigated. A further aim was to survey the response of the biological environment to these changes, by examining the attachment and proliferation of human epithelial cells after treatment of the Ti surfaces with these solutions. Materials and methods CP (grade 4) machined titanium discs (CAMLOG^{TM} Biotechnologies AG, Switzerland) were treated with 3% H_2O_2 (5 min), saturated citric acid (pH = 1; 1 min) or chlorhexidine gel (CHX, 5 min). The surface properties were followed through the use of X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). The epithelial cell attachment and proliferation was examined by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bicinchoninic acid (BCA) protein-content assays. Results XPS showed an intact TiO_2 layer on each sample and CHX was adsorbed by the surface, as C-O and/or C=O bond formation was revealed. AFM results gave no significant changes in the roughness after treating the surfaces with the cleaning solutions. While MTT and BCA assays did not show significant differences in epithelial cell attachments, the cell proliferation was significantly increased after H_2O_2 treatment as compared to CHX (not shown by BCA assays). Conclusions The applied decontaminating agents do not damage the Ti surface. H_2O_2 can be used effectively in decontaminating the implants affected by peri-implantitis, as the human epithelial cell growth was improved, in contrast with CHX.

Key words: peri-implantitis, implant surface, decontamination, epithelial cell culture

PÁLYÁZAT KÖRMÖCZI-PÁLYADÍJRA

Felhívjuk minden, a *Fogorvosi Szemle*ben publikáló, 35 évnél fiatalabb első szerzős cikk szerzőjét, hogy pályázzanak a 2010-es Körmöczi-pályadíjra.
Pályázni csak a 2010-ben, a *Fogorvosi Szemle*ben megjelent közleményekkel lehet.
Kérjük, a közlemény különlenyomatának egy példányát mellékelje a pályázathoz.

A pályázat beadási határideje: 2011. július 15.
A pályázatokat, kérem, postán juttassák el a címemre.

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Supplement 1.

To the request of opponent Dr. Csaba CSONKA (Department of Biochemistry, Faculty of Medicine, University of Szeged) we make the following additional/clarifying remarks to the thesis of Dr. Krisztina UNGVÁRI:

The article A. Stájer, **K. Ungvári**, I. K. Pelsőczi, H. Polyánka, A. Oszkó, E. Mihalik, Z. Rakonczay, M. Radnai, L. Kemény, A. Fazekas, K. Turzó: Corrosive effects of fluoride on titanium: investigation by X-ray photoelectron spectroscopy, atomic force microscopy and human epithelial cell culturing. *J Biomed Mater Res A* 2008; 87:450-458 is directly related to two different PhD theses as of the thesis of the first author Dr. Anette Stájer (2012) and the second author (Dr. Krisztina Ungvári, present). In the aforementioned paper, the first author A. Stájer was responsible for the atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), and the scanning electron microscope (SEM) measurements, while the second author K. Ungvári performed the human epithelial cell culturing experiments. Therefore, the results of this article were divided before the submissions of any of the PhD thesis as refined in a statement by the first author, which is attached to the present thesis. In agreement with the statement, A. Stájer utilized the AFM, XPS and SEM results in her PhD thesis (2012) and K. Ungvári presented the epithelial cell culture results in her present PhD thesis.

In order to a better understanding of the result of the thesis of K Ungvári, the paragraph *4.1.1. Surface characterization by AFM and XPS* had to be included in the *Results and Discussion* chapter because without these results the conclusions of the epithelial cell viability (MTT) and protein concentration cannot be understood. However, in this supplement we clearly state that the results represented in the first part of paragraph *4.1.1.* (page 37, including Fig 21 and 22, but excluding Fig 23) were presented in the thesis of A. Stájer and, therefore, are not considered as part of the results section of this thesis, and do not represent thesis points of this thesis.

In the body of the thesis in paragraph *5.1. Prophylactic agents used in dentistry* (as a part of chapter *5. Summary and Conclusions*), we also clearly stated which are the thesis points of the thesis and that the AFM, XPS and SEM results are presented in details in Stájer et al. and the PhD thesis of A. Stájer (2012).

The thesis points of the author refer only to the own results of the candidate K. Ungvári, which are in the second (**K. Ungvári**, I. K. Pelsőczi, B. Kormos, A. Oszkó, Z. Rakonczay, L. Kemény, M. Radnai, K. Nagy, A. Fazekas, K. Turzó: Effects on titanium implant surfaces of chemical agents used for the treatment of peri-implantitis. J Biomed Mater Res B Appl Biomater 2010;94:222–229) and third (**K. Ungvári**, I. K. Pelsőczi, B. Kormos, A. Oszkó, M. Radnai, K.

Nagy, A. Fazekas, K. Turzó: Dekontamináló anyagok hatása a titán felszín biointegrációs tulajdonságaira: *in vitro* humán epithel sejtkultúra vizsgálatok. Fogorvosi Szemle 2011;104:9-18) publications of the thesis (page 3) and the epithelial cell culture part of the first article (A. Stájer, **K. Ungvári**, I. K. Pelsőczi, H. Polyánka, A. Oszkó, E. Mihalik, Z. Rakonczay, M. Radnai, L. Kemény, A. Fazekas, K. Turzó: Corrosive effects of fluoride on titanium: investigation by X-ray photoelectron spectroscopy, atomic force microscopy and human epithelial cell culturing. *J Biomed Mater Res A* 2008; 87:450-458) where she is the second author.

Szeged, 22th of July, 2013

Dr. Krisztina Ungvári

Supplement 2.

Flowchart of cell culture examinations

O. hour	**1. hour**	**2.-3. hours**	**4. hours**	**5. hours**	**6. hours**	**7. hours**	**31. hour**	**79. hour**	**80. hour**
Receiving titanium discs	Cleaning with acetone and absolute ethanol	Treating with prophylactic and cleansing solutions	Washing with ultrapure water	Drying	Sterilizing the discs with UV-C	Plate the cells to the discs	Investigate the attachment of cells after 24h (MTT, protein content assay), microscopic images	Analysis the amount of proliferation after 72h (MTT, Protein content assay), microscopic images	Statistical analysis

Társszerzői lemondó nyilatkozat

Alulírott Dr. Sájer Anette és Dr. Turzó Kinga nyilatkozunk, hogy a: *Stájer A., Ungvári K., Pelsőczi-K. I., Polyánka H, Oszkó A, Mihalik E, Rakonczay Z., Radnai M., Kemény L, Fazekas A., Turzó K.: Corrosive effects of fluoride on titanium: Investigation by X-ray photoelectron spectroscopy, atomic force microscopy, and human epithelial cell culturing. J Biomed Mater Res A.; 2008 Nov;87(2):450-8.* tudományos publikáció humán epithél sejtkultúra vizsgálatok része teljes egészében Dr. Ungvári Krisztina munkája. Hozzájárulunk, hogy Ungvári doktornő felhasználja ezen részt a Ph.D értekezésében és téziseiben. Kijelentjük, hogy a publikáció ezen részét nem használjuk fel más személy Ph.D értekezéséhez.

Dr. Turzó Kinga

Dr. Turzó Kinga
felelős szerző

Dr. Stájer Anette
első szerző

2010-08-31, Szeged