Effect of Different Textured Surfaces on Osteoblast Attachment and Proliferation

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A Thesis presented to the Department of Periodontology and the Advanced Education Committee of the Oregon Health and Science University School of Dentistry in partial fulfillment of the Master of Science Degree in Periodontology

Portland, Oregon

May 2005

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ACKNOWLEDGMENTS

- Dr. Winthrop Carter
- Thomas Eshraghi
- Dr. J. Ernest Weinberg
- Dr. Marvin Levin
- Dr. Steven Hokett
- Dr. Paul Grady
- Dr. Xi Gong
- Dr. Byung Park
- Todd Hannon
- Dolores Judkins
- Justin Nelson
- Miranda Leasure

- This work was supported in part by Zimmer Dental ® (Previously Center Pulse Dental, Inc.)

DEDICATION

I would first and foremost like to thank my director, Dr. Winthrop B. Carter, for giving me the courage and support for completing my Master's Thesis. Dr. Carter was not only my director during the past 3 years, but also a good friend who has influenced me to strive for the best. I would also like to pay special thanks to my family for supporting me in my academic accomplishments.

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ABSTRACT

Considerable interest has been generated recently, concerning the effect of surface roughness on osteoblast attachment and proliferation.

Objectives: This study characterized attachment and proliferation of rat pre-osteoblast cells on discs of material having differing surface textures.

Methods: Discs were made of Ti-6A1-4V alloy and each disc measured 15.5mm in diameter and 1.5mm thick. Test disc samples were provided by Center Pulse Dental, Inc.® (now Zimmer Dental®) and were textured to evaluate 3 selected surface parameters: pit spacing, pit diameter, and pit depth (see Table 1 and 2). The test discs were designated as: D2 (close-small-deep); D3 (close-large-shallow); D6 (far-small-shallow); D7 (far-large-deep). D4 (blasted), D5 (HA coated), and D8 (machined) discs were used as control discs. D1 (polystyrene) consisted of empty cell culture treated wells that did not contain a disc were used as positive controls. The discs were used as substrates upon which the osteoblast cells were cultured onto 24-well culture polystyrene plates. To obtain pre-osteoblasts, calvarae from day 19 fetal Sprague Dawley rats were pooled and treated with collagenase to liberate the cells. Cells were allowed to recover for three days prior to plating. Quantification of cell viability within the test wells was measured at predetermined times of 4, 24, 48, 96, 120, 144, and 168 hours by observing the reduction of 10% Alamar Blue nuclear dye using a fluorescent colorimeter. The resulting measurements from the Alamar Blue dye analyses were then analyzed and reviewed for possible trends or differences between the different surface textures using ANOVA at 95% confidence interval ($p<0.05$).

Results: Discs with far parameters had statistical significant greater cell numbers than discs closer together at 72 hours ($p<0.05$). There were no observed differences in cell numbers between the shallow and deep pits. The 4 experimental discs resulted in greater cell numbers than D5 (HA coated) at all time points except 72 hours. D3 (cl-lg-sh) showed greater cell numbers than D4 (blasted) at 96 and 144 hours. Also, D3 (cl-lg-sh) resulted in greater cell numbers than D5 (HA coated) at all times except 72 hours. D4 (blasted) showed greater cell numbers than D5 (HA coated) at all time points except at 72 and 96 hours ($p<.05$).

Conclusions: Within the limitations of this study, it appears that the pitted surfaces generally have a greater cell attachment and proliferation than the HA coated; however, machined surface textures showed the greatest number of cells, suggesting that size of texture is not necessarily as important as type of surface.

INTRODUCTION

Osseointegrated dental implants are recognized as a “state-of-the-art” treatment modality in dentistry. Interest has been generated concerning the effect of surface roughness on osteoblast attachment and proliferation.[13] Modifying osseous responses at implant surfaces to enhance bone growth is a goal of clinical therapy.[37] However, the process by which surface texture affects osseointegration remains unclear.[1] Several studies showed that the mechanism of osteogenesis or bone formation at the bone-implant interface, induced by osteoblast-like cells, involves cellular attachment, proliferation, and differentiation of bone matrix.[27]

Considerable speculation on the possible toxicity of various texture treatment of implants included acid-etching and sand-blasting. A recent study showed that sandblasted and acid-etched implants had non-cytotoxic cellular effects and appeared to be biocompatible.[37]

More recent studies have attempted to optimize surface morphology for enhanced osteoblast responses.[13,15,*19,26*] *In vitro* cellular responses of osteoblast-like cells were studied on titanium surfaces with different surface morphologies.[36] Significantly greater cell numbers were found using rough, sandblasted surfaces with irregular morphologies. These results suggested that implants should be prepared with roughened surfaces at bony contact areas. To demonstrate the increases in cell growth of roughened implants, a comparative analysis between the bone response to machined and sandblasted implants in rabbits was performed.[19] The histomorphometric analysis showed that sandblasted implants

presented, from the third week onwards a significantly higher contact percentage ($P < 0.0001$). These values could point to higher osteoconductivity as a result of the higher surface roughness of sandblasted surfaces.

Another study evaluated the quality and the remodeling of bone around commercially pure titanium implants after 3, 6, 12, and 18 months in the femurs of the sheep.[26] Results suggested that roughened implants showed greater cell numbers than smooth implant surfaces. A comparative study of bone cell interactions with smooth titanium, titanium dioxide-blasted, titanium plasma-sprayed, and HA plasma-sprayed implants have been performed.[38] Implants were exposed to neonatal rat osteoblast cells in suspension for a 20-minute period and, by means of scanning electron microscopy, attached cells were classified according to stage of attachment. Quantitative analysis showed that cells spread most quickly on the titanium plasma-sprayed implants as compared to the other textured surfaces. Another study showed that surface topographic features of commercially pure titanium substrates can enhance cultured osteoblast extracellular matrix formation and mineralization.[11]

Some studies have been published to show that surface roughness does not enhance bone integration.[1,23,39] One investigation suggested that commercially pure Ti (cpTi) surfaces that were submitted only to machining treatment favor the final event of osteoblastic differentiation of the rat bone marrow cells as compared to sandblasted surfaces. [39] Another *in vitro* study had results which showed no significant difference in mineralized extracellular matrix formation on the roughened substrates as compared to the machined substrates.[23] Results of

another study suggested that cpTi would optimize osteoblastic differentiation by rat bone marrow cells, including increased cell proliferation, and increased alkaline phosphatase (ALP) activity as well as bone-like nodule formation, while surface roughness, within the parameters used, would not significantly affect the rat bone marrow cell response. [1]

The overall objective of this study is to improve osseointegration between implants and bone. The specific aim is to characterize cell attachment and proliferation on material disks with differing surface textures.

The working hypotheses are: (1) that discs containing the greatest pit diameters will result in a greater osteoblastic attachment and proliferation; (2) discs containing the greatest amount of spacing between pits will result in a greater cell numbers (as determined by the greater amount of Alamar Blue dye metabolism) than those discs containing least spacing; (3) those discs containing the greatest pit depths will result in a more greater cell numbers.

LITERATURE REVIEW FOR THESIS

The evolution of implant dentistry has provided a reliable and safe solution to replace missing teeth. However, an understanding of biologic response of bone cells to implant surfaces is critical because initial events that occur at bone-implant interface determine the success or failure of implants.[1] The purpose of the literature review below is to evaluate the various aspects of cell and tissue response to different surface textures.

Titanium has been a metal of choice for use in the human body.[2,3,6-8] It exists as a pure element listed in the periodic table with atomic number of 22 and atomic weight of 47.9. It is the fourth most abundant element in earth's crust following aluminum, iron, and magnesium. The use of titanium as the metal of choice has been well supported by the literature.[3] The type of orthopaedic implant surface (grit-blasted and acid-etched) affects the interaction between bone and the implant. Results of this study showed increased alkaline phosphatase levels produced by osteoblasts on rougher surfaces (grit-blasted).[4] Important points regarding titanium and its alloys are summarized as follows[2]: 1) Ti and its alloys, particularly the alpha-beta alloys (Ti6Al4V consisting of 6% Aluminum and 4% Vanadium), possess mechanical properties that make them ideal implant materials; 2) Ti and its alloys oxidize readily in air (this surface oxide is extremely chemically stable in the physiologic environment of the body); 3) The stability and inertness of this surface oxide layer acts to protect Ti and its alloys from corrosive breakdown when used in the body; 4) The elimination of surface irregularities and contaminants (ie casting inclusions or corrosion) by

adequately cleaning methods is important when preparing a metal for implantation; 5) Ti can be coupled with equally passive metals in the body without causing galvanic corrosion (galvanic corrosion is caused by coupling with other metals).

Passivity of an implant material occurs when an implant metal is oxidized and the oxide does not break down under physiologic conditions.[2] Titanium, both in its pure or alloy forms, is easily passivated, forming a stable titanium surface oxide (TiO_2) that makes the metal corrosion resistant. In comparing titanium with other metals such as cobalt chromium (CoCr), it was demonstrated that titanium surface oxide layer formed on titanium surfaces had superior passivity, thus making titanium an ideal metal for endosseous implants.[3] Unmineralized tissue (cartilage and osteoid) was observed more frequently on the CoCr surface than on the Ti6Al4V surface. The authors suggested that this lack of mineralization occurred because the bone around CoCr was less mature than the Ti6Al4V alloy. The decrease in osseointegration was possibly due to a slight difference in surface roughness. Some negative effects of CoCr on bone attachment such as ion release from the CoCr were also suggested for the decrease in mineralization of tissue.

There has been much controversy over the surface oxide and strength differences between commercially pure titanium (cpTi) and Ti-6A1-4V alloy. A series of surface characterization techniques (oxide thickness, corrosion resistance, alpha-beta alloys added to titanium) were used in conjunction with short term *in vitro* biological assays to assess the effects of materials selection

(cpTi and Ti alloy) on osteoblast-like cell responses.[7] Surface analysis indicates that with the exception of oxide thickness, there were no significant differences in surface characteristics (corrosion resistance, contaminant levels, and strength) between the two implant materials. These results are in general agreement with previous *in vivo* studies and continue to indicate that cpTi and Ti alloy are suitable, biologically compatible materials for fabrication of dental implants. A comparative study showed that CpTi provides greater biocompatibility (i.e. least corrosion and greater cell numbers) with regard to proliferation and differentiation.[6] This was suggested based on the relatively high alkaline phosphatase and osteocalcin levels which are produced by osteoblasts during cell differentiation.

Osseointegration was a term coined by Professor Per-Ingvar Brånemark when he discovered the ability of living tissue to integrate with titanium.[8] Osseointegration has been described as a direct structural and functional connection between living bone and the surface of a load carrying implant. During his original research, Professor Brånemark found a way of using commercially pure titanium as an anchoring unit and support for a variety of prosthetic reconstructions including what was then a new type of hearing device. This new method was to place an intermediate adapter between the prosthesis and titanium implant in the ear.

Cell contact with the substrate is maintained by the formation of adhesion sites called focal adhesions.[9] These adhesion sites are mediated by integrins which are proteins produced by osteoblasts upon interaction with the substrate

which the osteoblast will adhere to. The ability of osteoblasts to bind fibronectin and to support cell attachment, adhesion, migration and synthesis of extracellular matrix was tested on grit-blasted and titanium plasma sprayed (TPS) discs. This study suggests that the surface characteristics (blasted or TPS) of implants improves the growth and proliferation of osteoblasts leading to better wound healing, interfacial tissue morphogenesis (differentiation) and repair (mineralization of extracellular matrix). In an evaluation of the healing of bone around implants, it was observed that cells closest to the implant surface were nonviable.[10] This does not agree with previous studies which demonstrated that cells are viable as indicated by increased attachment and proliferation of osteoblast.[1,3,5-7]

The rationale of using a cell culture has been to perform preliminary *in vitro* studies on cellular responses to different textured substrates.[11] The interactions of osteoblasts with different substrates can be measured in terms of cytotoxicity, attachment, proliferation, and differentiation. The cytotoxity effects are included in this study to describe the various surface oxide and corrosion factors which have been shown.[3,5] Several culture systems have been categorized as: primary cultures (bone marrow stromal cells, etc); nontransformed clonal cell lines (MC3T3-E1); osteosarcoma cell lines; immortalized cell lines. Studies indicating that rough surfaces enhance osteoblast adherence are distinct from studies using gingival fibroblastic cells, in which smooth surfaces consistently promote greater attachment and spreading. Of these cell culture systems, the primary cultures are optimal because the primary

cells (i.e. pre-osteoblasts) undergo all stages of bone development, whereas the oseosarcoma cell lines do not differentiate; so, only proliferation can be assessed from the osteosarcoma cell lines.[11]

The effect of titanium (Ti) passivation on the response of rat bone marrow cells was evaluated, considering cell attachment, cell morphology, cell proliferation, total protein content, alkaline phosphatase (ALP) activity, and bonelike nodule formation.[12] Cells were cultured on both commercially pure titanium (cpTi) and titanium-aluminium-vanadium alloy (Ti-6Al-4V) discs, either passivated or not. The results did not show any effect of this surface treatment on *in vitro* biocompatibility of Ti as evaluated by osteoblast attachment, proliferation, and differentiation. Bone formation within porous hydroxylapatite (HA) implants in human periodontal defects was evaluated.[13] Three subjects who had periodontal defects were treated with a porous HA implant and their tissue samples were investigated using light and scanning electron microscopy. Results of this study showed evidence of continued bone formation with lamellar bone being the major component within the pores. It has also been shown that the ostoeblastic gene expression is enhanced by roughened implant surfaces *in vitro*.[14]

MG63 cells, a human osteoblast-like osteosarcoma cell line, responded to increasing surface roughness (HA-coated and grit-blasted) with decreased proliferation and increased osteoblastic differentiation.[15] Alkaline phosphatase activity and osteocalcin production were increased suggesting that the cells are in the differentiation stage. ALP and osteocalcin are indicators of cell differentiation indicating some calcification, thus forming bone. Local factor

production was also affected as well as an increase in production of both TGF-ß1 and PGE2, thus suggesting that prostaglandins may mediate the effects of surface roughness. In evaluating the initial healing of osteoblasts on roughened surfaces (grit-blasted with and without BMP), it was shown that an increase in BMP-2-positive cells was seen during the first 4 days of healing. [10]

Increased cp titanium implant surface topography improves the bone-to-implant contact and the mechanical properties of the enhanced interface.[15-17] Growing clinical evidence for increased bone-to-implant contact at altered cp titanium implants confirms the temporally limited observations made in preclinical studies. The aggregate experimental evidence supports the use of cp titanium implants with increased surface topography. In an effort to evaluate the clinical benefits of roughened titanium surface as they apply to osseointegration, a review demonstrated that rough implant surfaces have increased bone-to-implant contact and require greater forces to break the bone-implant interface compared to more smooth surfaces.[17]

In vitro cellular responses of osteoblast-like cells were studied on titanium surfaces with different surface morphologies.[18] Significantly higher levels of cellular attachment were found using rough, sandblasted surfaces with irregular morphologies. These results suggested that implants should be prepared with roughened surfaces at bony contact areas. To demonstrate the molecular enhancements of roughened surfaces on implants, a comparative analysis between the bone response to machined and sandblasted implants in rabbits was performed.[19] The histomorphometric analysis showed that sandblasted

implants presented, from the third week onwards a significantly higher contact percentage ($P < 0.0001$). These values could point to greater proliferation of osteoblasts as a result of the higher surface roughness of sandblasted surfaces.

A human *in vitro* study evaluated the effect of surface roughness on osteoblast proliferation, differentiation, and protein synthesis. [20] Human osteoblast-like cells (MG63) were cultured on titanium (Ti) discs that had been prepared by one of five different treatment regimens: electropolished (EP); fine sandblasted, etched with HCl and H2SO4, and washed (FA); coarse sandblasted, etched with HCl and H2SO4, and washed (CA); or Ti plasma-sprayed (TPS). When compared to confluent cultures of cells on polystyrene, the number of cells was reduced on the TPS surfaces and increased on the EP surfaces, while the number of cells on the other surfaces was equivalent to plastic. These results demonstrate that those elctroplished and basted textures showed greater osteoblast proliferation, differentiation, and matrix production (ALP and osteocalcin) that the TPS *in vitro*.

The results of an *in vitro* study looking at differences in rat calvarial osteoblast growth and proliferation on polystyrene demonstrated that the polystyrene itself caused increases in osteoblastic proliferation and differentiation in cell cultures.[21] An *in vitro* study of neonatal rat calvarial osteoblasts revealed an increase in osteoblastic responses to roughened titanium surfaces.[22]

Results of an animal *in vitro* study disagreed with the findings of previous studies. No significant difference in mineralized ECM formation was observed on the ground substrate compared to the machined substrates.[23] This study

suggests that there could be other factors playing a role in osteoblastic growth and proliferation to titanium. On the basis of these findings, it was concluded that this *in vitro* study could not clearly confirm the effect of surface roughness on the proliferation, differentiation and calcification of rat bone marrow cells. A higher percentage of human osteoblast-like cells attached to rough cpTi surfaces compared to smoother surfaces.[24] Cell attachment was directly related to the average surface roughness, with the highest levels of cell attachment observed on sandblasted and sandblasted-acid etched surfaces. [7]

Effect of surface roughness on cells can be the result of surface roughness alone or the result of the reaction which occurs as the material surface is conditioned by the media and serum.[20] So, the culture materials may also have an effect on the behavior of the cells in this project as indicated by the results which contradict some previous studies. For example, this study revealed that, at certain time points, smooth surfaces produced greater osseous responses than the roughened surfaces (acid-etched, pitted, etc) during the later stages of proliferation.

A histologic *in vivo* study on pigs evaluated the influence of smooth and TPS implant surfaces on bone integration. It was concluded that the extent of bone-implant interface is positively correlated with an increasing roughness (grit-blasted and acid-etched) of the implant surface.[25] An animal *in vivo* study suggests that the rougher implants seemed to be associated with greater bone growth.[26]

Significantly higher levels of cellular attachment were found using rough, sandblasted surfaces with irregular morphologies. [15] These results correlate with recent *in vivo* findings and suggest that implants should be prepared with roughened surfaces at bony contact areas.

The surface roughness of an implant to which osteoblasts attach has been shown to influence endogenous expression of growth factor and cytokines at the implant-tissue interface.[10,15-18] Cells derived from human mandibular bone were used to investigate the effect of varying roughness of titanium surfaces on production of transforming growth factor beta1 (TGF-beta1) and prostaglandin E2 (PGE2).[27] There were no significant differences among the three blasted surfaces with respect to production of the local factors. However, a synergistic effect of surface roughness and vitamin D was not observed on the production of both TGF-beta1 and PGE2. The hypothesis that estrogen modulates osteoblast response to implant surface morphology was tested.[28] Primary female human osteoblasts were cultured to confluence on three Ti surfaces (pretreatment, PT; sandblasted and acid-etched, SLA (roughest); and Ti plasma-sprayed, TPS) and treated for 24 h with 17beta-estradiol (E(2)). Results showed that normal adult human female osteoblasts had greatest osteoblastic growth on SLA and TPS and that E(2) was not shown to decrease this response of osteoblastic growth. Implant surface roughness may play a role in determining phenotypic expression of cells *in vivo*.[20]

A microscopic study found that macrophages exhibited “rugophilia,” or an affinity for rough surfaces.[31] This finding supports a previous study that indicated that rough surfaces promote inflammation via macrophage attraction.[32]

In addition to the biological events and aspects of implant dentistry, it is also very important to understand the biomechanical events that take place during the healing as well as the restorative phase of healing. A hallmark study by Branemark et al. examined torsion tests and pull-out tests on osseointegrated commercially pure titanium fixtures.[33] There were significant ($P<0.01$) correlations between torque and percentage of bone in contact with the fixture, and between pull-out load and the bone thickness around the fixture ($P<0.001$). Estimations of shear stresses and shear moduli (stiffness) in the bone tissue (pull-out test) and at the interface (torque test) indicated that the increase in bone volume around the implant substantially improved the mechanical capacity. Although an animal study, this study highlights the importance of optimal osseointegration around implants. An investigation used to study the use of resonance frequency analysis in search of the stability of the implant-tissue interface in vitro was performed.[34] The change in stiffness observed during bone healing was modeled by embedding implants in gypsum during the setting period. The results showed that there was an increase in resonance frequency (duration of sound) related to a stiffness increment during osseointegration.

The adhesive strengths of cells involved in osseointegration are a crucial factor in the cells ability to produce an extracellular matrix. It was suggested that differences in extracellular matrix production will be chiefly responsible for not

only differences in cellular phenotypes (morphotypes) among samples but also differences in adhesive strengths.[35]

MATERIALS AND METHODS

Isolation of rat pre-osteoblast cells:

Pregnant Sprague Dawley rats (day 18 of gestation) were asphyxiated in a carbon dioxide chamber. Fetuses were removed onto a sterile field and decapitated quickly. Heads of the fetuses were pooled in petri dishes containing sterile phosphate buffered saline (PBS) (Sigma®) and transferred to a tissue culture hood for calvarial dissection.

Calvariae were isolated by carefully removing all soft tissue from the inner and outer surfaces. The well-isolated calvariae were minced in a petri dish containing fresh PBS, and treated with collagenase (Sigma®) to liberate cells. All procedures were performed in accordance with the Oregon Health & Science University (OHSU) and Institutional Animal Care and Use Committee (IACUC) regulations.

The minced tissue was incubated with collagenase for 10 minutes 3 times. This was followed by 20-minute incubations twice. The protocol was as follows: (1) tissue was suspended in 3 ml collagenase; (2) incubated with agitation every minute; (3) the tissue was allowed to settle; and (4) supernatant was withdrawn.

The initial wash with collagenase was discarded, while the remainder was pooled into a container with an excess of Fetal Bovine Serum (FBS) (Sigma®) to attenuate the digestion. The tube containing the cell suspension was centrifuged for 5 minutes at 1200rpm. The supernatant was carefully removed and discarded, while the pellet, containing liberated pre-osteoblasts, was rinsed and suspended in Hanks Balanced Salt Solution (HBSS) (Sigma®). This was

performed twice. Cell counts were performed using a hemocytometer by measuring a small sample (50 μm) of cell suspension diluted with 50 μm trypan blue dye followed by taking the average of the 4 squares of the hemocytometer. The cell solution was distributed into T-75 culture flasks (approximately 1 million cells/flask). Flasks were allowed to incubate in a 37°C incubator for up to 5 days to allow for cell recovery. Media was replaced every other day to provide proper nutrients for the cells to grow.

Materials and textures:

Blasted, HA coated, and machined discs were used as experimental controls, while empty cell culture treated wells that did not contain a disc were used as positive controls. The discs were used as substrates upon which pre-osteoblast cells were cultured. This was performed in sterile 24-well culture plates. Each experimental run consisted of the following time intervals: 24, 48, 72, 96, 120, 144, and 168 hours.

Test disc samples were provided by Zimmer Dental® (formerly Centerpulse Dental, Inc.®) and were custom textured to evaluate 3 selected surface parameters: pit spacing, pit diameter, and pit depth (see Table 1 and 2). The test discs were designated as: (1) close-small-deep; (2) close-large-shallow; (3) far-small-shallow; and (4) far-large-deep.

Discs were made of Ti-6Al-4V alloy and each disc measured 15.5mm in diameter and 1.5mm thick, so that they fit snuggly into the bottom of the culture wells. Discs were sterilized with gamma radiation and handled similarly to

implants placed in humans according to Zimmer Dental® protocol. Discs fit the wells snuggly to cover the entire bottom. It was observed that bubbles filled the surface of pits upon immersion. Therefore, each disc was treated with ultra-sonication to remove bubbles present. The sonication was performed prior to placing the cells in the wells.

Cell plating experiments:

The experiments were performed under a sterile hood in the laboratory and the cells were cultured using 1ml media (5% FBS). Before plating the pre-osteoblasts onto the discs, each well containing the discs and media was ultra-sonicated in Hanks Balanced Salt Solution (HBSS) to remove any bubbles that may have developed during the immersion into liquid. The wells were viewed under light microscopy at 10X magnification to confirm the absence of bubbles.

The cells were then plated onto the 24-well plates containing the material discs. Each well received 1.0 ml of cell suspension in media containing 10,000 cells. The plates were stored in a 37°C incubator. The media was replaced every other day with sterile media to provide proper nutrients for the cells to grow optimally.

Table 1. All disc types

Disc Number	Disc Type
D1*	polystyrene
D2(Exp. #1)	close-small-deep
D3(Exp. #2)	close-large-shallow
D4	blasted
D5	HA-Coated
D6(Exp. #3)	far-small-shallow
D7(Exp. #4)	far-large-deep
D8	machined

*-Empty well containing cell suspension only; used as internal control to which all other discs were compared to.

Table 2. Dimensions of the experimental discs

Type	Space between pits(μm) ¥	Diameters(μm)∞	Depths(μm)¶
D2	Close	small	deep
D3	Close	large	shallow
D6	Far	small	shallow
D7	Far	large	deep

¥- Close (.004 inches) or Far (.008 inches)

∞- Small (.004 inches) or Large (.008 inches)

¶- Shallow (.003 inches) or Deep (.005 inches)

Quantifying cell growth:

The sample size (N) was 1248 observations. A total of 52 plates each containing 24 wells were used in our study. Cell growth was evaluated at the time intervals specified previously associated with attachment to (0-2 days) and proliferation (2-8 days) on the discs.[1] Cell proliferation and viability numbers at each time point were determined by measuring the reduction of Alamar Blue dye (Sigma®) placed on the cultures. Alamar Blue, which is reduced to metabolic intermediates, was used to monitor cell proliferation. The reduction is accompanied by a shift in color of the dye. A 10% Alamar Blue dye solution was placed into each well and the plates were incubated in complete darkness (at 37°C) for 2 hours.

After incubation time was completed, the alamar solution was withdrawn and replaced with standard media. The alamar samples were transferred to 96-well absorbance-reading plates and then read in a Fluoreskan® unit. Triplicate samples of each disc surface were analyzed. Fluorescence measurements were made by exciting samples at 540 nm and measuring emission at 590 nm. Four replicate measurements were taken for each well to confirm the accuracy of the Fluoreskan unit. The data was then standardized by reporting the emission intensity units as a ratio of intensity of experimental material to that produced by the polystyrene controls.

Statistical comparisons of the different textures:

2-way analysis of variance (ANOVA) was used to compare the ratios between different disc types and different time points. Eight disc types, including

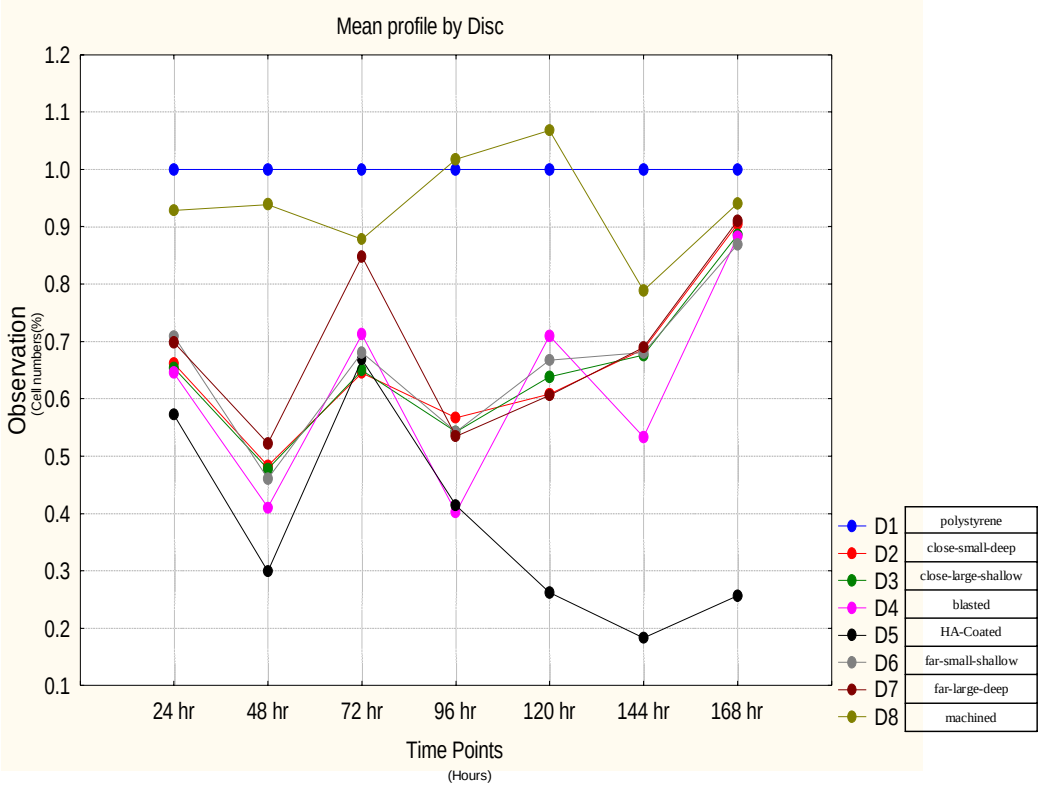
polystyrene were assessed at the 7 time points chosen. The individual absorbance values of each individual disc were divided by the polystyrene control wells. Because there were control and experimental discs, the experiment portion of the design looks very similar to the 2^{3-1} fractional factorial design. All reported p-values were two-tailed and results were considered significant at the $p < 0.05$ significance level. SAS® statistical package was used for the statistical analysis.

The measurements from the Alamar Blue dye analyses were then analyzed and reviewed for possible trends or differences between the different surface textures using ANOVA at 95% confidence interval ($p<0.05$). Both the differences between varying pit dimensions and between the pitted and non-pitted disc were performed.

RESULTS

In comparing the different parameters of the experimental discs with each other, several results were observed (Figure 1). Discs with far parameters had statistical significant greater cell numbers than discs closer together at 72 hours ($p<0.05$). There were no observed differences in cell numbers between the shallow and deep pits. Also, the only statistically significant difference among the experimental discs was that the D7 (far-lg-dp) showed greater cell numbers than D2 (cl-sm-dp), D3 (cl-lg-sh) and D6 (far-sm-sh) at the 72-hour time point. Calculated means and standard error of the means (SE) were determined for each sample (See appendix I).

Figure 1. Graph of comparisons of observations during attachment and proliferation



Several differences were observed when comparing the experimental disc types (D2 (cl-sm-dp), D3 (cl-lg-sh), D6 (far-sm-sh), and D7 (far-lg-dp)) to those control discs: D4 (blasted), D5 (HA coated), and D8 (machined). D2 (cl-sm-dp) showed greater cell numbers than D4 (blasted) at 96 and 144 hours. The 4 experimental discs resulted in greater cell numbers than D5 (HA coated) at all time points except 72 hours. D8 (machined) showed greater cell numbers than all experimental discs between 24-144 hours ($p<0.05$) with the exception of D7 (far-lg-dp) at 72 hours which has similar cell numbers to D8 (machined).

D3 (cl-lg-sh) showed greater cell numbers than D4 (blasted) at 96 and 144 hours. Also, D3 (cl-lg-sh) resulted in greater cell numbers than D5 (HA coated) at all times except 72 hours. D6 (far-sm-sh) showed greater cell numbers than D5 (HA coated) at all time points except 72 hours. D6 (far-sm-sh) also showed greater cell numbers than D4 (blasted) at 96 and 144 hours. D7 (far-lg-dp) showed greater cell numbers than D4 (blasted) at 48, 96, and 144 hours. Also, D7 (far-lg-dp) showed greater cell numbers than D5 (HA coated) at all time points.

In addition to comparing experimental vs. the controls, comparisons among the controls were also assessed. D4 (blasted) showed greater cell numbers than D5 (HA coated) at all time points except at 72 and 96 hours ($p<.05$). Also, D8 (machined) showed greater cell numbers than both D4 (blasted) and D5 (HA coated) at all time points except at the 168 hour time point.

DISCUSSION

It has been suggested that the success of implants depends on the early stages of osseointegration.[1,4,6,8,19] Many studies have been performed using a wide scope of methods to evaluate and simulate the early events of osteoblastic growth on various titanium surfaces. Primary cell culture systems, which are pre-osteoblasts harvested from various sources, including humans or animals, have the advantage of being able to undergo attachment, proliferation, and differentiation stages of development.[1,4,6,13,21-23,25-28,38-39] This study evaluated the rat calvarial cells which had undergone maturation into the pre-osteoblast cells during initial culturing. The pre-osteoblasts were monitored through a light microscope and media replaced every 2-3 days of culturing to provide optimal growth conditions of the osteoblasts.

Osteoblast like cell line (MG63) is a cell line isolated from a human osteosarcoma. [7,15,18,20,24,37,45] Cell line is typically used solely to assess only the first 2 stages of development (attachment and proliferation).[45] This cell line proliferates more rapidly than human bone derived cells but exhibits many of the same traits; however, the restriction of using osteosarcoma cell-derived culture systems is that, because they are cancerous cells, they do not reach differentiation stage. [45]

The results showed a sharp increase in cell numbers at the 72-hour time point followed by a sharp decrease in cell numbers at the 96-hour time point for all surfaces except the machined surfaces. This supports previous studies which showed the grooves formed by machining providing for greater attachment and

proliferation of osteoblasts. [38,39] In addition, the sharp decrease at the 96-hour time point for the HA-coated, blasted, and 4 experimental discs may be linked to a decrease in surface area for the osteoblasts to adhere to which may have been reached after proliferation of cells caused the newly formed cells to cover the substrate. This corresponds to another study which compared osteoblastic growth at different time points on different surface textures. It was shown that blasting Ti implants with Ti oxide particles is a good method of increasing surface roughness and enlarging surface area.[27] The study indicated that the surface area formed by the titanium oxide, which forms during passivation, occurs when this surface oxide does not disintegrate.[2]

Many factors, including biomechanical, have been suggested to play a role in the early stages of osseointegration.[31-35,46-49] Although this study solely based results on the Alamar Blue dye metabolism, it is important to understand that other factors may be playing a role in the osteoblastic growth patterns on the different surface textures evaluated in this study. In evaluating the growth patterns of the osteoblasts, it was observed that there were higher cell numbers for the experimental discs compared to the HA and blasted surfaces at the 96 and 144 hour time points, which may indicate a higher surface energy of the experimental pitted discs.[31-33,46]

The surface energy is defined by its general charge density and the net polarity of the charge.[46] Thus, a surface with a net positive or negative charge may be hydrophilic in character, whereas a surface with a neutral charge may be more hydrophobic. The net effect of the surface charge is to create a local

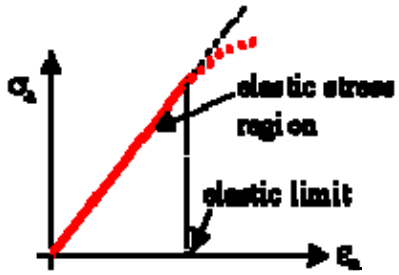
environment with a specific surface tension, surface free energy and energy of adhesion. The surface energy of the material can influence which proteins adhere to the material. This is governed by changes in the hydration of the material surface and the protein, Coulomb interactions between the material and the protein, and structural rearrangements in the adsorbing proteins.

Focal contacts are specialized microstructures anchored within the cell to cytoskeletal microfilaments which underlie the cytoplasmic membrane.[46] Components of the microstructure traverse the cytoplasmic membrane, permitting the cell to bind to its ECM via proteins called integrins. It is known that many ECM proteins, including fibronectin, contain a unique RGD peptide sequence of the amino acids arginine(R), glycine(G), and aspartic acid(D) which modulate attachment of fibroblasts and osteoblasts.

Osteoblasts interact with their substrate via integrin receptors and the type of substrate determines which integrins and ECM proteins are expressed.[47] Integrins are cell receptors found on osteoblasts which are mainly designed for cell-cell and cell-matrix interactions. The expression of these integrins is controlled by the ECM and growth factors, most notably the TGF-B.[48]

Upon evaluation of the biomechanical events taking place at the cell-substrate interface, the relationship between stress and strain must be considered.[49] When a material is loaded with a force, stress at some location in the material is defined as force per unit area. Strain is defined as a change in dimensions as a response to the force of stress. It turns out that for elastic materials, stress is linearly proportional to strain (Hooke's Law) (Figure 2).

Figure 2. Stress-Strain relationship (ε_a-stress; σ_a-strain)



Note that Hooke's law breaks down when the strain gets too high.[49] On a typical stress-strain diagram, Hooke's law applies only in the elastic stress region, in which the loading is reversible. Beyond the elastic limit (or proportional limit), the material starts to behave irreversibly in the plastic deformation region, in which the stress vs. strain curve deviates from linear, and Hooke's law no longer holds.

In this study, the different experimental discs were pressed producing pits of different dimensions. The other discs (blasted, machined, and HA coated) also received the changes in dimensions upon forces applied by the various treatments. The pressing of the discs or other treatments produced stress to the titanium discs, thus, resulting in a strain which caused a change in dimensions of the discs containing the different pitting. This phenomenon of stress and strain may cause some changes in the strength of the titanium discs, hence decreasing its modulus of elasticity, or stiffness. Modulus of elasticity is defined as the ratio of stress over strain.

In addition to the biomechanical aspects of osseointegration, the biological aspects are equally important.[5,9,14,17,50] Osteoblasts secrete a complex extracellular matrix (ECM) containing collagenous and noncollagenous proteins,

bone morphogenetic proteins (BMPs), and growth factors. Osteoblast-specific gene expression requires ascorbic acid (AA)-dependent assembly of a collagenous ECM. Matrix responsiveness requires an alpha2beta1 integrin-collagen interaction and mitogen-activated protein kinase (MAPK) activity, which phosphorylates and activates the osteoblast-specific transcription factor Cbfa1.) ECM deposition is dependent on surface microtopography.

Results of an *in vitro* study using the same cell used in this study, rat calvarial cells, showed a decrease in cell proliferation(at day 8) while there was an increase in ALP activity for the sand-blasted and acid-etched (SLA) and titanium plasma sprayed (TPS) compared to the smooth surfaces which showed lower ALP activity.[50] This indicated an increase in the ECM deposition which corresponds to the results which demonstrated a downward slope during late proliferation and differentiation. In all instances, Ca levels were elevated suggesting that mineralization was occurring.

Alkaline phosphatase (ALP) is a glycoprotein associated with the formation of calcified tissues. It catalyzes the hydrolysis of phosphate esters at an alkaline.[51] On a cellular level, ALP is the most widely recognized marker of osteoblast phenotypes during differentiation stage of osteoblastic development. ALP has many isoforms found in the blood, intestines, bone, and liver. While the *in vivo* function of ALP is unknown, their affinity for bone mineral constituents implies a role in bone formation. This study did not measure ALP levels because this study was purely interested in evaluating attachment and proliferation of

osteoblasts. Results of this study solely based the results on the amount of metabolism of the Alamar Blue dye (Sigma®) by the osteoblasts.

A recent study demonstrated the growth stages of rat osteoblasts.[1] Their study was carried out using fetal rat calvariae to evaluate the course of development of rat pre-osteoblasts via measuring the alkaline phosphatase (ALP) levels which have been shown to be hallmark indicators of osteoblast development. The results demonstrated 3 distinct stages which were shown to occur at different time intervals: attachment (0-2 days), proliferation (2-8 days), and differentiation (8-10 days). This experiment observed osteoblastic responses to the different textures only during the first 2 stages (attachment and proliferation).

During the proliferative stage of development, discs containing pits further apart (0.005 in.) showed greater cell numbers than those discs containing pits closer together (0.003 in.) at 72 hours. The results indicate that the osteoblasts were able to proliferate more readily because of greater surface area between the pits. No statistically significant differences were observed between the other parameters (large vs. small, shallow vs. deep). However, when comparing experimental discs D2 (cl-sm-dp), D3 (cl-lg-sh), D6 (far-sm-sh), and D7 (far-lg-dp), results showed that the D7 had statistically significant greater cell numbers than D2, D3, and D6 at the initial stages of proliferation (48 hours). The results indicate that growth and proliferation was possibly enhanced with greater surface areas, thus indicating that the particle size of the surface texture seems to be playing a greater role than the actual nature of the surface texture.

All four experimental discs D2 (cl-sm-dp), D3 (cl-lg-sh), D6 (far-sm-sh), and D7 (far-lg-dp) had significantly greater cell numbers than blasted discs during proliferation stage, suggesting that pitted discs can enhance the proliferation of rat osteoblasts compared to the blasted discs. This may have been linked to the increase in surface area caused by the strain produced by the pressing of the discs which has been shown to enhance growth and proliferation of the discs. Additionally, all 4 experimental discs had greater cell numbers than HA coated surfaces during attachment as well as proliferation.

In comparing the different control discs, it was observed that the machined surfaces showed superior cell numbers compared to the experimental as well as blasted and HA coated discs during attachment and proliferation. This may have been due to grooves produced during the turning process which have been shown to result in greater growth and proliferation of osteoblast cells.[38,39] However, the blasted discs showed greater cell numbers than HA coated discs only during the initial stage of proliferation, which disagrees with previous studies which proposed that HA offers optimal attachment due to increased roughness found in HA surfaces.[11,13,15,19,36-37]

CONCLUSIONS

This study demonstrated that pitted surfaces resulted in greater cell numbers than the blasted and HA coated surfaces at certain time points. Although the study indicated that there were differences between far and closer parameters at early proliferation time, the overall results showed no statistical significance ($F > 0.05$) thus rejecting the null hypothesis, suggesting that discs containing pits that are further apart, deeper, and larger in diameter do not have greater cell numbers during compared to the remaining experimental parameters. This may be due to limitations in the study such as absence of all of the possible combinations of the parameters (our study only evaluated 4 of the 8 possible combinations). Moreover, the pitted discs showed greater cell numbers than the controls during proliferation, suggesting that pitting plays an important role during the proliferative phase of osteoblast development.

The pitted surface texture used in this study has not been well studied. Therefore, further studies should be completed on animals as well as humans to evaluate the optimization of growth of osteoblast cells on pitted implant surfaces.

FUTURE STUDIES

Current research on dental implants and osseointegration has been directed toward enhancing the initial healing events. Osseointegration is defined as the direct connection from implant to living remodeling bone without any soft tissue component between implant and bone on the light microscopic level.[41] Many *in vitro* and *in vivo* studies have demonstrated that surface roughness does in fact enhance the initial stages of osteogenesis around dental implants. My experimental design was to assess the enhancement of attachment and proliferation of osteoblasts using a novel surface texture. Overall, the study demonstrated that the cell number was influenced by the surface texture. The rougher surfaces, including pitted and non-pitted surfaces seemed to have a pattern of greater cell number that machined and smooth surface textures. The results of this study are promising; however, according to many recent studies, newer types of modifications of surface textures of titanium implants are being considered.

The performance of nonresorbable and reactive sol-gel-derived nano-porous titania (TiO(2)) coatings in a soft tissue environment has been investigated.[42] A direct attachment between the soft tissue and the sol-gel-derived titania coatings was found *in vivo* after 2 days of implantation, whereas the titanium control implants showed no evidence of soft tissue attachment. The coated implants were in immediate contact with the connective tissue, whereas the titanium controls formed a gap and a fibrous capsule on the implant-tissue

interface. The good soft tissue attachment of titanium coatings may result from their ability to initiate calcium phosphate nucleation and growth on their surfaces (although the formation of poorly crystalline bonelike apatite does not occur). Thus, the formation of a bonelike Calcium Phosphate (CaP) layer is not crucial for their integration in soft tissue. The formation of bonelike apatite was hindered by the adsorption of proteins onto the initially formed amorphous calcium phosphate growth centers, thus preventing the dissolution/reprecipitation processes required for the formation of poorly crystalline bonelike apatite. These findings might open novel application areas for sol-gel-derived titania-based coatings.

Calcium ion implantation of titanium was previously reported to enhance osseointegration and bone formation *in vivo*, although the lack of consistent and reproducible effects highlight the need to understand the basic mechanisms involved in the response of target cells to such surfaces.[43] Measurements of the precise effects of ion implantation of titanium on bone cells were performed *in vitro*. Alveolar bone cells were seeded on the surface of polished titanium discs implanted with calcium, potassium, and argon ions. Using radioisotopically tagged bone cells, the results showed that although the calcium ion implanted surface reduced cell adhesion, it nevertheless significantly enhanced cell spreading and subsequent cell growth. In contrast, few differences in bone cell behavior were observed between the potassium- and argon-implanted titanium and the control nonimplanted titanium discs. These findings suggest the possibility that the calcium-implanted surface may significantly affect the

biocompatibility of titanium implants by enhancing bone cell growth. Surface modification by ion implantation could thus prove to be a valuable tool for improving the clinical efficacy of titanium for bone repair and regeneration *in vivo*.

CaTiO(3) is a strong candidate to form at the interface between hydroxylapatite (HA) and titanium implants during many coating procedures.[44] However, few studies have compared the cytocompatibility properties of CaTiO(3) to HA pertinent for bone-cell function. An *in vitro* study determined the ability of bone osteoblasts to adhere on titanium coated with HA, resulting in the formation of CaTiO(3). Results from cytocompatibility tests revealed increased osteoblast adhesion on materials that contained CaTiO(3) compared to both pure HA and uncoated titanium. the present *in vitro* study results imply that orthopedic coatings that form CaTiO(3) could increase osseointegration with juxtaposed bone needed for increased implant efficacy.

In this experimental study, the effect of surface roughness on rat pre-osteoblasts was evaluated using different surface textured titanium disks. The results of the study showed that the pitted surface textures have greater osseous responses that the conventional acid-etched, SLA, or HA surfaces. The next phase of this project will be to perform the surgical placement of the different surface textured implants on dogs.

BIBLIOGRAPHY

1. Rosa AL, Beloti MM. Rat bone marrow cell response to titanium and titanium alloy with different surface roughness. *Clinical Oral Implants Research. 14(1):43-8; 2003 Feb.*

2. Parr GR, Gardner LK, Toth RW. Titanium: the mystery metal of implant dentistry. *Journal of Prosthetic Dentistry. 54(3):410-14; 1985 Sept.*

3. Jinno T, Goldberg VM, Davy D, Stevenson S. Osseointegration of surface-blasted implants made of titanium alloy and cobalt-chromium alloy in a rabbit intramedullary model. *Journal of Biomedical Materials Research. 42(1):20-9; 1998 Oct.*

4. Sinha RK, Morris F, Shah SA, Tuan RS. Surface composition of orthopaedic implant metals regulates cell attachment, spreading, and cytoskeletal organization of primary human osteoblasts *in vitro*. *Clinical Orthopaedics & Related Research. (305):258-72; 1994 Aug.*

5. Gundberg CM, Hauschka PV, Lian JB, Gallop PM. Osteocalcin: isolation, characterization, and detection. *Methods Enzymol.; 107:516-44; 1984 May*

6. Schmidt C, Ignatius AA, Claes LE. Proliferation and differentiation parameters of human osteoblasts on titanium and steel surfaces. *Journal of Biomedical Materials Research. 54(2):209-15; 2001 Feb.*

7. Keller JC, Stanford CM, Wightman JP, Draughn RA, Zaharias R. Characterizations of titanium implant surfaces. III. *J Biomed Mater Res. 28(8):939-46; 1994 Aug.*

8. Brånemark PI, Hanson BO, Adell R. Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period. *Almqvist and Wiksell, Stockholm; 1977.*

9. Bagambisa FB, Kappert HF, Schilli W. Cellular and molecular biological events at the implant interface. *Journal of Cranio-Maxillo-Facial Surgery. 22:12-17; 1994.*

10. Eriksson C, Broberg M, Nygren H, Oster L. Novel *in vivo* method for evaluation of healing around implants in bone. *Journal of Biomedical Materials Research. Part A. 66(3):662-8; 2003 Sep.*

11. Cooper LF, Masuda T, Whitson SW, Yliheikkila P, Felton DA. Formation of mineralizing osteoblast cultures on machined, titanium oxide grit-blasted, and plasma-sprayed titanium surfaces. [Journal Article] *International Journal of Oral & Maxillofacial Implants. 14(1):37-47; 1999 Jan-Feb.*

12. Faria AC, Beloti MM, Rosa AL. Nitric acid passivation does not affect *in vitro* biocompatibility of titanium. *International Journal of Oral & Maxillofacial Implants. 18(6):820-5; 2003 Nov-Dec.*

13. Kenney EB, Lekovic V, Sa Ferreira JC, Han T, Dimitrijevic B, Carranza FA Jr. Bone formation within porous hydroxylapatite implants in human periodontal defects. *Journal of Periodontology. 57(2):76-83; 1986 Feb.*

14. Schneider GB, Perinpanayagam H, Clegg M, Zaharias R, Seabold D, Keller J, Stanford C. Implant surface roughness affects osteoblast gene expression. *Journal of Dental Research. 82(5):372-6; 2003 May.*

15. Schwartz Z, Lohmann CH, Oefinger J, Bonewald LF, Dean DD, Boyan BD. Implant surface characteristics modulate differentiation behavior of cells in the osteoblastic lineage. *Advances in Dental Research. 13:38-48; 1999 Jun.*

16. Cooper LF. A role for surface topography in creating and maintaining bone at titanium endosseous implants. *Journal of Prosthetic Dentistry. 84(5):522-34; 2000 Nov.*

17. Cochran DL. A comparison of endosseous dental implant surfaces. *Journal of Periodontology. 70(12):1523-39; 1999 Dec.*

18. Bowers KT, Keller JC, Randolph BA, Wick DG, Michaels CM. Optimization of surface micromorphology for enhanced osteoblast responses *in vitro*. *International Journal of Oral & Maxillofacial Implants. 7(3):302-10; 1992 Fall.*

19. Piattelli A, Manzon L, Scarano A, Paolantonio M, Piattelli M. Histologic and histomorphometric analysis of the bone response to machined and sandblasted titanium implants: an experimental study in rabbits. *International Journal of Oral & Maxillofacial Implants. 13(6):805-10; 1998 Nov-Dec.*

20. Martin JY, Schwartz Z, Hummert TW, Schraub DM, Simpson J, Lankford J, Dean DD, Cochran DL, Boyan BD. Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells(MG63). *Journal of Biomedical Materials Research 29: 389-401; 1995 May*.

21. Hatano K, Inoue H, Kojo T, Matsunaga T, Tsujisawa T, Uchiyama C, Uchida Y. Effect of surface roughness on proliferation and alkaline phosphatase expression of rat calvarial cells cultured on polystyrene. *Bone. 25(4): 439-451; 1999 Oct.*

22. Puleo DA, Holleran LA, Doremus RH, Bizios R. Osteoblast responses to orthopedic implant materials *in vitro*. *J Biomed Mater Res. 25(6):711-23; 1991 Jun.*

23. Castellani R, de Ruijter A, Renggli H, Jansen J. Response of rat bone marrow cells to differently roughened titanium discs. *Clinical Oral Implants Research. 10(5):369-78; 1999 Oct.*

24. Michaels CM. *In vitro* cell attachment of osteoblast-like cells to titanium. *J Dent Res. 1989.*

25. Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stich H. Influence of surface characteristics on bone integration of titanium implants. A histomorphometric study in miniature pigs. *J Biomed Mater Res. 25(7):889-902; 1991 Jul.*

26. Grizon F, Aguado E, Hure G, Basle MF. Chappard D. Enhanced bone integration of implants with increased surface roughness: a long term study in the sheep. *Journal of Dentistry. 30(5-6):195-203; 2002 Jul-Aug.*

27. Mustafa K, Wennerberg A, Wroblewski J, Hultenby K, Lopez BS, Arvidson K. Determining optimal surface roughness of TiO(2) blasted titanium implant material for attachment, proliferation and differentiation of cells derived from human mandibular alveolar bone. *Clinical Oral Implants Research. 12(5):515-25; 2001 Oct.*

28. Lohmann CH, Tandy EM, Sylvia VL, Hell-Vocke AK, Cochran DL, Dean DD, Boyan BD, Schwartz Z. Response of normal female human osteoblasts (NHOst) to 17beta-estradiol is modulated by implant surface morphology. *Journal of Biomedical Materials Research. 62(2):204-13; 2002 Nov.*

29. Schierano G, Bellone G, Manzella C, Preti G, Emanuelli G. *In vitro* effect of transforming growth factor-beta on adhesion molecule expression by human gingival fibroblasts cultured in the presence of a titanium abutment. *Journal of Periodontology. 72(12):1658-65; 2001 Dec.*

30. Kawahara H, Kawahara D, Hashimoto K, Takashima Y, Ong JL. Morphologic studies on the biologic seal of titanium dental implants. Report I. *In vitro* study on the epithelialization mechanism around the dental implant. *International Journal of Oral & Maxillofacial Implants. 13(4):457-64; 1998 Jul-Aug.*

31. Rich A, Harris AK. Anomalous preferences of cultured macrophages for hydrophobic and roughened substrata. *J Cell Sci. 1981*.

32. Murray DW, Rae T. The influence of the surface energy and roughness of implants on bone resorption. *J Bone Joint Surg. 1989.*

33. Brånemark R, Ohrnell LO, Nilsson P, Thomsen P. Biomechanical characterization of osseointegration during healing: an experimental *in vivo* study in the rat. *Biomaterials, v 18, n 14, p 969-78; July 1997.*

34. Lin SF, Pan LC, Lee SY, Peng YH, Hsiao TC. Resonance frequency analysis for osseointegration in four surgical conditions of dental implants. *Engineering in Medicine and Biology Society, 2001. Proceedings of the 23rd Annual International Conference of the IEEE, Pages: 2998-3001 vol.3;2001.*

35. Gianoli DJ, Kohles SS, Burnham NA, Clark MB, Brown CA, Kenealy JN. The feasibility of atomic force microscopy as a cytodetachment technique to quantify osteoblastic adhesion with implant surfaces. *Bioengineering Conference, 2001. Proceedings of the IEEE 27th Annual Northeast, Vol., Iss. 2001. Pages:5-6; 2001.*

36. Bowers KT. Keller JC. Randolph BA. Wick DG. Michaels CM. Optimization of surface micromorphology for enhanced osteoblast responses in vitro. *International Journal of Oral & Maxillofacial Implants. 7(3):302-10, 1992 Fall.*

37. Orsini G. Assenza B. Scarano A. Piattelli M. Piattelli A. Surface analysis of machined versus sandblasted and acid-etched titanium implants. *International Journal of Oral & Maxillofacial Implants. 15(6):779-84, 2000 Nov-Dec.*

38. Lumbikanonda N. Sammons R. Bone cell attachment to dental implants of different surface characteristics. *International Journal of Oral & Maxillofacial Implants. 16(5):627-36, 2001 Sep-Oct.*

39. Xavier SP. Carvalho PS. Beloti MM. Rosa AL. Response of rat bone marrow cells to commercially pure titanium submitted to different surface treatments. *Journal of Dentistry. 31(3):173-80, 2003 Mar.*

40. Gundberg CM, Hauschka PV, Lian JB, Gallop PM. Osteocalcin: isolation, characterization, and detection. *Methods Enzymol.;107:516-44. 1984 May*

41. P.I. Brånemark, Osseointegration and its experimental background. *J Prosthet Dent **50**:399–410; 1983.*

42. Areva S. Paldan H. Peltola T. Narhi T. Jokinen M. Linden M. Use of sol-gel-derived titania coating for direct soft tissue attachment. *Journal of Biomedical Materials Research. Part A. 70(2):169-78; 2004 Aug*.

43. Nayab SN. Jones FH. Olsen I. Human alveolar bone cell adhesion and growth on ion-implanted titanium. *Journal of Biomedical Materials Research. Part A. 69(4):651-7; 2004 Jun.*

44. Webster TJ. Ergun C. Doremus RH. Lanford WA. Increased osteoblast adhesion on titanium-coated hydroxylapatite that forms CaTiO3. *Journal of Biomedical Materials Research. Part A. 67(3):975-80; 2003 Dec.*

45. Clover J, Gowen M. Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype? *Bone 15(6):585-91; 1994 Nov-Dec*

46. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. *Biomaterials 17(2):137-46; 1996 Jan.*

47. Gronowicz G, McCarthy MB. Response of human osteoblasts to implant materials: integrin-mediated adhesion. *J Orthop Res 14(6):878-87.1996 Nov.*

48. Uitto VJ, Larjava H. Extracellular matrix molecules and their receptors: an overview with special emphasis on periodontal tissues. *Crit Rev Oral Biol Med. 2(3):323-54; 1991.*

49. American iron and steel institute(AISI), 2003. Washington D.C.

50. Boyan BD, Bonewald LF, Paschalis EP, Lohmann CH, Rosser J, Cochran DL, Dean DD, Schwartz Z, Boskey AL.Osteoblast-mediated mineral deposition in culture is dependent on surface microtopography. *Calcif Tissue Int. 71(6):519-29; Dec 2002.*

51. Sabokbar A, Millett PJ, Myer B, Rushton N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. *Bone Miner. 27(1):57-67. 1994 Oct.*

Appendix I. Data table

Time	DT	Dim	Dis	Dep	Mean	SE
1	1	0	0	0	1.0000	0.0283
1	2	1	1	1	0.6618	0.0283
1	3	1	2	2	0.6536	0.0286
1	4	0	0	0	0.6455	0.0283
1	5	0	0	0	0.5725	0.0283
1	6	2	1	2	0.7092	0.0283
1	7	2	1	1	0.6982	0.0283
1	8	0	0	0	0.9284	0.0283
2	1	0	0	0	1.0000	0.0283
2	2	1	1	1	0.4829	0.0279
2	3	1	2	2	0.4773	0.0272
2	4	0	0	0	0.4103	0.0272
2	5	0	0	0	0.2993	0.0276
2	6	2	1	2	0.4605	0.0276
2	7	2	1	1	0.5223	0.0283
2	8	0	0	0	0.9390	0.0276
3	1	0	0	0	1.0000	0.0588
3	2	1	1	1	0.6451	0.0624
3	3	1	2	2	0.6498	0.0588
3	4	0	0	0	0.7123	0.0588
3	5	0	0	0	0.6685	0.0624
3	6	2	1	2	0.6803	0.0588
3	7	2	1	1	0.8478	0.0588
3	8	0	0	0	0.8786	0.0624
4	1	0	0	0	1.0000	0.0385
4	2	1	1	1	0.5674	0.0385
4	3	1	2	2	0.5422	0.0385
4	4	0	0	0	0.4020	0.0385
4	5	0	0	0	0.4144	0.0385
4	6	2	1	2	0.5424	0.0395
4	7	2	1	1	0.5351	0.0385
4	8	0	0	0	1.0172	0.0385
5	1	0	0	0	1.0000	0.0624
5	2	1	1	1	0.6083	0.0588
5	3	1	2	2	0.6382	0.0588
5	4	0	0	0	0.7094	0.0588
5	5	0	0	0	0.2620	0.0588
5	6	2	1	2	0.6673	0.0588
5	7	2	1	1	0.6062	0.0588
5	8	0	0	0	1.0679	0.0588
6	1	0	0	0	1.0000	0.0532
6	2	1	1	1	0.6859	0.0510
6	3	1	2	2	0.6761	0.0510
6	4	0	0	0	0.5330	0.0510
6	5	0	0	0	0.1831	0.0510
6	6	2	1	2	0.6806	0.0510
6	7	2	1	1	0.6898	0.0558
6	8	0	0	0	0.7887	0.0532
7	1	0	0	0	1.0000	0.0721
7	2	1	1	1	0.9050	0.0721
7	3	1	2	2	0.8866	0.0721
7	4	0	0	0	0.8829	0.0721
7	5	0	0	0	0.2565	0.0883
7	6	2	1	2	0.8693	0.0721
7	7	2	1	1	0.9102	0.0721
7	8	0	0	0	0.9403	0.0721

Figure 1. Graph of comparisons of observations during attachment and proliferation

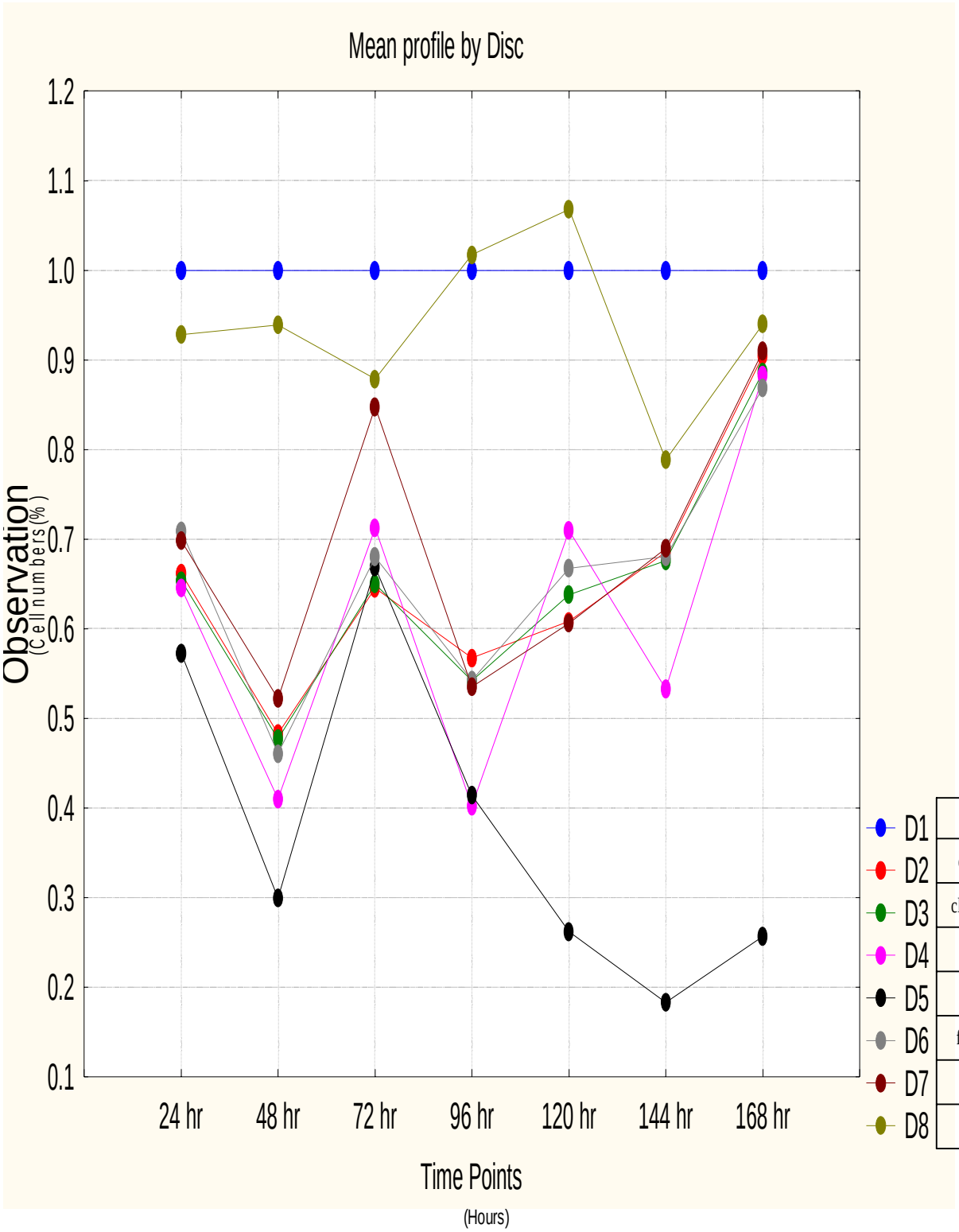

Mean profile by Disc
Observation
(Cell numbers(%))
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
24 hr
48 hr
72 hr
96 hr
120 hr
144 hr
168 hr
Time Points
(Hours)
D1
D2
D3
D4
D5
D6
D7
D8
polystyrene
close-small-deep
close-large-shallow
blasted
HA-Coated
far-small-shallow
far-large-deep
machined

Figure 2. Stress-Strain relationship (ε_a-stress; σ_a-strain)