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The effect of titanium topography features on mesenchymal human stromal cells' adhesion

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Key words: adhesion, cell culture, implant, scanning electron microscope, stromal cells, surface, wettability

Abstract

Objective: The aim of this study was to evaluate the effect of two kinds of dental implants surfaces with their own characteristics on human marrow stromal cells' adhesion.

Material and methods: Fifty-six titanium discs (28 machined and 28 acid etched) were used. Machined (MS) and acid-etched surfaces (ES) were analyzed using a scanning electron microscope, energy dispersing spectroscopy (EDS), contact angle analysis and human marrow stromal cells' culture.

Results: Significant differences were observed in the topography and wettability of the tested surfaces. However, etched surfaces presented a high level of wettability when compared with machined surfaces. Contact angles showed considerable differences between etched and machined surfaces (Friedman test $P < 0.05$). EDS analysis showed the same composition on both the surfaces tested. Counting of adhered cells on both types of surfaces showed that there is no statistical significance in human marrow stromal cells' adhesion after 18 h (Mann-Whitney test $P > 0.05$).

Conclusion: The present study concludes that modifications on the titanium implant surfaces roughness may promote differences in the morphology of bone marrow stromal cells. Nevertheless, in this microenvironment, no interference in the adhesion phenomenon was noted.

Biomaterials can be described as biocompatible materials that are used for replacing deteriorated bio-structures, increasing the size of existing ones or inducing the formation of a new tissue (Burg et al. 2000; Lim & Donahue 2004). Among these materials, commercially pure titanium (cpTi) is currently the most widely used one in cranium-maxillofacial surgeries as bone fixtures and dental implants.

Titanium is a reactive metal and, when exposed to the environment (air and/or water), a fine hydrated layer of oxide of approximately 4–5 nm can be observed over the metal (Kasemo & Lausmaa 1994). The

biological significance of this oxide is still arguable (Lundstrom & Tengvall 2005). However, it is believed to be one of the reasons for the excellent biocompatibility of the metal (Cooper 2000; Rupp et al. 2006).

The term osseointegration was introduced by Per-Ingvar Branemark in 1952 in a study of bone marrow circulation in rabbits (Branemark et al. 2001). Currently, this phenomenon is considered when there is no movement between the implant and bone in direct contact (Rigo et al. 2004).

Along with these evidences, knowledge of macro- and microstructures of osseointegrated implants has become increasingly

Date:

Accepted 22 June 2009

To cite this article:

Carneiro-Campos LE, Fernandes CP. The effect of titanium topography features on mesenchymal human stromal cells' adhesion.
Clin. Oral Impl. Res. 21, 2010; 250–254.
 doi: 10.1111/j.1600-0501.2009.01811.x

more important in the quest for a system that fulfills every biological purpose. However, the behavior of cellular population in contact with a metal depends directly on the implant microscopic properties, such as topography, chemicals and surface energy (Albrektsson & Wennerberg 2004).

Several studies advocate the superior features of rough surfaces on osseointegration (Carlsson et al. 1988; Brunski 1999; Cochran et al. 2002; Bischof et al. 2004; Brunski et al. 2005; Zhao et al. 2005). Along with these advantages, the significance of smooth surfaces is currently being questioned. However, microphotographies (Fig. 1) show microroughness in surfaces considered to be 'smooth,' which may represent a changing paradigm.

Although several surface treatments have provided promising results, there is no agreement in the literature as to the ideal surface (Sammons et al. 2005).

Albrektsson & Wennerberg (2005), alert on systems developed without proper technical and scientific scrutiny, merely copying other accepted methods.

Cellular cultures have been accepted as excellent methods to observe the behavior of the biomaterial in relation to living tissue. However, it seems that protocols may lead to many results and interpretations (Schwartz et al. 1999). Because of this and other assumptions, in our study we analyze the physical-chemical and biological characteristics of two implant surfaces in relation to their adhesion to the mesenchymal human stromal cells of healthy donors, after 18 h.

Material and methods

Preparation of titanium discs

Fifty-six cpTi discs grade II (ASTMF67) measuring 3 mm in diameter and 1 mm

in thickness were prepared by the conventional process as outlined for machined implants for 'Neodent Implante Osteointegrável' (Curitiba, PR, Brazil).

Twenty-eight discs remained with the original machined surface (MS discs), while the rest of the samples had their surfaces acid etched (ES discs). The detailed processes used to produce and sterilize surfaces have not been provided by the manufacturer (proprietary processing). Therefore, the discs were classified into three groups for analysis:

- (i) Twelve discs (6 MS and 6 ES) were used for topographic qualitative analysis and chemical analysis [scanning electron microscope (SEM)/energy dispersing spectroscopy (EDS)].
- (ii) Twenty discs (10 MS and 10 ES) were used for measuring contact angles and wettability.
- (iii) Twenty-four discs (12 MS and 12 ES) were used for analyzing biological response by mesenchymal human stromal cells' attachment.

Surface characterization

Titanium surface characterizations were performed qualitatively and quantitatively using SEM (JEOL JSM 6460 LV, Tokyo, Japan), EDS (Noran, Tokyo, Japan).

Contact angles were measured with a goniometer (SEO Phoenix 300, Surface Electro Optics, Suwon-si, Gyeonggi-do, Korea). In our study, two liquids were used for contact angle determination: deionized water and glycerol. The images of these liquids over the samples were captured by software DROPImage[®] (Oslo University, Oslo, Norway) and then analyzed for hydrophilicity or hydrophobicity.

Cell culture

Mesenchymal human stromal cells were obtained from the bone marrow cavities of healthy donors after hip arthroplasty, average between 20 and 50 years of age, without HIV serology, hepatitis B and C or thromboembolic diseases (Ethical Committee, National Institute of Traumatology-Orthopaedics, RJ, Brazil, protocol 092), following a protocol described previously by Lumbikanonda & Sammons (2001) and Sader et al. (2005). This material was rinsed and homogenized in a calcium- and magnesium-free saline solution (BSS-CMF,

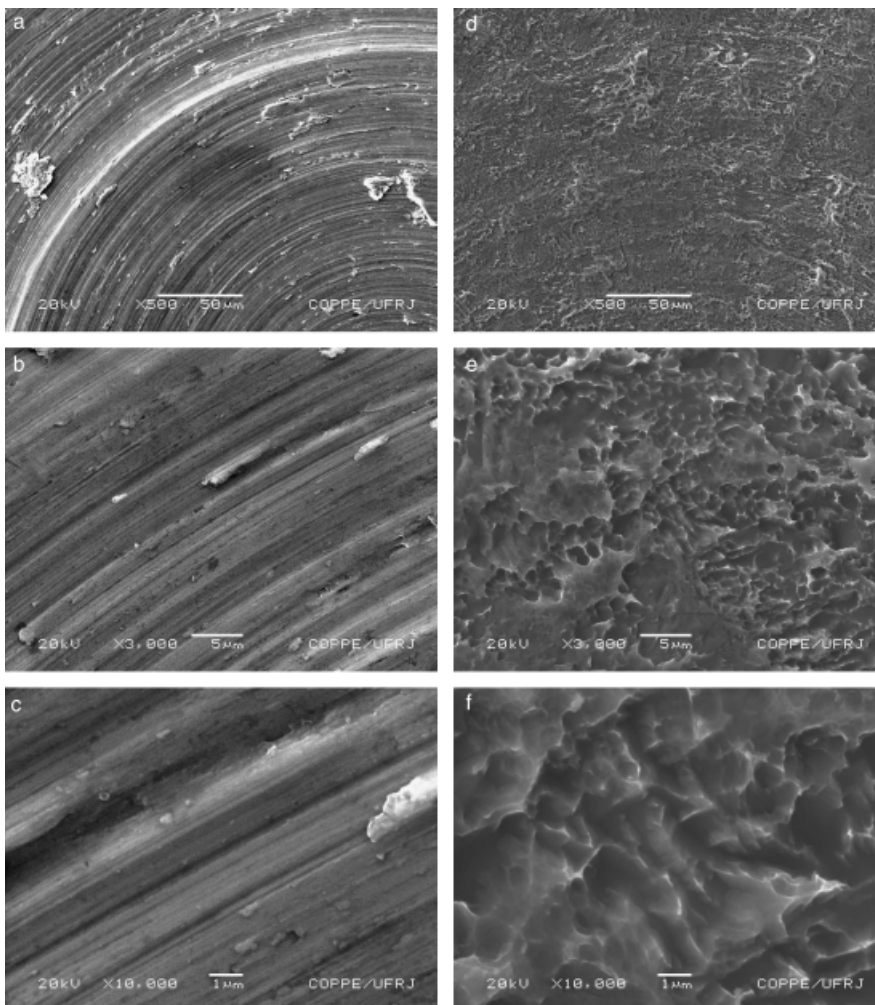


Fig. 1. Machined surface (a, b and c) and etched surface (d, e and f) at $\times 500$, $\times 3000$ and $\times 10000$ magnifications.

Sigma, São Paulo, SP, Brazil) with a culture medium (Dulbecco's modified Eagle's medium, PerBio Science Company, Helsingborg, Sweden) supplemented with 10% fetal bovine serum (FBS, Laborclin, Pinhais, PR, Brazil) with ciprofloxacin (Bayer Health Care Pharmaceuticals, Wayne, NJ, USA) to remove the pellet and then suspended.

After 13 s, the suspension was removed and transferred to a culture flask. This process was repeated five times in a laminar flow booth (Trox Technoc, Curitiba, PR, Brazil). The cell suspensions were centrifuged (Excelsa 4 Mod 280R, Fanen, São Paulo, SP, Brazil) and the supernatant was discarded.

The cell number was analyzed on an inverted microscope (Nikon Eclipse 100, Nikon, Tokyo, Japan), adjusted to 1.2×10^5 cells/mm² in the culture medium and the unattached cells were pipetted off. Subsequently, these cells were incubated for 2 h, at 37°C, in 5% CO₂. The samples were rinsed with culture medium and incubated for 18 h. After the incubation, the samples were then rinsed three times with phosphate-buffered saline (PBS) to remove any unattached cells, fixed for 1 h in 2.5% glutaraldehyde (EM Grade, Agar Scientific, Stansted, UK) in 0.1 mmol/L sodium cacodylate buffer pH 7.4, rinsed twice in the same buffer, post fixed with osmium tetroxide solution and dehydrated in ethanol. Samples were then incubated with HMDS (hexametil di-silane), coated in a sputter gold coater and viewed using an SEM (JEOL) operating at a beam current of 15 kV.

Cell attachment

After 18 h, all attached cells were identified and counted by SEM with retro spread analysis.

Each image was divided into 56 quadrants with 0.018 mm². Twelve quadrants were selected randomly. The numbers of cells presented per quadrants were quantified by two independent evaluators.

Statistical analysis

Wetability data were assessed using the Friedman non-parametric test of variance. Differences were considered statistically significant when $P < 0.05$. Cell attachment data were assessed using the Mann-Whitney test. Differences at $P < 0.05$ were considered significant.

Results

Surface characterization

Machined discs presented parallel grooves. Acid-etched surfaces demonstrated roughness as observed in presented micropits and valleys that were observed in acid-etched surfaces (Tavares et al. 2007). Both samples were observed at $\times 500$, $\times 3000$ and $\times 10,000$ magnifications (Fig. 1). EDS demonstrates equivalent chemical composition on both surfaces (Fig. 2).

Both surfaces showed hydrophilic behavior (Groessner-Schreiber et al. 2006). It was also observed that MS and ES demonstrated different wetability between them ($P < 0.05$). Contact angles varied from 46.77 to 112.6, with an average of 79.04 on MS, and from 20.62 to 122.3, with an average of 53.76, on ES. The Friedman test showed higher rank values for MS/G (3.3) and MS/W (2.8) than for ES/G (2.4) and ES/W (1.5). These results can be better evaluated in Table 1 and Fig. 3.

Cell attachment

Human mesenchymal stromal cells were analyzed with SEM ($\times 33$, $\times 300$ and $\times 500$), with a beam current of 15 kV, after 18 h. All cells presented adhesion and spread. However, both surfaces demonstrated different and dependent behavior with respect to the surface topography. Machined surfaces showed attached cells with the spread morphology oriented according to the surface grooves. Acid-etched surfaces presented stromal cells with a polygonal morphology and less spread on the samples tested, with many focal adhesion points (Fig. 4).

Discussion

The result of the present study shows that cpTi with the same chemical composition may show different biological responses after physical modifications to its topography. The human stromal mesenchymal cells' geometry tends can be distinguished

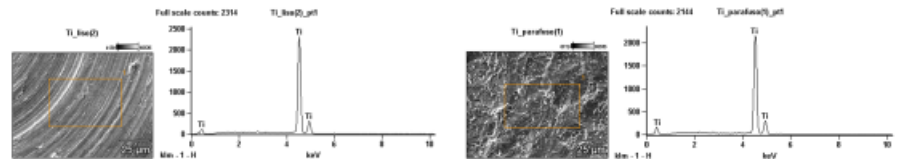


Fig. 2. Energy dispersing spectroscopy analysis shows the same chemical composition on MS and ES surfaces.

Table 1. Mean, standard deviation and standard error of contact angle values

	MS/G	ES/G	MS/W	ES/W
Mean	79.04	60.95	76.16	53.76
Standard deviation	15.06	19.27	13.49	4.166
Standard error	1.978	2.371	1.967	0.7365

MS/G, machined surfaces with glycerol; ES/G, acid-etched surfaces with glycerol; MS/W, machined surfaces with water; ES/W, acid-etched surfaces with water.

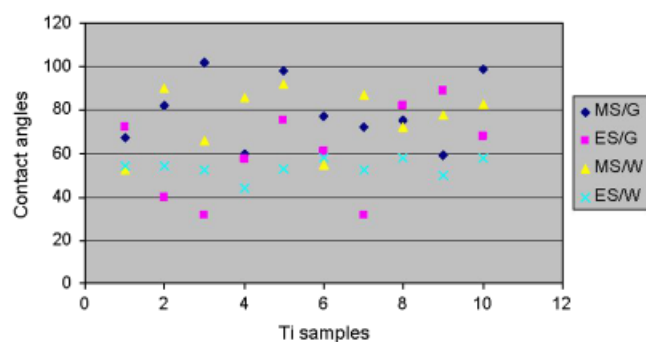


Fig. 3. Scatter plot of wetability data obtained for each titanium sample according to the surface treatment and contact angle liquid: MS/G, machined surface with glycerol; ES/G, etched surface with glycerol; MS/W, machined surface with water; ES/W, etched surface with water.

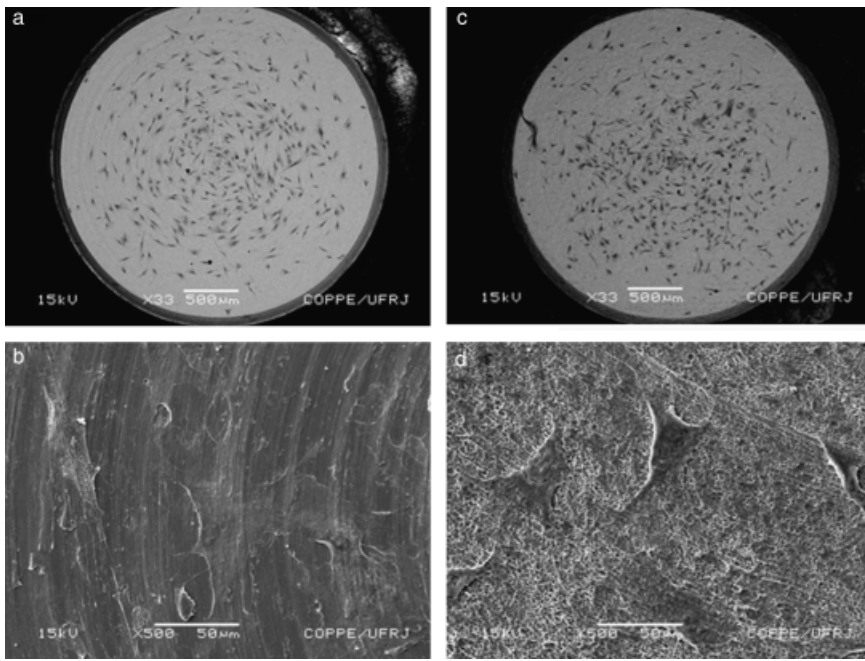


Fig. 4. Retro spread analysis shows the same pattern of adhesion (A and C), but etched surfaces presented stromal cells with a polygonal morphology (D) and less spread than machined surfaces (B) on the samples tested, with many focal adhesion points.

under these conditions; however, the number of adhered cells did not change after the 18th hour.

After 18 h, the cells that adhered to the MS presented fibroblastic characteristics, while the ones that adhered to the ES were characterized by a polygonal cytoskeleton with the presence of adhesion focal points.

Topographic studies, both on a macro- and a microscale, as well as the ones using osteoblast-like cell cultures, have demonstrated that average surface roughness is an important factor to achieve stable osseointegration, thus promoting fast adhesion, proliferation and differentiation, with the formation of bone nodules and, therefore, an increase in bone–implant contact.

According to Boyan et al. (2003), differences characterized by topographic modifications on distinct titanium samples with the same chemical composition tend to, as seen previously, alter the biomaterial–cell interaction. Furthermore, according to the same authors, there will be a tendency towards alterations in protein adsorption, clot formation and integrin expressions. However, care should be exercised when interpreting these factors, for errors may occur if the observations are evaluated individually (Morra et al. 2003). Apart from what has been proposed by several studies

on titanium implant topography, the increases in average roughness, as well as the chemical composition of the metal, are not the only factors responsible for osteoid matrix deposition and success in implantological practice. Factors such as the macro design and care during surgical procedures, as much as bone density are highly relevant for the final therapy results (Uehara et al. 2004; Dimitriou & Babis 2007). According to Albrektsson & Wennerberg (2004), implant systems from the same manufacturers may yield different results with different professionals. Some may obtain better results than others.

Titanium surface wettability is another topic that has received attention recently from the industry, generating high costs for research and manufacturing technologies. Surfaces generated and kept under controlled atmospheres can decrease titanium contamination after exposure to the environment, thus conserving a stable surface energy and optimizing wettability capacity (Rupp et al. 2006). Among the tested surfaces, both presented as hydrophilic (Groessner-Schreiber et al. 2006), whereas ES surfaces presented a higher wettability.

Some studies have demonstrated that high wettability tends to optimize the osteoid matrix deposition (Buser et al. 2004; Rupp et al. 2006). However, Taborelli et al.

(1997), suggested that hydrophobic surfaces may achieve a better result in relation to osteoid matrix deposition (Taborelli et al. 1997; Schwartz Fo et al. 2007), which questions the previous assumption.

In terms of the biological behavior, stromal cells presented a spread distribution on machined surfaces and a polygonal form on etched surfaces after 18 h. This characteristic is in accordance with the previous studies on osteoblast-like cells (Sader et al. 2005), which means that the higher the smoothness the higher the spread. As the topography starts to show the formation of peaks and valleys, the cell forms tend to be modified, clearly showing cell adhesion focal points (Fig. 4). These observations were also made on nanotopography studies (Schwartz Fo et al. 2007).

In 1952, during his first observations on the biocompatibility of titanium, Per Ingvar Branemark classified osseointegration as the direct and structural connection between live and ordered bone and the implant surface under load (Branemark 1959). Afterwards, in a histological analysis, Albrektsson & Johansson (2001) considered the classification as the direct bone–implant connection forming a free fibrous tissue interface. These observations were then corroborated by Larsson et al. (1997), when, during a long-term *in vivo* study, the author noted that the bone–implant interface showed little differences, independent of the physical–chemical characteristics of titanium implants after 1 year of service.

The results of this study indicate that the physical–chemical analysis should be aligned to the bone environment, as well as to the surgical procedures (Albrektsson & Wennerberg 2005), so that the cell responses are precise. However, long-term *in vivo* studies with the same surfaces are still necessary to analyze the actual advantages of the several modifications titanium has been going through in the search for the ideal surface.

Acknowledgements: This study was supported by Neodent Implante Osteointegrável; the National Institute of Traumatology and Orthopaedics (INTO, Rio de Janeiro, Brazil); Federal University of Rio de Janeiro (COPPE-UFRJ, Rio de Janeiro, Brazil); and Pontifícia

Universidade Católica do Rio de Janeiro (Rio de Janeiro, Brazil). The author also wishes to thank Dr Fátima Maria Namen,

Dr Márcia Sader, Dr João Galan Júnior, Dr Rodrigo Cabrera Derossi, Dr Geninho Thomé and Dr José Henrique Cavalcanti

Lima for their assistance, and without them this study would not have been possible.

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