HISTOMORPHOMETRIC AND BIOMECHANICAL ANALYSES OF OSSEOINTEGRATION OF FOUR DIFFERENT ORTHODONTIC MINI IMPLANT SURFACES

Sumit Yadav

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W. Eugene Roberts, Jr., D.D.S., Ph.D., Chair

Jie Chen, Ph.D.

Doctoral Committee

June 24, 2010

Thomas R. Katona, Ph.D., D.M.D

Sean S. Liu, Ph.D.

Sarandeep S. Huja, D.D.S., Ph.D.

DEDICATION

To my parents, who supported my education.

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ABSTRACT

Sumit Yadav

Histomorphometric and Biomechanical Analyses of Osseointegration of Four Different Orthodontic Mini Implant Surfaces

Objective: To evaluate the osseointegration potential of four different surfaces of mini-implants. We hypothesized that mini-implants surface roughness alters the intrinsic biomechanical properties of the bone integrated to titanium. Materials and Methods: Mini implants and circular discs were made from alloy Ti6Al4V grade 5. On the basis of surface treatment study was divided into 4 groups: Group 1: Machined: no surface treatment, Group 2: Acid etched: with hydrochloric acid, Group 3: Grit Blasted with alumina and Group 4: Grit blasted +Acid etched. Surface roughness parameters (mean surface roughness: Ra and Quadratic Average roughness: Rq) of the four discs from each group were measured by the optical profilometer. Contact angle measurement of 3 discs from each group was done with a Goniometer. Contact angle of liquids with different hydrophobicity and hydrophilicity were measured. 128 mini implants, differing in surface treatment, were placed into the tibias and femurs of 8 adult male New Zealand white rabbits. Biomechanical properties (Removal torque and hardness) measurements and histomorphometric observations were measured. Results: Ra and Rq of groups were: Machined (1.17±0.11, 2.59±0.09) Acid etched (1.82±0.04, 3.17±0.13), Grit blasted (4.83±0.23, 7.04±0.08), Grit blasted + Acid etched (3.64±0.03, 4.95±0.04) respectively. Group 4 had significantly

(p=0.000) lower Ra and Rq than Group 3. The interaction between the groups and liquid was significant. Group 4 had significantly lower contact angle measurements (40.4°, 26.9°), both for blood and NaCl when compared to other three groups (p≤0.01). Group 4 had significantly higher torque than Group 3 (Tibia: 13.67>9.07N-cm; Femur: 18.21>14.12N-cm), Group 4 (Tibia: 13.67>9.78N-cm; Femur: 18.21>12.87N-cm), and machined (Tibia: 13.67>4.08N-cm; Femur: 18.21>6.49N-cm). SEM analysis reveals significantly more bone implant gap in machined implant surfaces than treated implant surfaces. Bone to implant contact had significantly higher values for treated mini implant surface than machined surface. Hardness of the bone near the implant bone interface is 20 to 25% less hard than bone 1mm away from it in both Femur and Tibia. Conclusion: Surface roughness and wettability of mini implants influences their biological response. Grit blasted and acid etched mini implants had lowest contact angle for different liquids tested and highest removal torques.

W. Eugene Roberts, Jr., D.D.S., Ph.D., Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Anchorage

Anchorage control plays a pivotal role in the effective management of orthodontic cases for attaining both structural and facial esthetics (Burstone, 2007a, 2007b; Roberts, 2002; Roberts, Engen, Schneider, & Hohlt, 2004). Assuming ideal treatment goals, anchorage requirements should be evaluated in all three planes of spaces: anterior-posterior, transverse, and vertical. Attaining maximum or absolute anchorage has always been an arduous goal for the practicing orthodontist, often resulting in a condition, dreaded by most, called 'anchor loss'.

To address this problem numerous appliances and techniques have been devised such as: Nance holding arch, transpalatal bars, extraoral traction, use of multiple teeth as the anchorage segment, and applying differential moments (Geron, Shpack, Kandos, Davidovitch, & Vardimon, 2003; Hart, Taft, & Greenberg, 1992; Rajcich & Sadowsky, 1997). However all these methods have inherent disadvantages: complicated designs, need for exceptional patient cooperation, elaborate wire bending etc.

Although the idea of using a temporary device to establish orthodontic skeletal anchorage was introduced in a 1945 animal study, the first published case report did not appear until 1983 (Creekmore & Eklund, 1983). In recent years titanium mini-screws have gained enormous popularity in the orthodontic

community and are being considered as ‘absolute’ sources of orthodontic anchorage (Costa, Raffainl, & Melsen, 1998; Lee, Park, & Kyung, 2001; Melsen & Costa, 2000; H. S. Park, Bae, Kyung, & Sung, 2001). The primary advantages of the mini implants are: easy insertion and removal, immediate loading, placement at numerous anatomic locations and no need for patient compliance (Deguchi, et al., 2003; Kanomi, 1997). The primary deterring factors for mini implants are their inability to resist the rotational tendency of heavy dynamic loads and to control three dimensional tooth movements, which is best achieved at present by osseointegrated endosseous implants (3-4 mm in diameter x 6-8 mm in length)(Miyawaki, et al., 2003).

Researchers report 51% to 90% success rate with mini implants as orthodontic anchorage. However, the clinical application of a mini implant does not guarantee treatment success, and its stability is essential before it can be used (Hung, Oliver, Kim, Kyung, & Buschang, 2010). The two factors, which usually affect the success of the mini implants, are implant biocompatibility and host resistance (Florvaag, et al.; S. H. Kim, Lee, Cho, Kim, & Kim, 2009; Mo, et al.; Motoyoshi, et al.; Ren, 2009; Tseng, et al., 2006). Common implant variables are implant diameter, length, surface treatment, and thread design. The host factors include the quality and the quantity of the bone. Due to the large size of osseointegrated prosthetic implants, they cannot be placed in a variety of anatomic locations. Furthermore, they usually require a 2 stage placement protocol as well as a variable healing period (4-6 months) before they can be used as anchorage. These factors limit their use. The topography of the implants

surface has been widely studied, and various surfaces have been proposed for titanium dental implants and mini implants. However, the results of these studies are inconclusive.

Mini Implant Surfaces

Mini Implant surface characteristics such as its micro topography, chemistry, surface charge, wettability, and surface design have been found to be critical factors for enhanced biomimetic response (Guo, Zhou, Rong, Zhu, & Geng; He, Yang, Wang, & Zhao, 2009; Lucchini, Aurelle, Therin, Donath, & Becker, 1996; Stubinger, et al.). Implant surface roughness may influence osteoblast proliferation, differentiation, and local factor production (Galli, et al., 2005; H. K. Kim, Jang, & Lee, 2004; Schweikl, et al., 2007).

The success or failure of an implant is largely dependent on the degree to which it integrates with the host bone and the physical, chemical, and biological events at the bone–implant interface play a major role in its ability to do so. Titanium alloys are widely used as implants because of their excellent mechanical properties and anticorrosion behavior (resistance to deterioration due to formation of titanium oxide layer on its surface) in the physiological environment. Nevertheless, titanium is a bio-inert material with non-bone-bonding ability, which leads to an absence of rapid enhanced bone formation following implantation. Inadequate bone cell-surface interactions associated with existing materials have hindered the development of biologically active osseous implants. Altering the surface topography of the implant surface can enhance its bioactivity,

thus allowing it to form a more rapid and complete integration to the surrounding bone (J. W. Park, Jang, & Suh, 2008).

The surface topography of the dental implant has been widely studied, and various surfaces have been proposed. One of the two approaches to altering surface topography are additive methods such as: titanium plasma spray, hydroxy apatite coating, titanium oxide coating, niobium oxide coating, and covalently bonded bone morphogenic protein 2 coating (Brama, et al., 2007; Liu, de Groot, & Hunziker, 2005; Liu, Huse, de Groot, Buser, & Hunziker, 2007; Saju, Reshmi, Jayadas, James, & Jayaraj, 2009; Yan, et al., 2007). Titanium implant surfaces can also be modified by subtractive methods such as: acid etching (hydrochloric acid (HCL), Hydrofluoric acid (HF), a combination of sulphuric acid (H_2SO_4) and HCL, combination of HCL, H_2SO_4 and phosphoric acid (H_3PO_4) and chromo sulphuric acid), grit blasting (non-resorbable blasting media such as alumina, silica, and titanium oxide or resorbable blasting media such as hydroxyapatite, tricalcium phosphate, or a combination of Ca-P phases), combination of blasting and acid etching, and laser ablation (Bornstein, Wittneben, Bragger, & Buser; Calvo-Guirado, et al. 2006; Degidi, Piattelli, Shibli, Perrotti, & Iezzi, 2009).

Sandblasted, acid-etched and other moderately roughened implants show a stronger bone response than turned implants. Such surfaces can increase the rate and amount of bone formation on the implant surface and enhance mechanical fixation (Degidi, et al., 2009). The implant surface may be contaminated by residue from the grit blasting material. This may interfere with

the osseointegration of the titanium dental implants (Jeong, et al.; Meirelles, Currie, Jacobsson, Albrektsson, & Wennerberg, 2008) and even after etching with strong acids; blasting materials such as alumina may persist.

Cooper et al. suggested that surface topography may directly or indirectly affect the amount of bone formed at the interface (Cooper, 2000). A number of in vivo studies have demonstrated that altered surface topography, leading to increased surface area of implants, results in increased bone-to implant contact early after implant placement (Buser, et al., 2004; Le Guehennec, Soueidan, Layrolle, & Amouriq, 2007; Schwartz, et al., 2008). However, increased bone-to-implant contact, gained by increasing surface roughness (surface area) of an implant, may not always increase the biomechanical interaction with the surrounding bone. The character of surrounding bone and the nature of the interface formed bone may be factors in developing a positive biomechanical interaction (Stanford & Brand, 1999).

However, results are inconclusive concerning the best implant surface for obtaining clinical success. A consensus now favors a moderately rough implant surface with average roughness (Ra) values ranging from 1 to 5 mm (Buser, et al., 2004; Schwartz, et al., 2008).

Surface Energy and Wettability

Implant surface properties (surface energy, roughness and wettability) play a significant role in determining the host cellular responses to implant materials (Guo, et al.; He, et al., 2009). The combination of altered microstructure and high surface energy further enhances host cellular response (osteoblast

differentiation) on titanium surfaces. Implant surface wettability indicates the degree of contact with the host physiological environment. Increased wettability improves the interaction between the implant surface and the host biological environment (Elias, Oshida, Lima, & Muller, 2008). Surface energy and hydrophilicity of implant surfaces may play an important role during initial conditioning by proteins and initial cell adhesion. Studies have shown that hydrophobicity of the implant surfaces leads to reduced protein adsorption and insufficient wettability influences the initial conditioning of the surface by blood components and it affects subsequent cellular reactions (Elias, et al., 2008).

Several in vitro and in vivo results showed that, to increase the implant surface area for human osteoblast adhesion, it is necessary to increase the surface wettability (Lim & Oshida, 2001; Lim, Oshida, Andres, & Barco, 2001; L. Yang, Sheldon, & Webster, 2009). Research has also shown that proliferation of the cells increases with increase in surface wettability (Lim & Oshida, 2001; Lim, et al., 2001). Rupp et al. showed that implant surface roughness induces initial hydrophobicity, but subsequently increases hydrophilicity compared to smooth/machined titanium implant surfaces (Rupp, et al., 2006; Rupp, Scheideler, Rehbein, Axmann, & Geis-Gerstorfer, 2004). This initial hydrophobicity was thought to be due to air entrapped in the micro pores that are created to increase the surface roughness/surface area, leading to a heterogeneous surface, which cannot be spontaneously wetted (Rupp, et al., 2004).

However, surface energy of different treated implant surfaces, to enhance wettability to physiological fluid (blood, proteins) in both, in vitro and in vivo conditions are an area of active research.

Novel Concept, Clinical Significance and Hypothesis

With this phase 1 proposal, there is an attempt to develop a new surface, which shows enhanced bioactivity with bone, and thus better integration. The implants will be both chemically modified and blasted with other particles, leading to changes in the micro texture of the implant surface and thus making it more bioactive. Nano indentation will be used to measure the hardness of the integrated bone; a technique that has been used in the past with dog femoral bone integrated to machined endosseous implants. Thus with this technique we will assess the intrinsic biomechanical properties of ex vivo bone tissue integrated to different surface topographies of titanium.

Implant surface morphology (micro geometry and roughness) has been shown to have a significant effect on implant integration, which directly affects anchorage potential. Worldwide market for implant based dental products is forecasted to approach $3.5 billion by the end of 2010. Although only 2% of the global orthodontic market is allocated to mini implants, at a rate of 18% per annum it is believed to be the fastest growing segment. These projections imply that “Anchorage Control” still remains a significant problem. Adaptability, longevity and performance of the mini implants under heavy dynamic loads are an area of consideration.

It is expected that the outcome of this study will provide useful evidence regarding the osseointegration potential of different implant surfaces. The long-term goal of this phase 1 research endeavor is to develop a unique implant surface on existing machined implant that forms strong interfacial bonds with the surrounding bone, while stimulating rapid osseointegration when implanted in vivo for heavy dynamic loads, and acts as a stable source of anchorage. Thus, this phase 1 project will help in making evidence-based decisions on the effectiveness of different surface treated mini-implants. The immediate goals for this project were to understand and evaluate the: 1) osseointegration potential of four different surfaces of mini-implants, 2) mechanical properties of bone integrated to mini-implants, and 3) lack of optimal integration for some devices.

We hypothesize that “Mini-implants surface roughness alters the intrinsic biomechanical properties of the bone integrated to titanium”. The Hypothesis will be tested with following specific aims:

Specific Aim1: Determine if rough surface implants integrate better than machined implants. Integration will be measured both by histomorphometry (bone to implant contact, scanning electron microscopy) and mechanical analysis (removal torque).

Specific Aim2: Determine if the implant surface roughness affects the biomechanical quality of osseointegrated bone, and hardness of bone integrated to treated surfaces of implant is different than bone integrated to machined surface.

Materials and Methods

The primary objective of this phase 1 project was the development of a unique implant surface that could function in harmony and induce rapid biomimetic processes to provide a significant public health benefit.

Implant Surfaces Characterization

All mini implants (Dentaurum Co., Ispringen, Germany) and circular discs (3mm diameter and 3mm thick) (Dentaurum Co., Ispringen, Germany) were made from Titanium alloy Ti6Al4V grade 5. Its composition is C<0.08%, Fe<0.25%, N_2<0.05%, O_2<0.2%, Al=5.5%-6.7%, V=3.5%-4.5%, H2<0.140%, and the rest, titanium. The 6 mm long mini implants were self-drilling. The outer thread diameter was 1.6mm; inner core diameter of shank was 1.3mm (threads are 0.3mm deep). The screw shank and threads are cylindrical for the top 2/3 of the threads, and the lower 1/3 is tapered, Figures 1A and 1B. Machined “mini implant” surfaces were modified by subtractive method, acid etching with hydrochloric acid (HCL), blasting with non-resorbable blasting material (alumina) combination of blasting first with alumina particles and then acid etching with HCL.

On the basis of surface treatment, the implants and the circular discs were categorized into four types: Type-A: Machined- Smooth surface mini implants (no surface treatment). Type-B: Acid etched- Mini implants were acid etched with 0.11mol/L HCL at 65° centigrade for 20 minutes. After etching implants were

dried in an oven for 24 hours. Type-C: Grit blasted- Mini implants were blasted with alumina particles of grain size 25µm-50µm. Type-D: Grit blasted with acid etching- “Mini implants” were blasted first with alumina particles of grain size 25µm-50µm and then etched with 0.11mol/L HCL at 65° centigrade for 20 minutes. After etching implants were dried in an oven for 24 hours.

In Vitro Experiments

Surface Roughness

The implant surface roughness was quantified using an “Optical Profilometer” (Proscan 2000, Scantron, London). Surface roughness parameters (Mean Surface Roughness: Ra and Quadratic Average roughness: Rq) of four discs (3mm in diameter,3mm in height) from each group were measured (2 square millimeter area) two dimensionally in noncontact mode by the optical profilometer, Figures 2A and 2B. The discs (Dentaurum Co., Germany) received the same surface treatment as the “mini implants” (Dentaurum Co., Germany) and were sterilized according to manufacturer’s instructions. Additionally, the surface morphology of two “mini implants” and two discs from each group (Figures 1 and 2) were observed with a low vacuum scanning electron microscope (SEM, JEOL, JSM 5310LV, Japan).

Wettability/Contact Angle Measurement

The contact angle measurement of 3 discs from each group was done with a “Contact Angle Goniometer” (B.P Medical Supplies, Brooklyn, New York, USA). Distilled water contact angle was used as a reference measurement, and the results were compared with the contact angle of liquids with different

hydrophobicity and hydrophilicity: (i) highly hydrophilic liquid NaCl (0.150M NaCl); (ii) lightly hydrophobic dimethylsulfoxide (DMSO); and (iii) human blood. Institutional review board (IRB) clearance (NS1004-08) was obtained for the human blood. Single reading was measured from each disc at room temperature using a droplet of liquid. Height (h) and diameter (d) of the droplet was measured to calculate the contact angle ($\Theta = 2\tan^{-1}(2h/d)$), Figure 3.

In Vivo Experiments

Animal Justification

Rats are the smallest animals in which mini implants can be surgically placed, but rats do not usually display secondary remodeling of bone similar to humans. Therefore, for this phase 1 proposal, adult male New Zealand white rabbits were used. Animal committee approval (IACUC-Indiana University School of Dentistry) was obtained before the placement of “mini implants”.

Methodology

A total of 128 mini implants, differing in surface treatment, were placed into the tibias and femurs of 8 (4 to 5 months of age) male New Zealand white rabbits. On the basis of surface treatment, the mini implants were divided into the four types listed above: Type-A: Machined; Type- B: Acid etched; Type-C: Grit blasted and Type-D: Grit blasted with acid etching.

Each rabbit received a total of sixteen implants, four each in the mid diaphyseal regions of the tibia and the femur of each hind leg. The distance between adjacent implants was 20mm, Figures 4 and 5. Thus the study was

divided into 8 groups according to the surface finish of the implants and their locations (tibia or femur), Table1

Randomization and Mini implant placement

Implants placement was randomized according to the site of placement and type of implant, Table 2.

Type A: Machined

Type B: Grit blasted

Type C: Acid etched

Type D: Grit blasted with acid etching

Anesthetic/Analgesic Procedure

The rabbits were induced with an Acepromazine/Torbugesic (50/50 mixture at 0.15ml/kg given IM, not to exceed 0.45ml total) to tranquilize as a pre-anesthetic. The tranquilizer was administered 30-60 minutes prior to administering profound surgical anesthesia with isoflurane and oxygen. The analgesic Buprenex® (Reckitt Benckiser Pharmaceuticals, Richmond, Virginia, USA) was administered subcutaneously (SQ) 5-6 hours after the pre-anesthetic at a dose of 0.01 to 0.05mg/kg and subsequent doses were administered every 8-12 hours as needed, based on how the rabbit was behaving/acting and eating/drinking.

One dose of the broad-spectrum antibiotic Baytril® (Bayer pharmaceuticals, Pittsburgh, Pennsylvania, USA) at 4mg/kg SQ was given at the time of surgery. For three days after surgery, additional doses of the antibiotic

were administered; the dosage varied for each rabbit, depending on the probability or sign of infection in each rabbit, as determined by the caretakers. Prior to any surgical procedures, local anesthetic solution consisting of 50/50 mixture of 2% lidocaine + 0.5% bupivacaine with a total dose of no more than 1ml per 4.5kg were injected over the area of “mini implant” placement.

Surgery

All procedures were performed under sterile conditions. The rabbit’s legs were shaved using an electric razor, the remaining hairs were removed using Nair® lotion (Church & Dwight Co., Princeton, New Jersey, USA) and the legs were surgically prepared and draped. An incision approximately 5cm in length was made along the medial surface of the femur and the bone surface was surgically exposed by blunt dissection. In the tibia, because of decreased muscle mass and soft tissue thickness, a tissue punch supplied by the manufacturer of the implants, was used to expose the skin and the periosteum. All mini implant preparations (holes) were drilled 20mm apart with an internally irrigated, twist drill of 0.3mm in length and 1mm in diameter. The implants were then screwed into prepared holes. All rabbits were intramuscularly injected with fluorescent intravital bone labels as follows.

Mixing instructions for the bone label dyes

The injectable bone labels were prepared in the Mineralized Tissue Histology and Research Laboratory and stored in a desiccated container. All labels were mixed with sterile water, filtered and sterilized prior to intramuscular injection. Tetracycline is a routine animal medication supplied as IM-250mg per

vial and was combined with 275mg ascorbic acid, 40mg procaine and 46.84mg magnesium chloride for a total weight of 612mg per vial and IV-500mg per vial and was combined with 1250mg ascorbic acid for a total weight of 1,750mg per vial, Table 3. Calcein green was mixed by adding 2/3 of the calculated total fluid amount of 2% Sodium Bicarbonate (pH of 7.4). The dyes were mixed thoroughly with the use of a magnetic stir plate and bar and then adjusted to a pH of 7.4 with hydrochloric acid (HCl) and sodium hydroxide (NaOH), Table 3.

Euthanization

Rabbits were euthanized eight weeks after the surgical procedure by first anesthetizing with a ketamine 25mg/kg, xylazine 5mg/kg mixture and then the administration of B-euthanasia 1ml/4.5Kg IV. B-euthanasia (Schering-Plough Animal Health Corp., Union, New Jersey, USA) is a mixture of active ingredients of sodium salts of phenytoin, pentobarbital and inactive ingredients such as; ethyl alcohol, propylene glycol, rhodamine B, benzyl alcohol (preservative) and purified water. Euthanasia was due to cerebral death in conjunction with respiratory arrest and circulatory collapse. Cerebral death occurred prior to cessation of cardiac activity. The chief ingredient pentobarbital sodium produced rapid anesthetic action, where as phenytoin produced cardiovascular collapse and cerebral death.

After death was confirmed, the femur and tibia of the rabbits were dissected free and each specimen was assigned an identifying number and the principal investigator was blinded. Equal number of specimens (56 each) from the femurs and tibias were used for mechanical testing (Nano-indentation +

Torque) and histomorphometric analysis. Both the histomorphometric and mechanical testing were done separately, but specimens were randomized together. The femur and tibia were divided into blocks of tissue containing an individual implant after the torque testing.

Tissue Preparation for Histomorphometric Analysis

The bone blocks containing the mini implants were fixed in 10% neutral buffered formalin in the refrigerator for at least 48 hours. They were then transferred to 70% Ethyl Alcohol (ETOH) and held until processing was started.

Histology Procedures

The specimens were dehydrated in an ascending series of ethyl alcohol for 8 hours each, cleared in xylene for 2 changes for 13 hours each and infiltrated with methyl methacrylate (MM) for 20 hours. A second change of MM containing 2% dibutyl phthalate was performed 20 hours later in a Shandon Hypercenter XP™ (Shandon; Pittsburgh, PA) automatic tissue processor. Then, the tissues were placed in MM containing 0.5% initiator (Perkodox 16, AKZO; Chicago, Illinois, USA), oriented in their labeled containers and polymerized in a water bath that started at room temperature and gradually increased over 24 hours to 34 C° to polymerize the blocks. The specimens were then 2D x-rayed using the Skyscan® MicroCT (model: 1072 Skyscan®, Aartselaar, Belgium) to determine implant orientation within the bone block. The bone block was ground on model trimmer (Model No: 03413, Tuscon, Arizona, USA) to prepare a flat surface that was parallel to the plane of the mini implants.

The specimen was then mounted onto a plastic slide for further grinding with different grinding papers (K320, K500, K800, K1000, and K1200) on an Exakt® grinding system (Exakt Medical Instruments, Oklahoma City, OK) until one side of the implant was exposed completely, Figure 6A. The exposed side was then polished on the Exakt® grinding machine with a polishing paper and observed under the microscope to quantify scratches. It was then mounted to a second slide using the Exakt light cure resin. The first slide was popped off and then the block was ground on the Exakt until the 2nd side was exposed completely, Figure 6B. Once the section reached the desired thickness (50 to 70microns), the sections were polished as described above and readied for bright field, fluorescent and polarized light microscopy analysis, Figure 6C.

Parameters evaluated

Osseointegration is the structural and the functional contact between the bone and implant. To evaluate the osseointegration potential of the implant, certain gold standard parameters have been consistently used by researchers over the last three decades (Arisan, Anil, Wolke, & Ozer; Calvo-Guirado, et al. 2009; Degidi, Piattelli, Shibli, Perrotti, & Iezzi, 2009; Lian, et al.; Stadlinger, Hennig, Eckelt, Kuhlisch, & Mai, 2003). We used three common histomorphometric parameters to evaluate the histomorphometric and mechanical properties of the integrated bone:

Bone to Implant contact (BIC)

This parameter tells about the actual bone contact with the implant surface. All the sections were stained with toluidine blue (protocol attached) to perform this analysis.

$$\text{BIC\%} = \frac{\text{Implant surface length in contact with osseous tissue}}{\text{Total length of implant}} \times 100$$

Mineral apposition rate (MAR) (Parfitt, 1988a, 1988b)

This parameter tells us about the rate of bone formation with the aid of fluorochrome labeling. It was the distance between the two markers, tetracycline and calcein, divided by the number of days between the administrations of 2 markers (7 days), expressed in µm/day.

$$\text{MAR} = \frac{\text{Width between the fluorochrome labels}}{\text{Number of days}}$$

Scanning electron microscopy of interface:

The interface bone was examined by scanning electron microscopy (SEM, JEOL, JSM 5310LV, Japan). Three randomly selected specimens of each mini implant type in both femur and tibia were analyzed. The area and distance between the bone and the mini implant were measured using the Bioquant osteo® (Bioquant Image Analyses Corporation, Nashville, Tennessee, USA) software.

The histomorphometric parameters were evaluated (at 20X) with a Nikon microscope (Model No: 59920, Tokyo, Japan) and Bioquant Osteo software (Bioquant image analyses corporation, Nashville, Tennessee, USA) using the

appropriate filters for tetracycline yellow and calcein green. All the measurement was done at 20X magnification.

Tissue preparation for mechanical testing

For mechanical testing equal number of specimens were divided both for torque testing and nano-indentaion (Hardness) testing. Torque testing was completed immediately after euthanisation. The block of specimens for hardness measurement were wrapped in physiological saline soaked material and refrigerated.

Torque testing

Removal torque strength measurement (Gauge Tohnichi® model 6BGT, 0-150 N-cm range, Tohnichi Mfg. Co., Tokyo, Japan) was done immediately after the bone was harvested, before sectioning the individual specimen for histomorphometric and nano-indentation analysis. The gauge was positioned on the hexagonal implant head and an increasing torque was applied and removed at the first “give.” The peak torque registered by the instrument was recorded.

Nano-indentaion testing

Polishing procedure

The bone blocks were x-rayed using the Skyscan® MicroCT (model: 1072 Skyscan®, Aartselaar, Belgium) for mini implant orientation within the bone block. The specimens were cut through the center of the mini implant , perpendicular to the bone using the Exakt saw (Exakt® Medical Instruments, Oklahoma City, Oklahoma, USA).The specimen was glued to the polycarbonate

specimen holder using super glue® (Loctite Co., Avon, Ohio, USA), Figure 7. The sectioned specimens were wet-polished on a rotary wheel (Ecomet; Buehler, LakeBluff, Illinois, USA) at 120rpm with 2,400 grit Silicon Carbide papers under gentle pressure. Polishing was continued on a napless cloth (OPChem; Struers, Rodovre, Denmark) with diluted 1ml and 0.3ml alumina oxide pastes (Micropolish C alpha Alumina, Buehler) (Huja, Beck, & Thurman, 2006). Each specimen was examined under the microscope for polishing ability and sonicated in deionized water for 5 minutes.

Nano-indentation Calibration

Fused silica was used as a standard to ascertain calibration of the machine and diamond tip. This was done prior to polishing of the bone specimen so that there was minimal time lapse between polishing of the specimen and start of the indentation procedure, to avoid desiccation of the specimen. The known mean indentation Modulus for silica is 72 gigapascal (GPa). The silica readings suggested that the machine and indenter were properly calibrated.

Indentation protocol

Before starting the indentation on the bone specimen, mini implants were popped out from the bone specimens using a thread. Intravenous bag containing a mixture of distilled water and 0.5mg/mL of gentamicin sulfate (Sigma, Missouri, USA) was connected to the sample tray (contains specimen holder). The liquid flowed into the polycarbonate specimen holder and kept the bone specimens moist during the entire test period. A drip rate of 1 drop/6-8 minutes was adjusted by inserting a needle valve into the intravenous line.

12 indents were made within 200 microns on each side of the implant and an additional 12 indents were made parallel and 1mm away from the first 12 indents on each side of the implants, at a rate of10 nm/second, at room temperature, Figure 8. Software for making the indent was customized for bone (e.g., to allow for adjustment in loading and unloading rates and peak hold times) according to established protocol. The bone was loaded to reach a depth of 500 nm with a Berkovich diamond indenter (Huja, et al., 2006; Rho, Roy, Tsui, & Pharr, 1999; Zysset, Guo, Hoffler, Moore, & Goldstein, 1998, 1999). A 30-second hold period was imposed at each peak depth. The bone was subjected to a trapezoid-shaped load-hold unload cycle. The objective of the 30 second hold period was to allow the settling of the viscoelastic response in the bone specimens (Huja, et al., 2006).

Parameter Evaluated

Micro hardness was calculated as Pmax/Ac, where Pmax is peak load and Ac is the contact area (Hoffler, et al., 2000; Huja, et al., 2006; Zysset, et al., 1999).

Statistical Analysis

The statistical analyses were performed with Statistical Analysis System (SAS software), version 9.2 (SAS Institute, Inc., Cary, North Carolina, USA).

In-vitro

Comparisons between surface treated implant groups for differences in surface roughness measurements (Ra) were made using one-way analysis of variance (ANOVA). The effects of implants group and liquid on contact angle measurements were evaluated using two-way ANOVA

In-vivo

Comparisons among implant surfaces for differences in the study outcomes were performed using repeated measures analysis of variance (ANOVA). The repeated measures models were necessary to account for multiple (4 different types) implants within a bone and two bones (tibia and femur) within a leg from the same rabbit. The ANOVA included factors for implant type, bone type, the bone-by-implant interaction, and left or right side.

Results

Characterization of Mini Implant Surfaces

Machined

Figures 9A and 10A show the surface morphologies of machined implant surfaces. The tool marks were created during manufacture. The unidirectional surface irregularities indicate the direction of the turning process.

Acid Etched

Figures 9B and 10B show the surface morphology of the acid etched implant surface. A fine roughened isotropic surface was noted with regular elevations and depressions, but without any pits.

Grit Blasted

Figures 9C and 10C of grit blasted implant shows highly irregular surface with elevations, depressions, and irregular shaped cavities (pits).

Grit Blasted with Acid Etching

Figures 9D and 10D shows the surface morphology of grit blasted, followed by acid etching, implant surfaces. A much more uniform surface roughness was observed, when compared to grit blasted surface, with numerous elevations, depressions, and micro pits. Compared to grit blasted implants, the pits were more uniform and smaller in size.

Profilometer

The mean surface roughness (Ra) and quadratic average roughness (Rq) of different implant groups are listed in Figures 11A, 11B, 11C, 11D and 12,

Tables 4A and 4B. The acid etched and machined group had significantly lower ($p=0.0001$) mean value of surface roughness and quadratic average roughness than grit blasted and grit blasted and acid etched, Figure 12, Tables 4A and 4B. Grit blasted with acid etching had significantly ($p=0.0001$) lower surface roughness (Ra and Rq) than grit blasted, Tables 4A and 4B. Comparison of acid etched to machined showed no statistically significant ($p=0.35$) differences in mean and quadratic surface roughness values, Figure 12, Tables 4A and 4B.

Contact Goniometer

Implant surface treatment directly affects its wettability. The interaction between group and liquid was significant ($p=0.0002$). The grit blasted with acid etched group had significantly lower contact angle measurements, both for blood and sodium chloride (NaCl) (40.4°, 26.9°) when compared to other three groups ($p\leq0.01$). For both blood and NaCl, acid etched (50.4°, 33.7°) and grit blasted (46.3°, 32.9°) were significantly lower than machined ($p<0.05$), but not significantly different from each other ($p>0.08$). For dimethylsulfoxide (DMSO) and water, all groups were significantly different from each other ($p<0.005$), with grit blasted with acid etching (33.5°, 50.5°) < grit blasted (43.1°, 57.4°) < acid etched (55.8°, 65.5°) < machined (64.3°, 72.2°), Table 5.

For acid etched and machined implant group, all liquids were significantly different from each other ($p\leq0.02$), with NaCl < blood < DMSO < water. For grit blasted, NaCl had significantly lower contact angle measurements than the other liquids ($p=0.0001$), and DMSO and blood were significantly lower than water ($p=0.0001$), but DMSO and blood were not significantly different from each other

(p=0.16). For grit blasted with acid etching implant group, all liquids were significantly different, with NaCl < DMSO < blood < water, Table 5.

Removal Torque

The side (right vs. left) of the rabbit was not a significant factor (p=0.86) for the removal torque. There was no significant (p=0.67) interaction between bone type and implant surface. Torque was significantly higher in the femur than the tibia (p=0.0062) for all four different types of implants. Grit blasted with acid etching had significantly higher torque than grit blasted (Tibia: 13.67>9.07N-cm; Femur: 18.21>14.12N-cm) (p=0.0075), acid etched (Tibia: 13.67>9.78N-cm; Femur: 18.21>12.87N-cm) (p=0.0035), and machined (Tibia: 13.67>4.08N-cm; Femur: 18.21>6.49N-cm) (p=0.0001), Tables 6 and 7, Figures 9 and 10. Grit blasted (p=0.0009) and acid etched (p=0.0007) had significantly higher torque than machined, but were not significantly different from each other (p=0.82), Tables 6 and 7, Figures 13 and 14.

Bone to Implant Contact

The side (right vs. left) of rabbit was not a significant factor for the bone-to-implant contact outcome (p=0.37). There was no significant interaction between bone type (femur or tibia) and mini implant surface (p=0.70). The femur and tibia did not have significantly different BIC% (p=0.87). Grit blasted with acid etching had significantly higher BIC than grit blasted (Tibia: 66.34>53.07%; Femur: 68.94>49.10%) (p=0.0003), acid etched (Tibia: 66.34>50.64%; Femur: 68.94>48.30%) (p=0.0001), and machined (Tibia: 66.34>39.30%; Femur: 68.94>45.28%) (p=0.0001), Tables 8 and 9, Figures 15, 16, 17A, 17B, 17C and

17D. Grit blasted, acid etched, and machined were not significantly different from each other (p=0.08 for grit blasted vs. machined, p=0.16 for acid etched vs. machined, p=0.74 for grit blasted vs. acid), Tables 8 and 9 and Figures 15, 16 17A, 17B, 17C and 17D.

Scanning Electron Microscopy of Interface

Gap Area

The side (right vs. left) of the rabbit was not a significant factor for the interface area at the bone implant interface outcome (p=0.50). The interaction between bone type (tibia or femur) and implant surface (four different implants) was significant (p=0.0038). For acid etched (p=0.0031) and grit blasted (p=0.0015) surfaces, femur had significantly higher area than tibia, Table 10, Figures 18 and 19. In contrast for machined surfaces, femur had significantly lower area than tibia (p=0.0195). However for grit blasted with acid etching surfaces there was no significant difference in area between femur and tibia (p=0.32). Machined surfaces had significantly higher area than acid etched (p=0.0001), grit blasted (p=0.0001), and grit blasted with acid etching (p=0.0001) surfaces, Table 10, Figures 18,19, 20A-20D, 21A-21D and 22A-22B. Acid etched (p=0.0006) and grit blasted (p=0.0001) surfaces had significantly higher area than grit blasted with acid etching surfaces but were not significantly different from each other (p=0.45), Table 10, Figures 18,19, 20A-20D, 21A-21D and 22A-22B.

Gap Distance

The side (right vs. left) of the rabbit was not a significant factor for the distance at the bone implant interface outcome (p=0.50). The interaction between bone type (tibia or femur) and implant surface (four different implant) was significant (p=0.0172). For acid etched (p=0.0094) and grit blasted (p=0.0090) implant surfaces, femur had significantly higher distance than tibia, Table 1, Figures 23,24,25A-25E and 26A-26B. However for grit blasted with acid etching (p=0.64) and machined (p=0.08) surfaces there was no significant difference in distance between femur and tibia. Machined surfaces had significantly higher distance than acid etched (p=0.0001), grit blasted (p=0.0001), and grit blasted with acid etching (p=0.0001) surfaces, Table 11, Figures 23,24,25A-25E and 26A-26B. Acid etched (p=0.0040) and grit blasted (p=0.0023) surfaces had significantly higher distance than grit blasted with acid etching surfaces but were not significantly different from each other (p=0.59).

Mineral Apposition Rate

The side (right vs. left) of rabbit was not a significant factor for mineral apposition rate outcome (p=0.50). There was no significant interaction between bone type (femur or tibia) and implant surface (p=0.69). The femur and tibia did not have significantly different mineral apposition rate (p=0.73). Grit blasted with acid etching (Tibia: 1.89>1.47μm/day; Femur: 1.86>1.51μm/day), grit blasted (Tibia: 1.78>1.47μm/day; Femur: 1.79>1.51μm/day) and acid etched (Tibia: 1.75>1.47μm/day; Femur: 1.80>1.51μm/day) had significantly different mineral apposition rate from machined group both in tibia and femur, Tables 12 and 13,

Figures 27 and 28. Grit blasted with acid etching, grit blasted and acid etched groups were not significantly different from each other (p=0.062 for grit blasted with acid etching vs. grit blasted, p=0.054 for grit blasted vs. acid etched, p=0.071 for acid etched vs. machined), Tables 12 and 13, Figures 27 and 28.

Hardness

Hardness near the implant

The side (right vs. left) of rabbit was not a significant factor for the hardness near the bone implant interface outcome (p=0.89). There was no significant interaction between bone type (tibia or femur) and implant surface (p=0.28). The femur and tibia did not have significantly different hardness near the implant (p=0.32). Implant surface did not significantly affect hardness near the implant (p=0.31). The hardness near the bone interface for different implant surfaces was, machined (Tibia: 0.51± 0.03Gpa, Femur: 0.44± 0.11Gpa); acid etched (Tibia: 0.54± 0.03Gpa, Femur: 0.61± 0.06Gpa); grit blasted (Tibia: 0.64± 0.06Gpa, Femur: 0.48± 0.15Gpa) and grit blasted with acid etching (Tibia: 0.56± 0.09Gpa, Femur: 0.56± 0.12Gpa), Tables 14 and 15, Figures 29, 30, 31A-31D and 32A-32D.

Hardness away from the implant

The side (right vs. left) of the rabbit was not a significant factor for the hardness 1mm away from the bone- implant interface outcome (p=0.50). There was no significant interaction between bone type and implant surface (p=0.18). The femur and tibia did not have significantly different hardness away from the implant (p=0.07). Implant surface did not significantly affect hardness away from

the implant (p=0.51). There was a significant difference when we compared the hardness near the interface and 1mm away from the interface, far > near, (p=0.0001) for all surface types and both bones. The hardness near the bone interface for different implant surfaces was, machined (Tibia: 0.84± 0.11Gpa, Femur: 0.65± 0.12Gpa); acid etched (Tibia: 0.80± 0.05Gpa, Femur: 0.85± 0.02Gpa); grit blasted (Tibia: 0.89± 0.05Gpa, Femur: 0.70± 0.20Gpa) and grit blasted with acid etching (Tibia: 0.87± 0.07Gpa, Femur: 0.82± 0.04Gpa), Tables 16 and 17, Figures 29, 30, 31A-31D and 32A-32D.

Discussion

Surface roughness of implants has been considered an important parameter for more than a decade because it may influence cell (osteoblast and fibroblast) adhesion, adsorption and differentiation. Mean surface roughness (Ra) is the arithmetic average of the roughness profile; whereas quadratic surface roughness (Rq) is the root mean square deviation of the roughness profile. Suzuki et al.(Suzuki, Aoki, & Ohya, 1997) showed that machined implants usually have a surface roughness between 0.5μm to 1.2μm. Branemark machined implant (stainless steel) had a mean surface roughness 0.5μm - 1μm, but by the late 90's, evidence showed that increased roughness generated more bone response (Albrektsson, et al., 1988; Eckert, Parein, Myshin, & Padilla, 1997). In the present research, machined implants had a mean roughness of 1.17μm. Suzuki et al. (Suzuki, et al., 1997), Albrektsson et al.(Albrektsson, et al., 1988) and Guehennec et al. (Le Guehennec, Soueidan, Layrolle, & Amouriq, 2007)showed that acid treated implants have roughness between 0.54μm and 1.97μm, depending on concentration and type of acid (Hydrochloric acid, sulphuric acid, nitric acid, hydrofluoric acid or a combination of any of these), and etching time. Our implants were etched with 0.11mol/L HCL for 20 minutes, yielding a 1.82μm roughness.

Grit blasted roughness is a function of particle type and size and the blasting pressure. Conventional grit blasted dental implants have mean roughness between 2μm and 6.2μm (David, et al., 1995; Svehla, et al., 2000).

Grit blasted mean Ra = 4.83μm in this study. The roughness (3.64μm in this study) of the grit blasted with acid etching implants depends on the combination of both procedures.

Among the surface properties affecting the quality of bone healing surrounding implants, the micro-rough/ or nano rough surface property is a potential factor for achieving favorable bone implant healing (Trisi, Lazzara, Rao, & Rebaudi, 2002; Trisi, et al., 2003; Wennerberg, Hallgren, Johansson, & Danelli, 1998). Ra values above 1.2μm to 1.5μm are considered favorable for osseointegration (Wennerberg, Albrektsson, & Lausmaa, 1996; Wennerberg, et al., 1998; Wennerberg, Hallgren, Johansson, Sawase, & Lausmaa, 1997). Furthermore, severe roughening of implant surface may lead to peri-implantitis and risk of ionic leakage, thus hindering osseointegration. In the present research, Ra > 3μm for grit blasted and grit blasted with acid etching implants. The Ra value for acid etched and machined group was less than 2μm. There is only one publication involving the Rq of dental implants(Elias, Oshida, Lima, & Muller, 2008) . Rq along with Ra gives an important estimate regarding the surface roughness of the implant surfaces. Rq value can be 20% to 150% more than Ra value.

Surface energy and wettability are usually quantified by the contact angle of liquid with surface (Lim & Oshida, 2001; Lim, Oshida, Andres, & Barco, 2001). Values of contact angles indicate whether an implant surface is hydrophilic or hydrophobic, lesser the contact angle, and more the hydrophilicity. The present research shows that grit blasted with acid etching is the most hydrophilic surface

for all the liquids tested, Table 5. The grit blasted and acid etched group were equally hydrophilic for all liquids tested (statistically insignificant). Machined implant group was the least hydrophilic (contact angle values highest for the liquids tested) group among all the groups, Table 5. Numerous in-vitro and in-vivo studies concluded that, to increase the implant surface area for human protein adsorption and cell (osteoblast and fibroblast) adhesion, it is necessary to increase the hydrophilicity of the implant surface (Brunette, 1999; Brunette & Chehroudi, 1999; Chou, Firth, Uitto, & Brunette, 1998; Schuler, et al., 2009).

Shibata et al. (Buser, et al., 2004; Shibata, Hosaka, Kawai, & Miyazaki, 2002) and Buser et al. (Buser, et al., 2004) concluded that, increased wettability of the implant surfaces can enhance the adsorption of cell adhesion promoting proteins containing an Arginine–Glycine–Asparginine (RGD) sequences on their surfaces. Researchers further stated that increased adsorption of RGD-containing extracellular matrix proteins contributes to cell adhesion and differentiation of osteoblasts (Buser, et al., 2004; Eriksson, Nygren, & Ohlson, 2004; Eriksson, et al., 2007; Shibata, et al., 2002; Yamamoto, Shibata, & Miyazaki, 2005).

Removal torque test have been used consistently over the time, to evaluate osseointegration potential of implants (Buser, et al., 2004; Buser, et al., 1991; David, et al., 1995; Elias, et al., 2008; Feighan, Goldberg, Davy, Parr, & Stevenson, 1995; Gotfredsen, Nimb, Hjorting-Hansen, Jensen, & Holmen, 1992; Guo, Zhou, Rong, Zhu, & Geng). Removal torque has been correlated with the amount of bone in contact with implant, leading to changes in biomechanical

characteristic of the bone implant interface. Implant surface properties are one of the major factors affecting osseointegration, but the mechanism involved in this process has not been clearly elucidated. Theoretically, rough implant surfaces (Ra > 1.5μm) are capable of establishing stronger biomechanical interactions with the peri-implant bone tissue than machined implant surface. The removal torque values obtained in this study are consistent with the results from previous studies (Arisan, Anil, Wolke, & Ozer, 2010; Brama, et al., 2007; Brunette & Chehroudi, 1999; Buser, et al., 2004; Buser, et al., 1991; Calvo-Guirado, et al.; Chou, et al., 1998; Cooper, 2000; Elias, et al., 2008; Feighan, et al., 1995; Gotfredsen, et al., 1992), which have shown a significant increase in bone retention of implants with increasing Ra values, except that in our study, both in tibia and femur, grit blasted with acid etching (Ra = 3.64μm) implants had higher removal torques when compared to those of grit blasted implants (Ra = 4.83μm), Figures 13 and 14. Surprisingly, despite statistically different Ra values, in our study, we were unable to statistically differentiate between the removal torques of acid etched implants (Ra: 1.82) and grit blasted implants (Ra: 4.83μm). Possibly, the blasting material used for developing grit blasted implants, alumina (Al_2O_3), often remains embedded in the implant material, even after the ultrasonic cleaning of the implants, and these alumina particles are released into the surrounding bone and interferes with osseointegration (Le Guehennec, et al., 2007).

This problem can be overcome by passivating the implant surfaces using different acids, and probably this could have been the main reason of getting

higher removal torque values in the grit blasted with acid etching implants.. Another possible reason could be enhanced osteoconductive (migration and differentiation of osteoblasts precursor) process through the attachment of fibrin and osteogenic cells, resulting in bone apposition on the surface of the acid etched implant, when compared to grit blasted implant having different Ra values (Davies, 1996, 1998, 2003, 2007).

Previous studies showed that the strength of the bone-implant interface of rough surface titanium is greater than that of a relatively smoother machined implant and results in more stable bone-implant interface (Feighan, et al., 1995; Goldberg, Stevenson, Feighan, & Davy, 1995; Martin, et al., 1995). The present data favors these conclusions i.e. all the rough surface implants (Ra > 1.5) were having significantly higher removal torque than the machined surface implants. Some studies have even speculated that higher biomechanical fixation of rougher surface implants, compared to machined surfaces, was primarily due to mechanical interlocking between the implant surface and the surrounding bone.

However, it is very difficult to compare studies, particularly because the techniques used for altering the surface topography (different types of acid used, particle size of alumina, different types of particles used for blasting, blasting pressure) of machined implant vary considerably, and even more, the techniques used for surface topographical characterization (2D (Ra) Vs. 3D (Sa), laser profilometer Vs. optical profilometer) vary considerably; hence, a surface that is termed rough in one study may be termed smooth in another. In reality, even a machined surface may vary considerably in roughness as is the case for grit

blasted, acid etched, and a combination of grit blasted and acid etched. Even more, the animals used in studies are different (changes the healing process, bone remodeling activity, cortical to trabecular bone ratio) and the surgical techniques of placing the implants vary from study to study.

A prerequisite for a successful integration is the establishment of a direct bone-to-implant contact without the interposition of fibrous tissue. For clinical success with mini implant assisted treatment, a direct contact between implant and surrounding bone is necessary. Research has shown that the specific surface properties of implants may have an impact on the adsorption of proteins and subsequently the initial regulation of cell adhesion (Davies, 1996, 1998, 2003, 2007). Additionally, it has also been shown that the surface properties of implants control the type of tissue, which develops at the bone-implant interface (Curtis & Wilkinson, 1997; Eriksson, Lausmaa, & Nygren, 2001; Nygren, Eriksson, & Lausmaa, 1997). Buser et al. (Buser, et al., 1991) suggested a tendency for an increased bone-to-implant contact with increasing roughness/ or changing the micro topography of the implant surface. In contrast, London et al.(London, Roberts, Baker, Rohrer, & O'Neal, 2002) and Novaes et al. (Novaes, Souza, de Oliveira, & Souza, 2002) did not find any significant change in bone-to-implant contact with different surface treated implants, but treatments that added roughness to the implant surface were having superior bone-to-implant contact, than found for the machined surface. Other studies did not report any significant effect between rough surfaced and machined implants (Gotfredsen, et al., 1992; Vercaigne, Wolke, Naert, & Jansen, 1998a, 1998b, 2000a, 2000b).

This research showed significantly higher bone-to-implant contact with grit blasted with acid etching implant when compared to the other three implant surfaces, Figures 15, 16 and 17A-17D. It is important to observe that increased bone-to-implant contact on grit blasted with acid etching implants compared to the other three implants was due to direct bone apposition along the implant surfaces, as evident by toluidine blue staining, Figures 17A, 17B, 17C and 17D. There were no statistical differences among the other three groups of implant, but numerically both in tibia and femur machined group had least bone-to-implant contact.

Another possible reason for the grit blasted implant with highest Ra could be, leaching of alumina particles either during the attachment of fibrin clots (necessary for the release of growth factors) on the implant surface or during the osseoconduction, both of them must have jeopardized the initial healing process (Davies, 2003, 2007). It has been suggested that vascularization and initial stabilization of implants play essential roles in the early stages of peri-implant wound healing (Long, Robinson, Ashcraft, & Mann, 1995; Reilly, Seldes, Luchetti, & Brighton, 1998).

However, whether the increased stability of rough surfaced implants is due to mechanical interlocking, increased contact, or modified bonding, or a combination of these, is still controversial and unknown. Removal torque is a dynamic test of the three dimensional (3D) relationship between implant and bone, but bone-to-implant contact measurement is a two-dimensional static

parameter. Thus, more research is needed to exactly determine the parameters evaluating the 3D bone structure relationship to adjacent implant.

In this study, osseointegration of machined implants and implants treated with different subtractive procedures were investigated at the ultra structural level using scanning electron microscopy (SEM). Ultra structural analysis of the bone-implant interface revealed significant differences between the machined and surface treated implants. Grit blasted with acid etched group had relatively small gap area and gap distance at the interface i.e. the bone was closely approximated to the implant, whereas with our interface analysis (gap area and gap distance), we were not able to differentiate between the acid etched and grit blasted groups, Figures 18, 19, 23 and 24. Our interface results are consistent with our bone-to-implant contact results and we speculate these may be due to, increased surface area of treated implants, which promote the attachment of fibrin clot (affecting the osseoconduction), and thus bone apposition. Our SEM analyses are in agreement with the findings of previous studies, confirming that the surface roughness positively influences bone integration(Schupbach, et al., 2005; Sennerby, Dasmah, Larsson, & Iverhed, 2005).

Mineral apposition rate (MAR) is the amount of bone formed per day. Implant micro roughness is an important parameter for bone response, but it may not be the only factor. Osseointegration and bone formation at the bone-implant interface is accomplished by the recruitment of mesenchymal cells by growth factors and cytokines, and the terminal differentiation of these cells into mature osteoblasts (Eghbali-Fatourechi, et al., 2005). Implants with surfaces that present

retentive features have both, increased protein adsorption, as well as osteoblast adhesion (Thevenot, Hu, & Tang, 2008).

Bone response to an implant surface can be attributed to the physiochemical and micro/nano roughness properties of the surface. The MAR results show statistically significant differences between the rough surface and machined implants, but we were not able to statistically differentiate between the different rough surfaces. We speculate that more than one factor modulates the bone response, as grit blasted group had a highest mean surface roughness, but MAR for the grit blasted group was less than grit blasted with acid etched group, Figures 27 and 28. Possibly, rate of bone formation was affected both by hydrophilicity and surface roughness of the implant. This study shows that grit blasted with acid etching group has the highest MAR, because it is the most hydrophilic for all the liquids tested and has mean surface roughness of (3.64μm), which may have lead to maximum protein adsorption on the surface and in turn increased osteoblast adhesion, which lays down the bone matrix. Machined implants have a least bone response due to less hydrophilicity and mean surface roughness, Table 5 and Figures 12, 27 and 28.

Hardness measurement at the micro structural level provides material/mechanical properties for individual bone constituents such as lamellae and osteons. The biomechanical properties (especially hardness) of bone integrated to different mini implant surfaces have not been sufficiently addressed in the literature. Hoffler et al. (Hoffler, et al., 2000) stated that the biomechanical properties of bone are primarily determined by the collagen and mineral

deposition. In vitro studies by Takeuchi et al. (Takeuchi, Saruwatari, Nakamura, Yang, & Ogawa, 2005) assessed intrinsic biomechanical properties of mineralized tissue cultured on titanium having different surface topographies, and concluded, that the mineralized tissue on the acid-etched surface shows 3-3.5 times greater hardness than that on the machined surface.

Our research shows that bone hardness near (200μ) the interface is significantly lower than 1mm away from it, both in tibia and femur, regardless of implant surface. It has been reported by Roberts et al.(Roberts, 1988) and Garetto et al. (Garetto, Chen, Parr, & Roberts, 1995) that remodeling activity is observed adjacent to the interface 4-6 months after implantation in rabbits and at 12 months after implantation in dogs. Our findings coincided with these reports, that bone constantly remodels at interface and does not undergo secondary mineralization and that's why lack of hardness adjacent to interface is significantly less than 1mm away from it.

We were not able to find any statistically significant differences in bone hardness (near or at 1mm) associated with the implant groups. Mineral apposition rate data shows significantly more bone formation per day against rough surfaces when compared to the machined group, and our hardness results are in agreement with it i.e. machined group having the least MAR, has the least hardness both in tibia and femur, Figures 27, 28, 29 and 30.

Limitations

Despite the rabbit model's widespread use in implant research, its size (vs. dogs, pigs and sheep) is a major drawback because the number of implants per animal is limited. In addition, its commonly utilized bones (the tibia and femur) possess significantly different macrostructure, especially when compared to the trabecular bone in the human alveolar bones, but resemble cortical bone in human mandible. Thus, direct extrapolation of rabbit study results directly to humans is a challenge and should be carefully performed. As this study was the phase 1 trial, we used rabbit model to elucidate the effect of bone to treated implant surfaces.

Secondly, there is a real need for the development of standardized methods for measuring and characterizing surface roughness and generating defined surface topographies to allow data comparison between different researches.

Conclusions

The range of biomechanical properties that promote an optimal bone-implant interface are not all known, surface roughness is thought to be one of the more important consideration for investigation, therefore with this research we conclude that:

1. The present study indicates that surface roughness parameters (Ra and Rq) were significantly more for Grit blasted implants > Grit basted and acid etched > Acid etched > Machined.
2. Contact angles for liquids tested: Machined > Grit Blasted = Acid Etched > Grit blasted with acid etching.
3. The Removal torque of the mini implants both in tibia and femur were in following order: Grit blasted and acid etched > Grit blasted = Acid etched> Machined.
4. Hardness of bone is significantly lower at the bone implant interface than hardness 1mm away from it.
5. Our histomorphometric results showed a significantly higher percentage of bone-implant contact with the rough surface implant than the machined. It must also be stressed that higher bone-implant contact percentage found around rough surface mini implants could be especially useful in exacting clinical conditions like poor quality bone and early or immediate loading.
6. Mineral apposition rate was significantly greater for the treated mini implants than machined mini implant.

Clinical Extrapolation

1. Grit blasted and acid etched screws can be used as an effective anchor source for orthopedic effects.
2. Rough surfaces mini implants offer better anchorage potential than machined surface mini implants.
3. Avoid placing Grit blasted screws in patients with poor bone quantity and quality (periodontally compromised patients).

Table 1- Depicting the number and type of implant placement in tibia and femur of rabbit

GROUPS	TYPE OF IMPLANT	PLACEMENT SITE	NUMBER OF IMPLANTS
Group-1	Machined	Tibia	16
Group-2	Machined	Femur	16
Group-3	Grit Blasted	Tibia	16
Group-4	Grit Blasted	Femur	16
Group-5	Acid Etched	Tibia	16
Group-6	Acid Etched	Femur	16
Group-7	Grit Blasted and Acid Etched	Tibia	16
Group-8	Grit Blasted and Acid Etched	Femur	16

Table 2- Intra-vital labels administered in rabbits

Labels	Dose	Time of Administration	Time interval	Number of dosages
1)Tetracycline Yellow	10mg/kg	10 days before euthanasia	8hours	2
2) Calcein Green	5mg/kg	3 days before euthanasia.	8 hours	2

Table 3A- Mean surface roughness (Ra) of four different surface treated titanium discs

Group	N	Mean	SD	SE	Min	Max
Machined	4	1.17	0.11	0.02	1.00	1.27
Acid Etched	4	1.82	0.04	0.05	1.77	1.88
Grit Blasted	4	4.83	0.23	0.13	4.59	5.14
Grit Blasted + Acid Etched	4	3.64	0.03	0.03	3.61	3.78

Table 3B- Quadratic surface roughness (Rq) of four different surface treated titanium discs

Group	N	Mean	SD	SE	Min	Max
Machined	4	2.59	0.09	0.02	2.50	2.69
Acid Etched	4	3.17	0.13	0.05	3.09	3.38
Grit Blasted	4	7.04	0.08	0.13	6.96	7.16
Grit Blasted + Acid Etched	4	4.95	0.04	0.02	4.89	4.99

Table 4- Contact angle (°) of four different surface treated titanium discs

Group	Liquid	N	Mean	SD	SE	Min	Max
Machined	Blood	3	55.2	0.3	0.2	54.9	55.5
Machined	DMSO	3	64.3	2.2	1.3	62.0	66.3
Machined	NaCl	3	43.3	3.3	1.9	39.7	46.1
Machined	Water	3	72.2	3.7	2.1	68.5	75.9
Acid Etched	Blood	3	50.4	0.8	0.5	49.7	51.3
Acid Etched	DMSO	3	55.8	1.3	0.8	54.5	57.1
Acid Etched	NaCl	3	33.7	1.1	0.7	32.8	35.0
Acid Etched	Water	3	65.5	3.9	2.2	62.1	69.7
Grit Blasted	Blood	3	46.3	1.8	1.0	44.3	47.6
Grit Blasted	DMSO	3	43.1	3.1	1.8	39.6	45.4
Grit Blasted	NaCl	3	32.9	4.4	2.5	29.2	37.7
Grit Blasted	Water	3	57.4	1.8	1.0	55.4	58.8
Grit Blasted + Acid Etched	Blood	3	40.4	0.7	0.4	39.8	41.1
Grit Blasted + Acid Etched	DMSO	3	33.5	4.4	2.5	30.2	38.5
Grit Blasted + Acid Etched	NaCl	3	26.9	0.7	0.4	26.1	27.4
Grit Blasted + Acid Etched	Water	3	50.5	4.0	2.3	47.2	54.9

Table 5- Removal torque (N-cm) in tibia

Implant Surface	N	Mean	SD	SE	Min	Max
Machined	3	4.08	0.75	0.43	3.43	4.90
Acid Etched	3	9.78	2.58	1.49	6.86	11.77
Grit Blasted	4	9.07	1.98	0.99	7.35	11.77
Grit Blasted + Acid Etched	4	13.67	1.01	0.50	12.50	14.81

Table 6- Removal torque (N-cm) in femur

Implant Surface	N	Mean	SD	SE	Min	Max
Machined	4	6.49	1.23	0.61	4.90	7.84
Acid Etched	4	12.87	2.38	1.06	9.81	15.79
Grit Blasted	3	14.12	0.54	0.39	13.73	14.50
Grit Blasted + Acid Etched	3	18.21	3.81	2.20	15.49	22.56

Table 7- Bone to implant contact (%) in tibia

Implant Surface	N	Mean	SD	SE	Min	Max
Machined	7	39.30	12.79	4.83	19.80	52.10
Acid Etched	8	50.64	14.92	5.27	29.70	71.30
Grit Blasted	9	53.07	14.41	4.80	36.70	78.00
Grit Blasted + Acid Etched	9	66.34	9.12	3.04	55.30	80.90

Table 8- Bone to implant contact (%) in femur

Implant Surface	N	Mean	SD	SE	Min	Max
Machined	6	45.28	10.72	4.38	35.10	61.20
Acid Etched	8	48.30	19.48	6.89	13.00	76.30
Grit Blasted	9	49.10	11.04	3.68	35.90	63.30
Grit Blasted + Acid Etched	9	68.94	7.58	2.40	56.90	83.10

Table 9- Gap area (μ^2) at bone implant interface using SEM

Bone	Implant Surface	N	Mean	SD	SE	Min	Max
Tibia	Machined	3	9211	312	180	8948	9556
	Acid Etched	3	3512	572	330	2922	4064
	Grit Blasted	3	3056	661	382	2363	3680
	Grit Blasted + Acid Etched	3	2214	170	98	2062	2398
Femur	Machined	3	8181	533	307	7639	8704
	Acid Etched	3	5209	848	489	4518	6155
	Grit Blasted	3	5306	229	132	5053	5498
	Grit Blasted + Acid Etched	3	1703	163	94	1542	1869

Table 10- Gap distance (μ) at bone implant interface using SEM

Bone	Implant Surface	N	Mean	SD	SE	Min	Max
Tibia	Machined	3	89.43	5.37	3.10	85.53	95.55
	Acid Etched	3	24.61	6.48	3.74	18.65	31.50
	Grit Blasted	3	24.54	2.52	1.45	21.75	26.65
	Grit Blasted + Acid Etched	3	19.12	1.94	1.12	17.80	21.34
Femur	Machined	3	82.60	4.53	2.62	79.37	87.78
	Acid Etched	3	36.48	3.65	2.11	32.40	39.46
	Grit Blasted	3	38.01	2.06	1.19	35.86	39.96
	Grit Blasted + Acid Etched	3	16.77	2.45	1.42	15.35	19.60

Table 11- Mineral Apposition Rate (μm/day) tibia

Implant Surface	N	Mean	SD	Min	Max
Machined	7	1.47	0.23	1.19	1.76
Acid Etched	8	1.75	0.06	1.73	1.81
Grit Blasted	9	1.78	0.18	1.57	1.93
Grit Blasted + Acid Etched	9	1.89	0.12	1.70	1.99

Table 12- Mineral Apposition Rate (μm/day) femur

Implant Surface	N	Mean	SD	Min	Max
Machined	6	1.51	0.10	1.27	1.65
Acid Etched	8	1.80	0.31	1.44	1.92
Grit Blasted	9	1.79	0.12	1.61	1.89
Grit Blasted + Acid Etched	9	1.86	0.27	1.66	2.03

Table 13- Mean hardness (Gpa) for indents at the bone implant interface in tibia

Implant Surface	N	Mean	SD	SE	Min	Max
Machined	3	0.51	0.03	0.02	0.49	0.54
Acid Etched	3	0.54	0.03	0.02	0.51	0.56
Grit Blasted	3	0.64	0.06	0.03	0.58	0.70
Grit Blasted + Acid Etched	3	0.56	0.09	0.05	0.48	0.66

Table 14- Mean hardness (Gpa) for indents at the bone implant interface in femur

Implant Surface	N	Mean	SD	SE	Min	Max
Machined	4	0.44	0.11	0.05	0.35	0.59
Acid Etched	3	0.61	0.06	0.03	0.54	0.66
Grit Blasted	3	0.48	0.15	0.09	0.37	0.65
Grit Blasted + Acid Etched	3	0.56	0.12	0.07	0.42	0.65

Table 15- Mean hardness (Gpa) for indents at the 1mm distance from bone implant interface in tibia

Implant Surface	N	Mean	SD	SE	Min	Max
Machined	3	0.80	0.11	0.06	0.74	0.96
Acid Etched	3	0.80	0.05	0.03	0.75	0.84
Grit Blasted	3	0.89	0.05	0.03	0.84	0.93
Grit Blasted + Acid Etched	3	0.84	0.07	0.04	0.80	0.92

Table 16- Mean hardness (Gpa) for indents at the 1mm distance from bone implant interface in femur

Implant Surface	N	Mean	SD	SE	Min	Max
Machined	4	0.65	0.12	0.06	0.51	0.81
Acid Etched	3	0.85	0.02	0.01	0.83	0.86
Grit Blasted	3	0.70	0.20	0.11	0.58	0.93
Grit Blasted + Acid Etched	3	0.82	0.04	0.02	0.78	0.86

Figure 1A- Mini implant size and shape

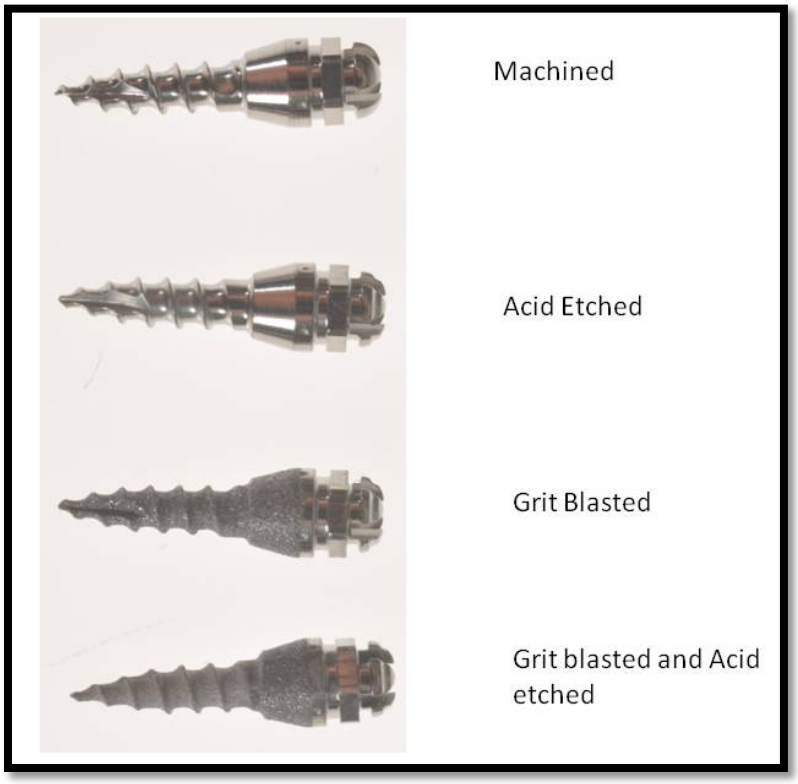


Figure 1B- Four different surfaces of mini implants

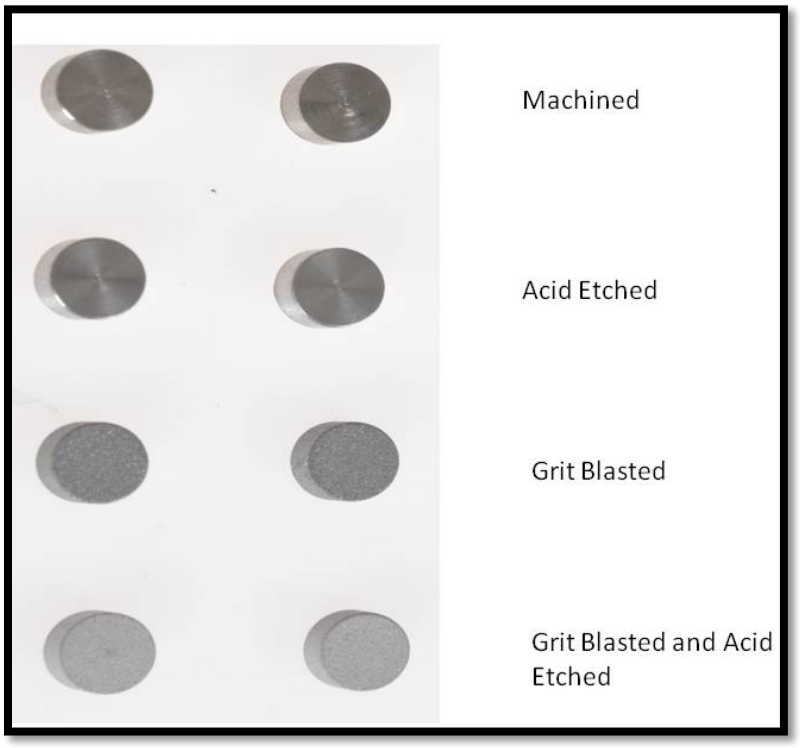


Figure 2A- Four different surfaces of circular discs

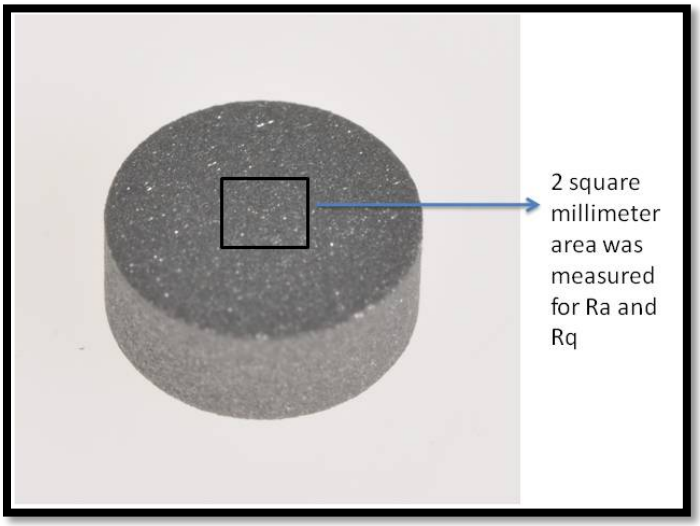


Figure 2B- Area used to measure the roughness parameters

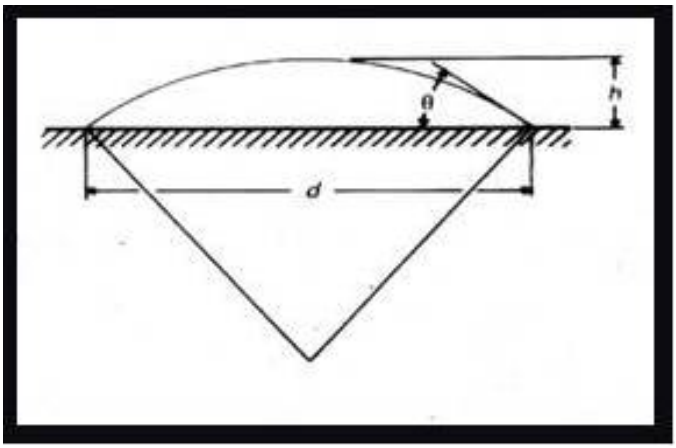
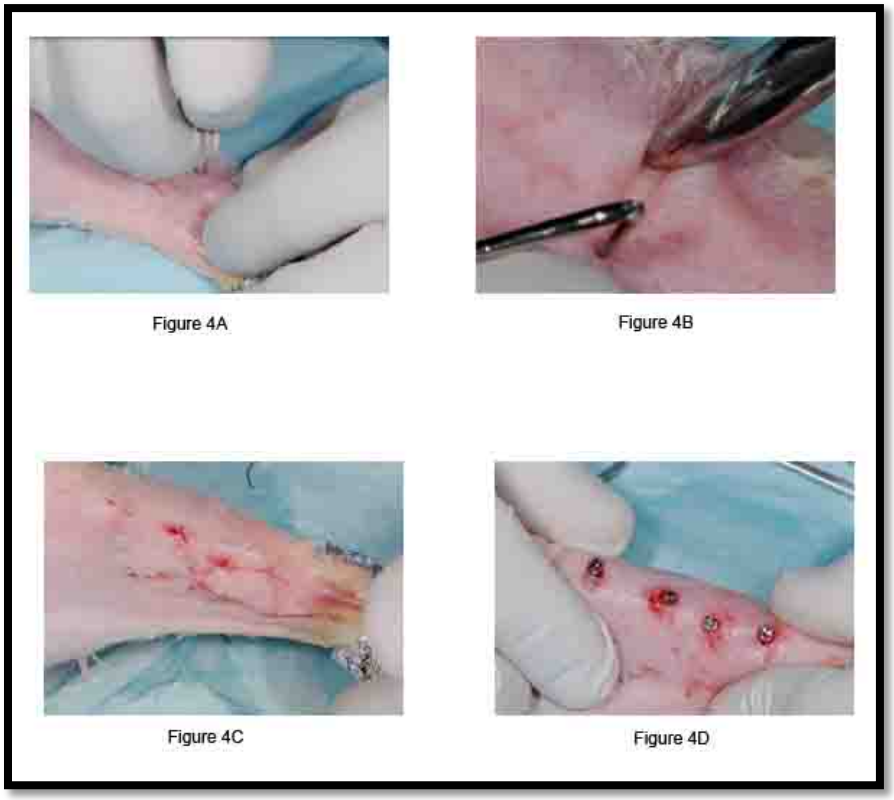
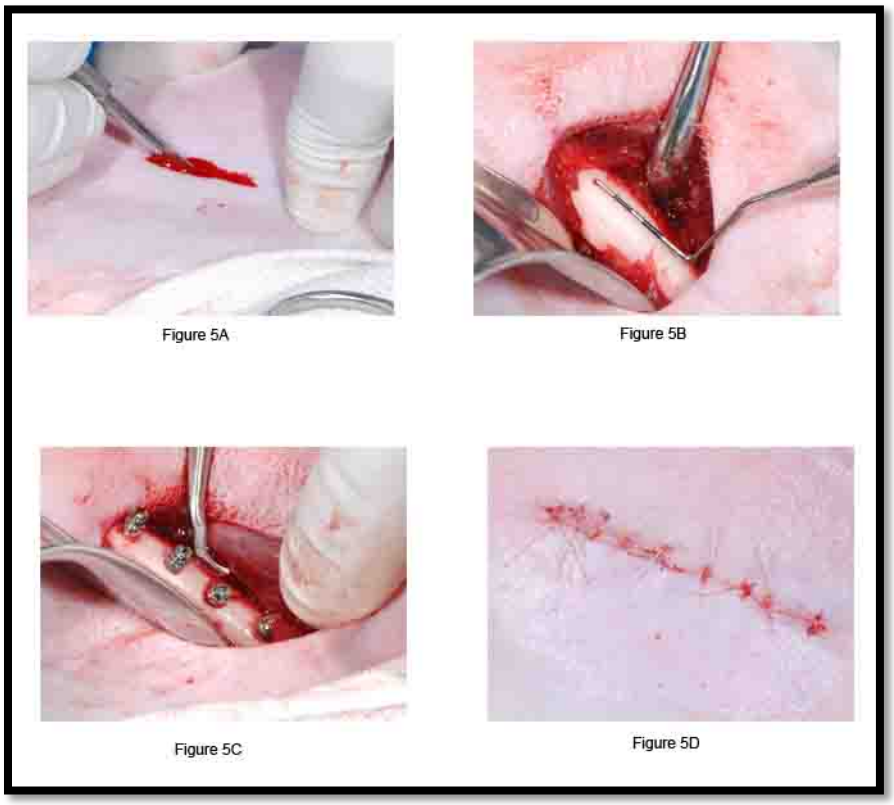


Figure 3- Contact angle measurement of a drop of a liquid



Figures 4A-4D- Surgical placement of mini implant in the tibia of rabbit



5A-5D- Surgical placement of mini implant in the femur of rabbit

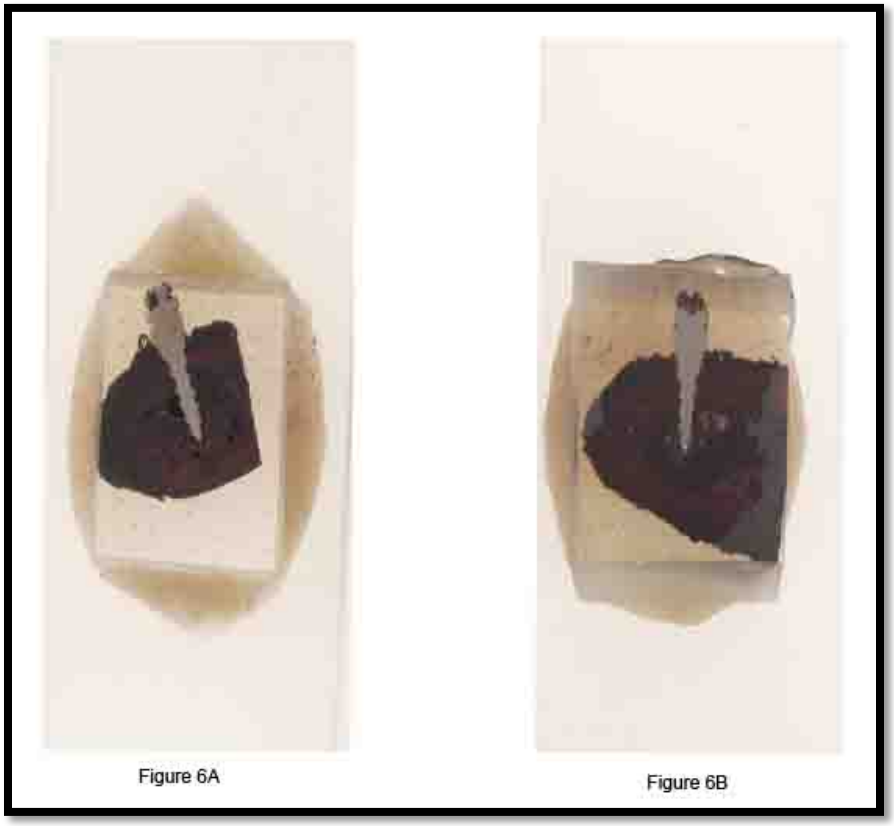


Figure 6A- Completely exposed first side of implant and Figure 6B- Exposed surface of implant mounted on plastic slide using light cure clear acrylic

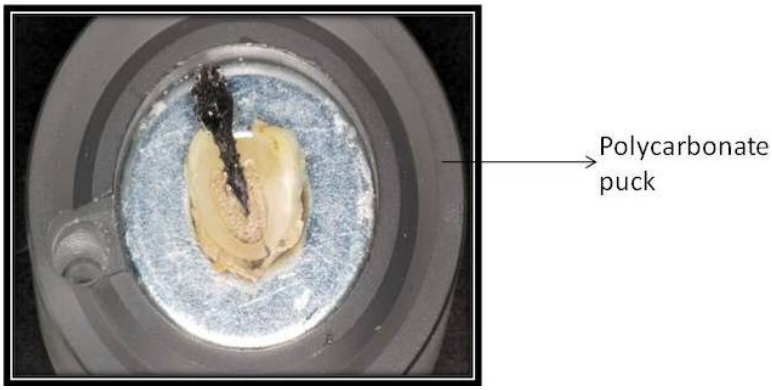


Figure 7- Specimen preparation before micro hardness testing

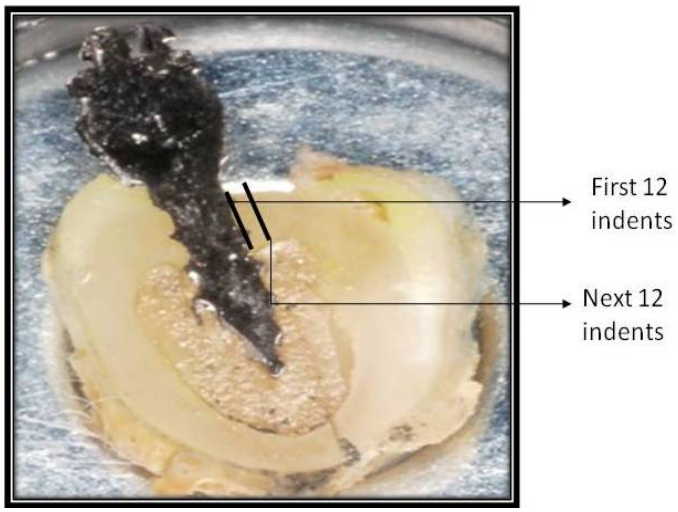
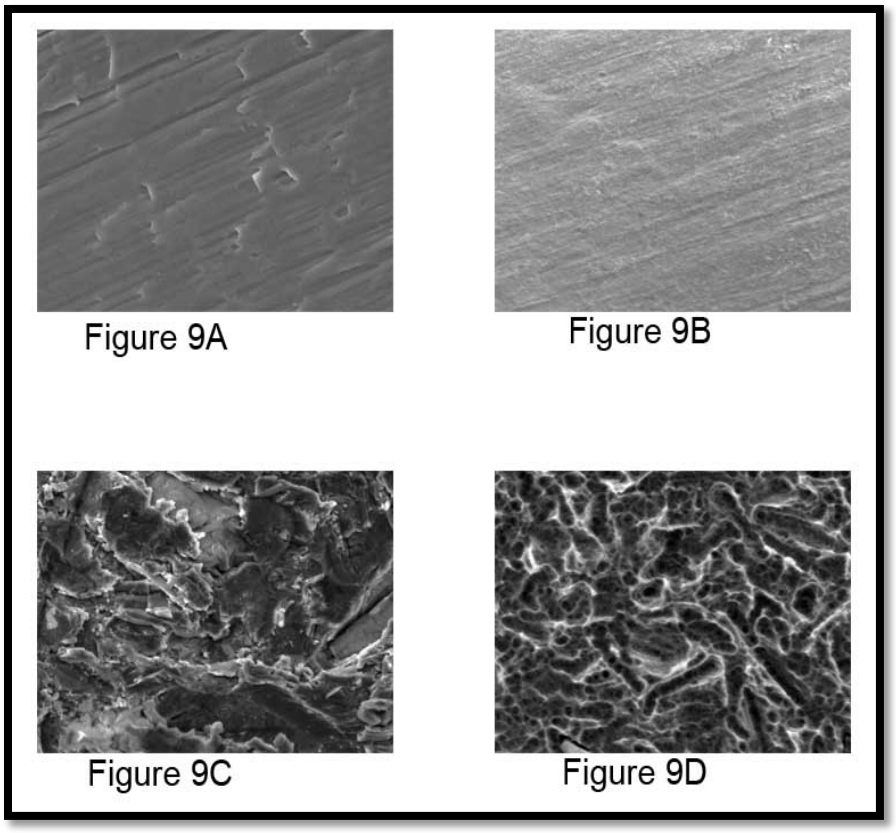
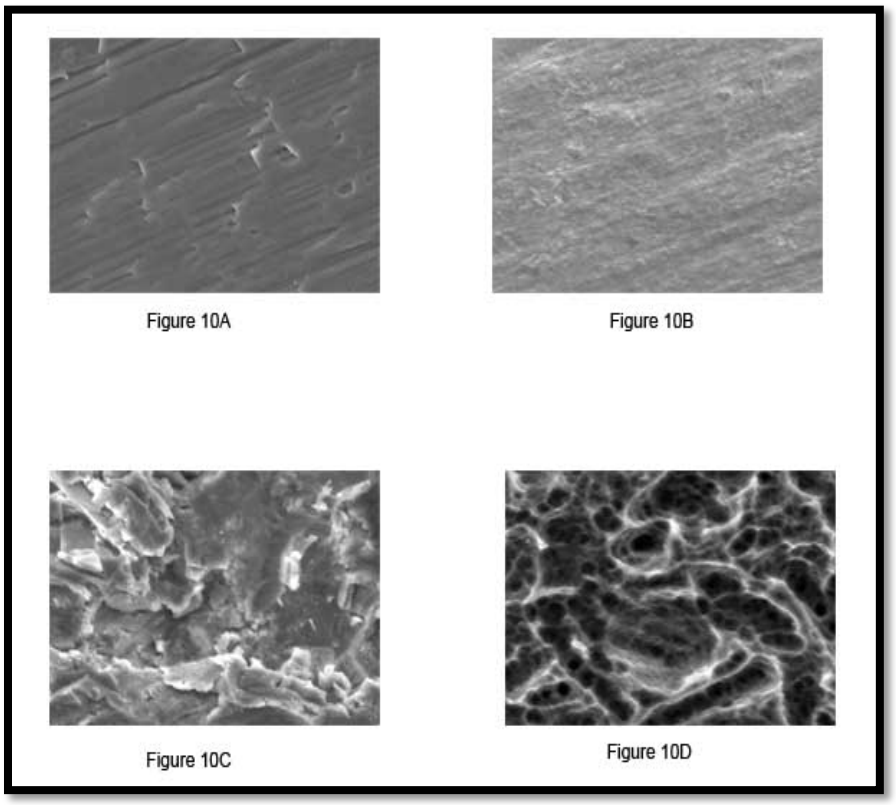


Figure 8- Specimen showing the area of indentation



Figures 9A- Machined; 9B- Acid etched; 9C- Grit blasted;
9D- Grit blasted and acid etched- SEM images of titanium discs at 1000 magnification



Figures 10A- Machined; 10B- Acid etched; 10C- Grit blasted; 10D- Grit blasted and acid etched- SEM images of titanium discs at 2000 magnification

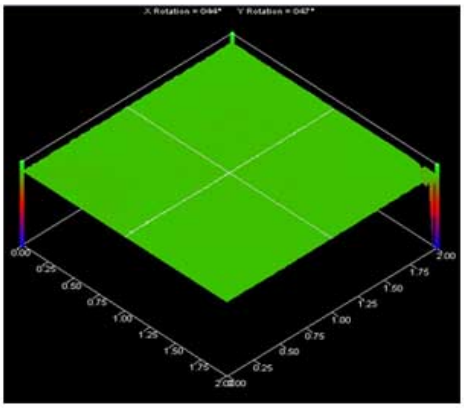


Figure 11A

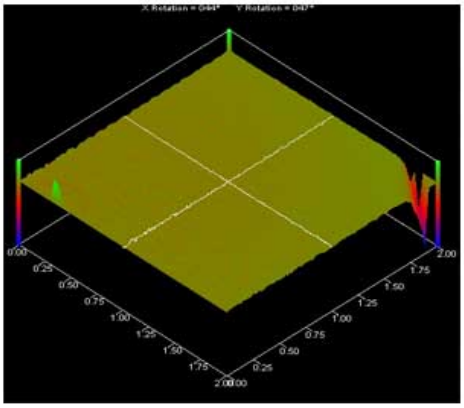


Figure 11B

Figures 11A- Machined; 11B- Acid etched- Profilometric images of titanium discs

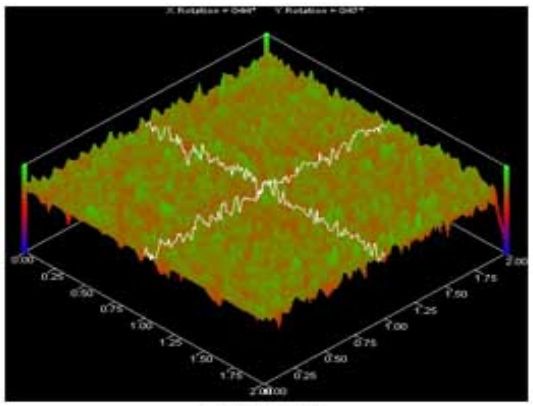


Figure 11C

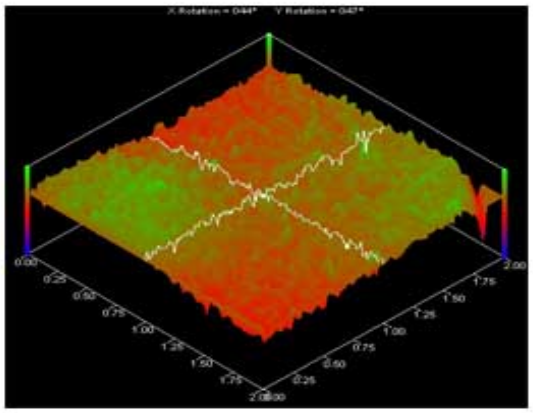


Figure 11D

Figures 11C- Grit blasted; 11D- Grit blasted and acid etched- Profilometric images of titanium discs

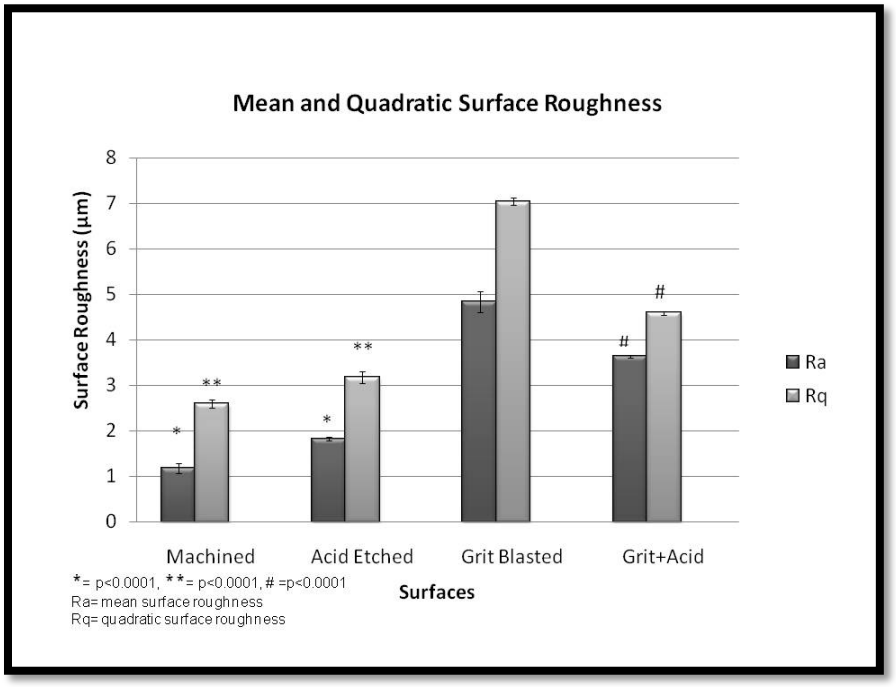


Figure 12- Mean and quadratic surface roughness of 4 differently treated titanium discs

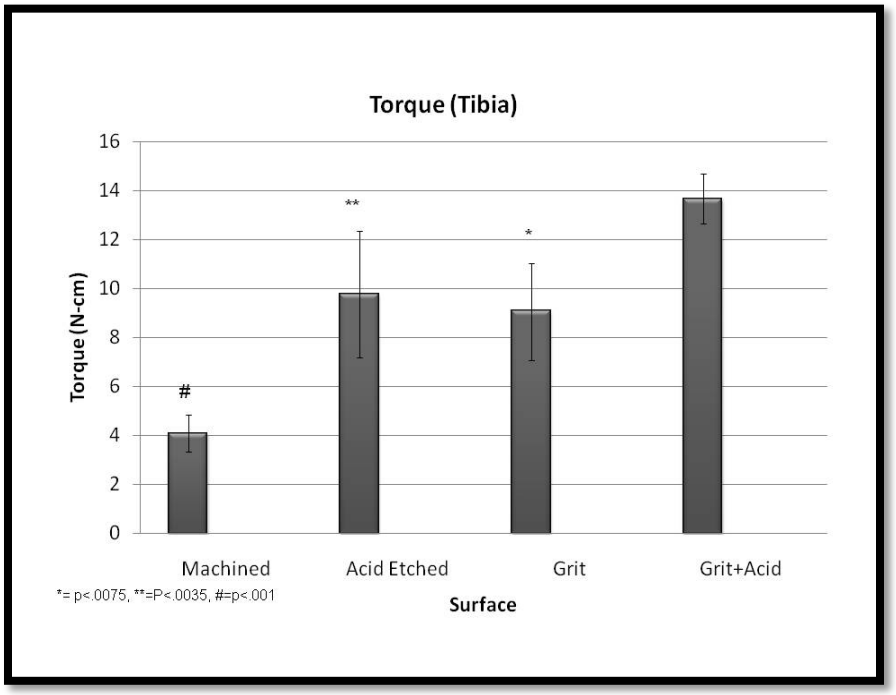


Figure 13- Removal torque of different surface treated implants in tibia

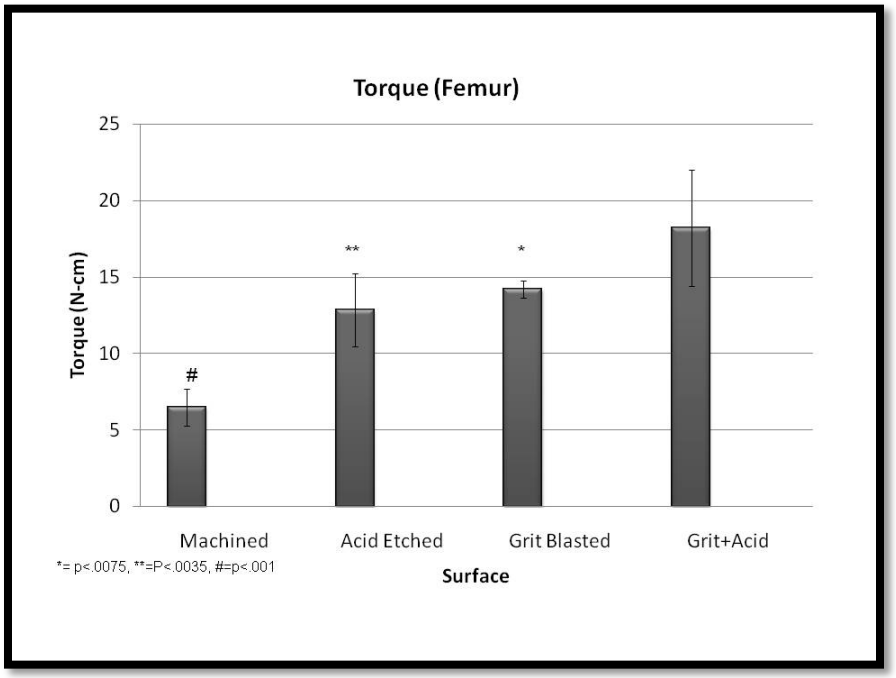


Figure 14- Removal torque of different surface treated implants in femur

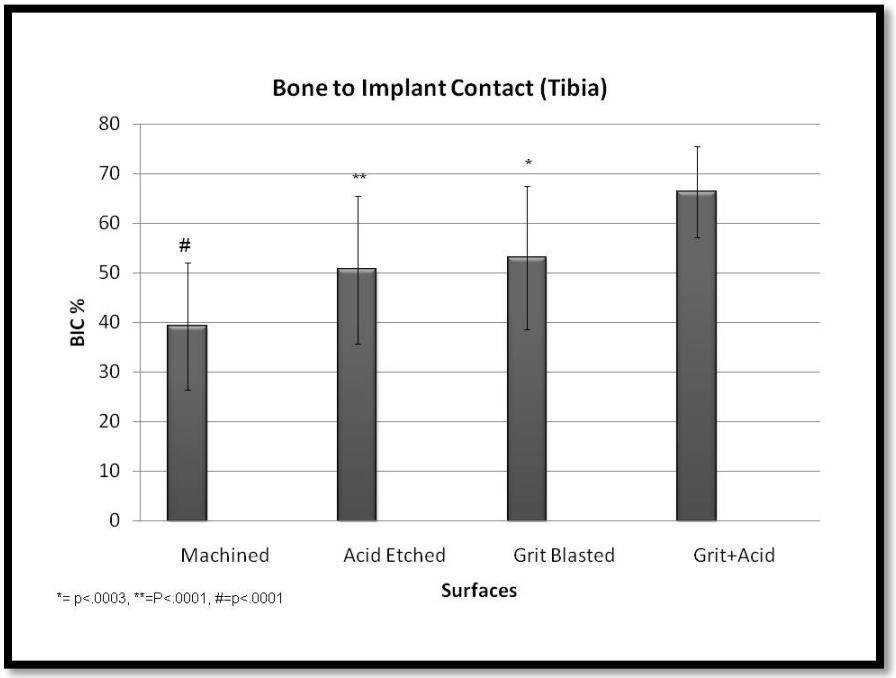


Figure 15- Bone-to-implant contact of different surface treated implants in tibia

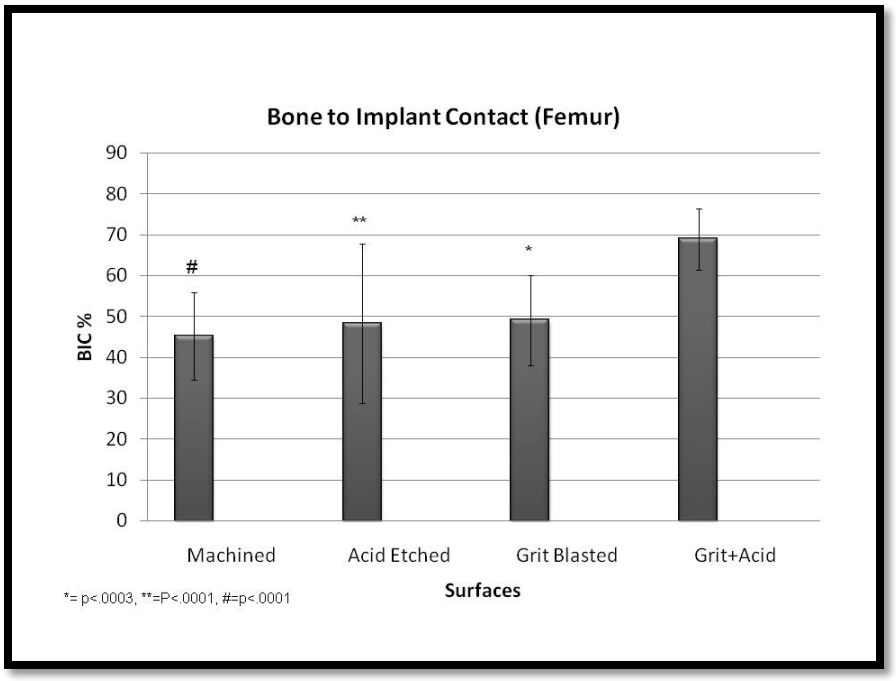


Figure 16- Bone-to-implant contact of different surface treated implants in femur

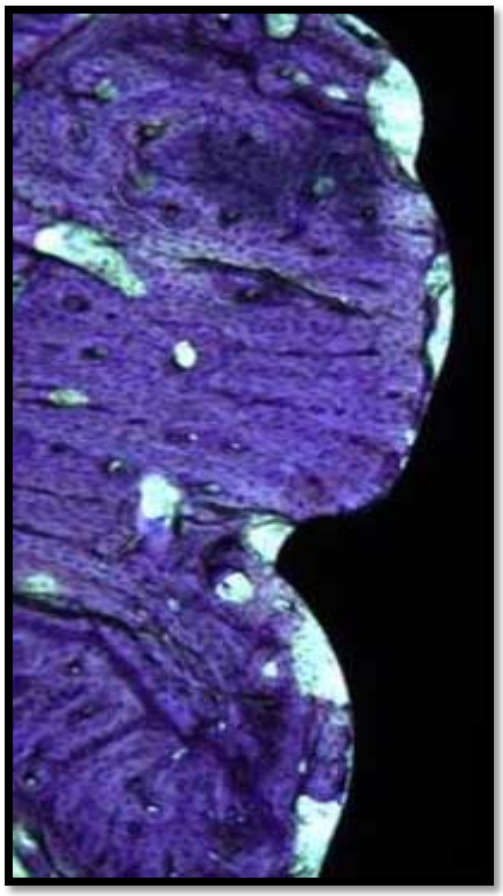


Figure 17A- Bone-to-implant contact of machined implant in femur (toluidine blue staining; original staining X5)

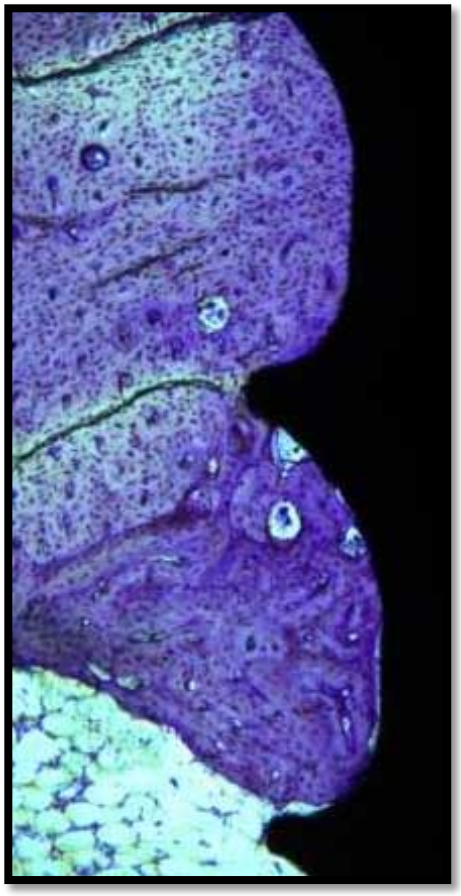


Figure 17B- Bone-to-implant contact of acid etched implant in femur (toluidine blue staining; original staining X5)

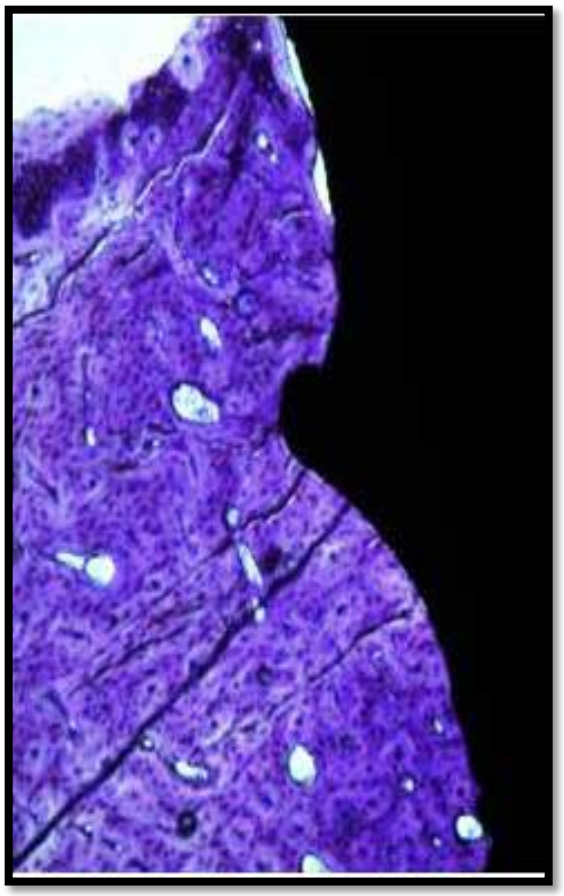


Figure 17C- Bone-to-implant contact of grit blasted implant in femur (toluidine blue staining; original staining X5)

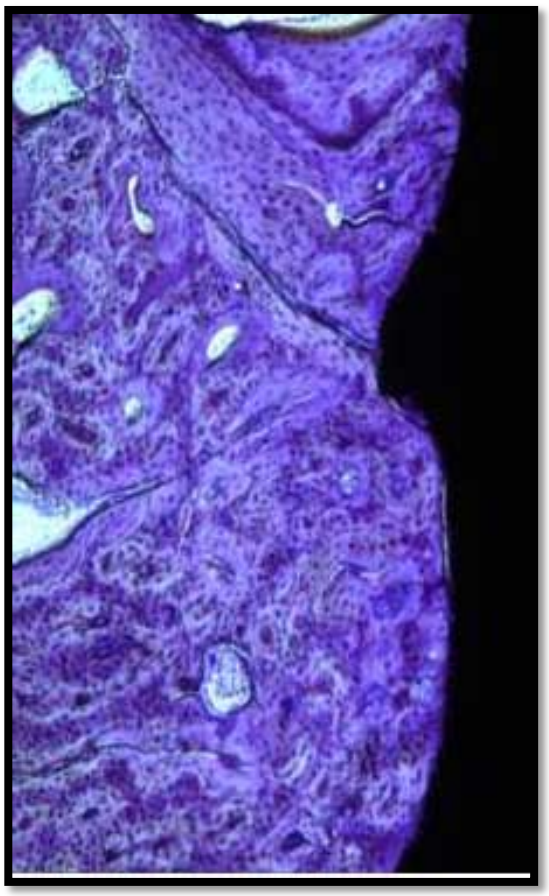


Figure 17D- Bone-to-implant contact of grit blasted and acid etched implant in femur (toluidine blue staining; original staining X5)

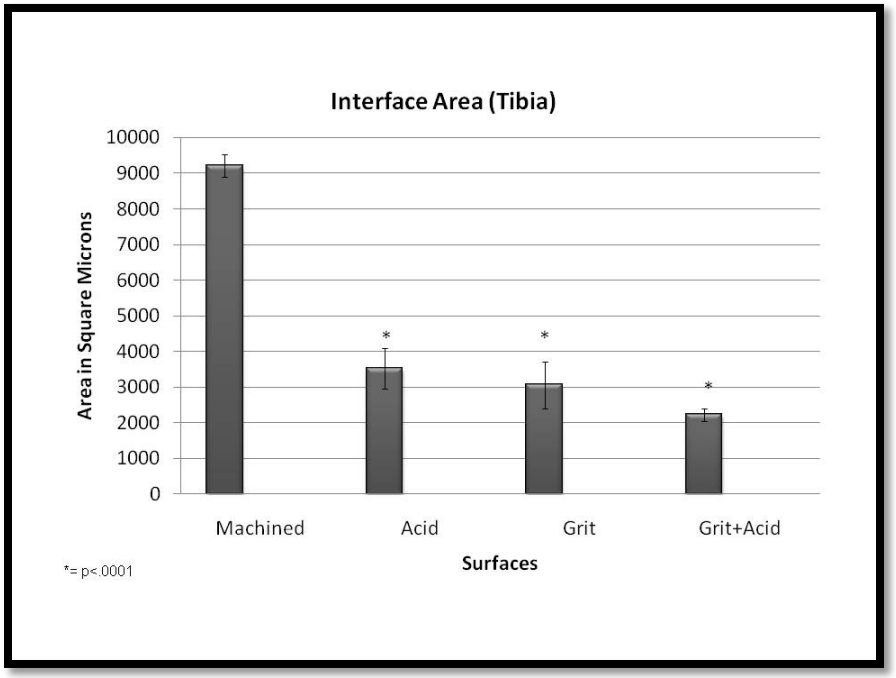


Figure 18- SEM analysis of interface area in tibia

(original magnification X2000)

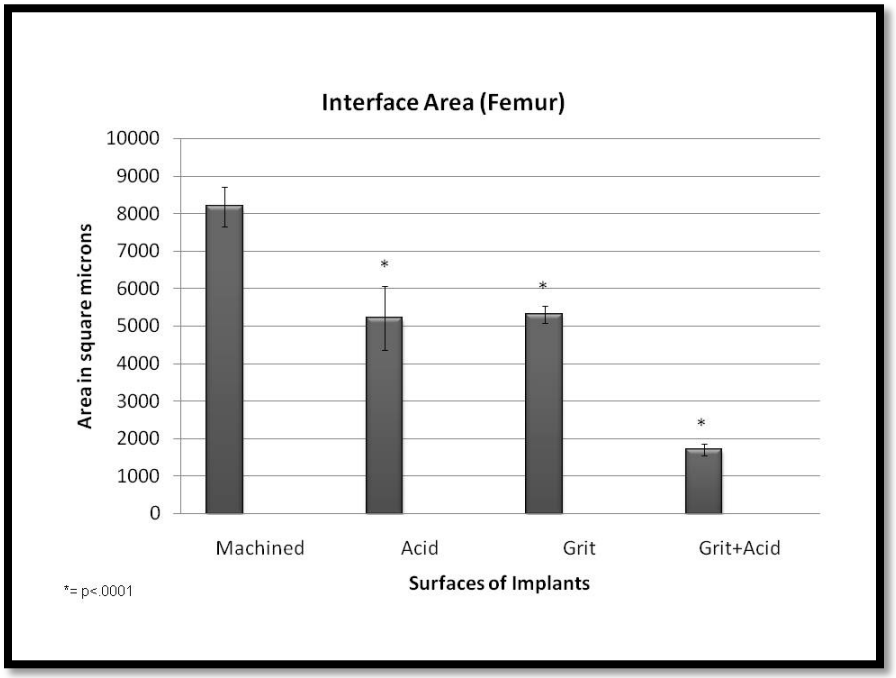
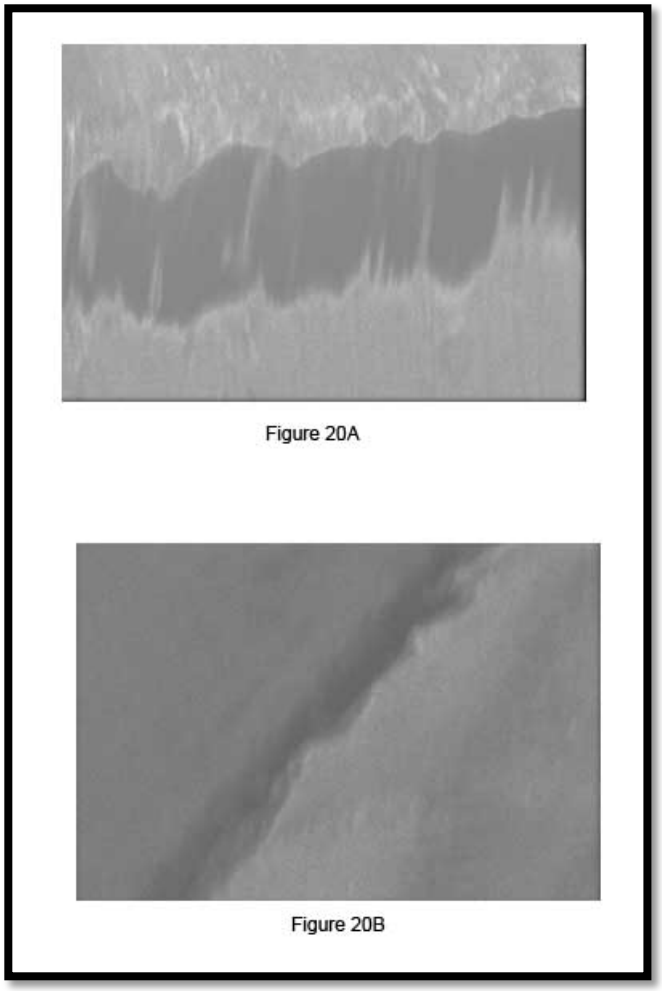


Figure 19- SEM analysis of interface area in femur

(original magnification X2000)



Figures 20A- Machined; 20B- Acid etched - SEM depiction of interface area in tibia (original magnification X2000)

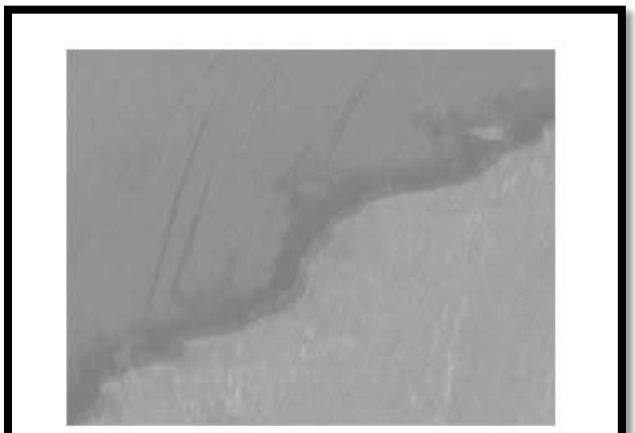


Figure 20C

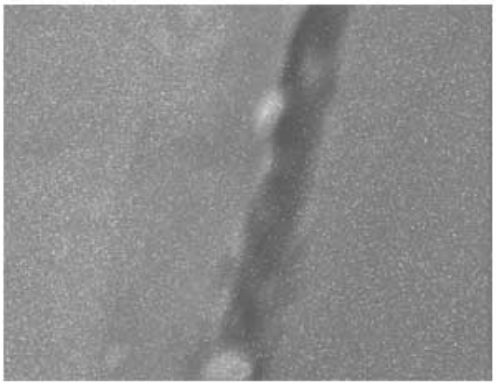


Figure 20D

Figures 20C- Grit blasted; 20D- Grit blasted and acid etched - SEM depiction of interface area in tibia (original magnification X2000)

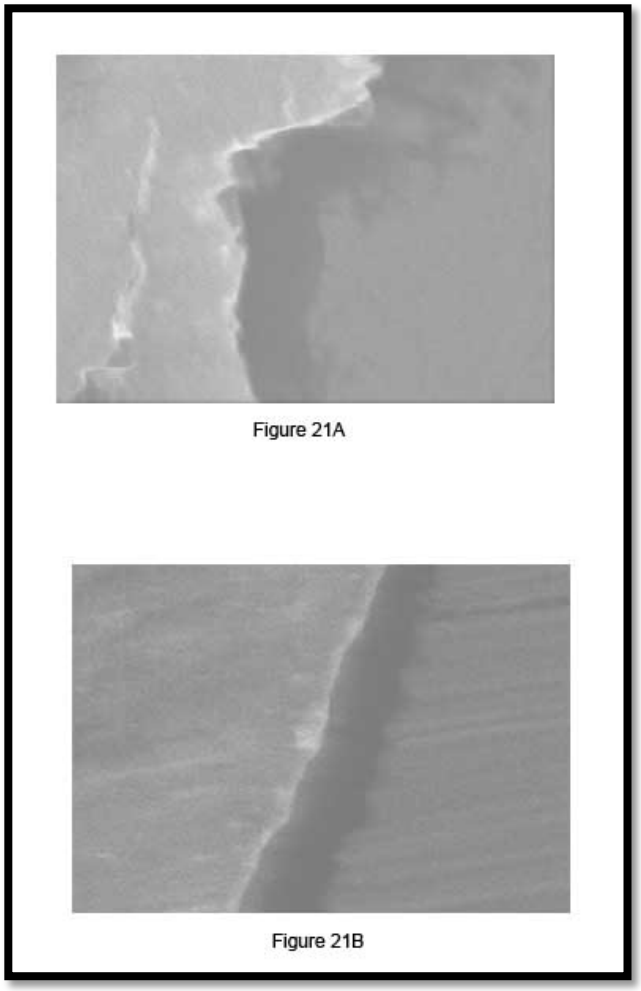


Figure 21A

Figure 21B

Figures 21A- Machined; 21B- Acid etched - SEM depiction of interface area in femur (original magnification X2000)

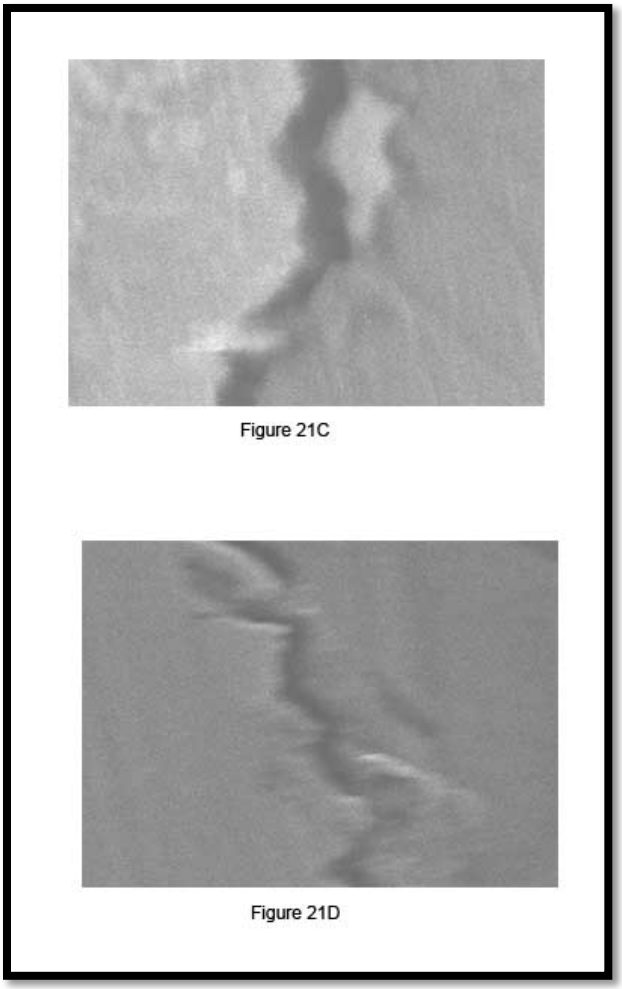


Figure 21C

Figure 21D

Figures 21C- Grit blasted; 21D- Grit blasted and acid etched - SEM depiction of interface area in femur (original magnification X2000)

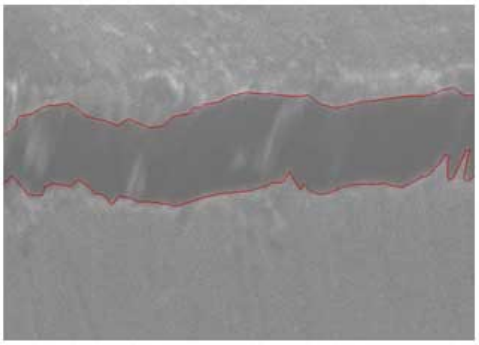


Figure 22A

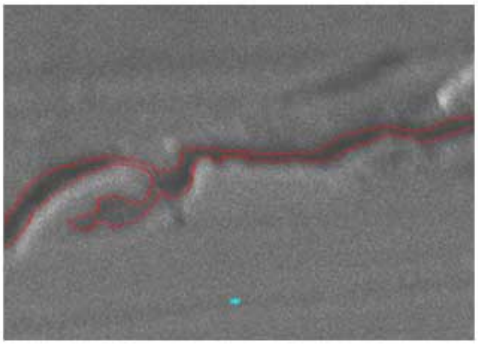


Figure 22B

Figures 22A-22B- Measurement of interface area

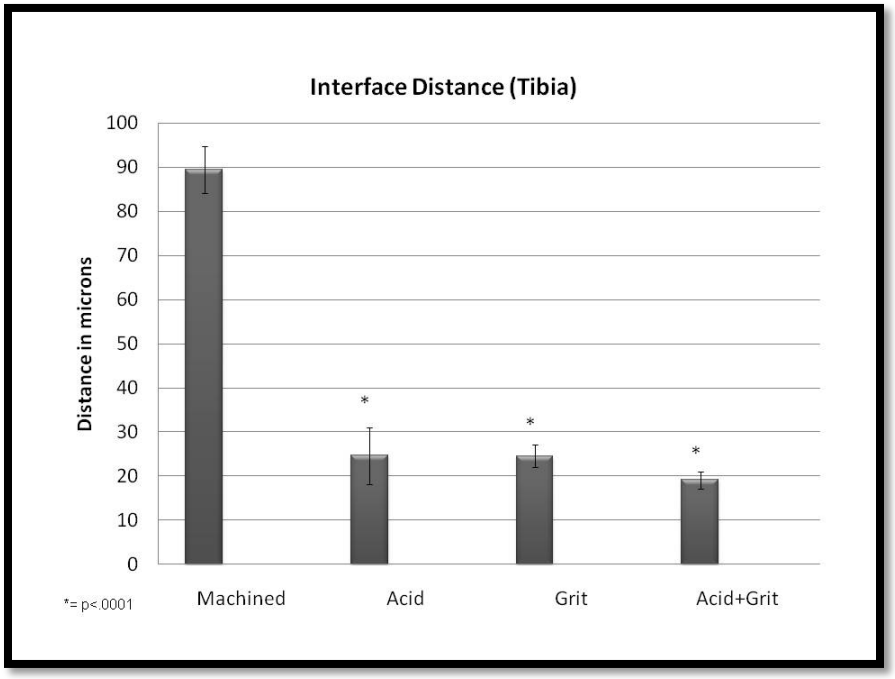


Figure 23-SEM analysis of interface distance in tibia

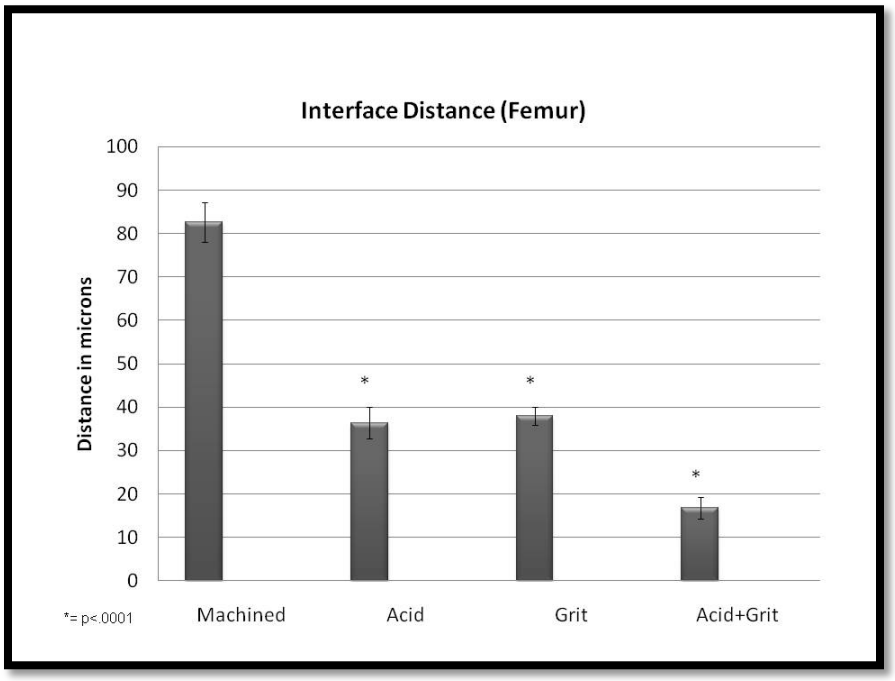
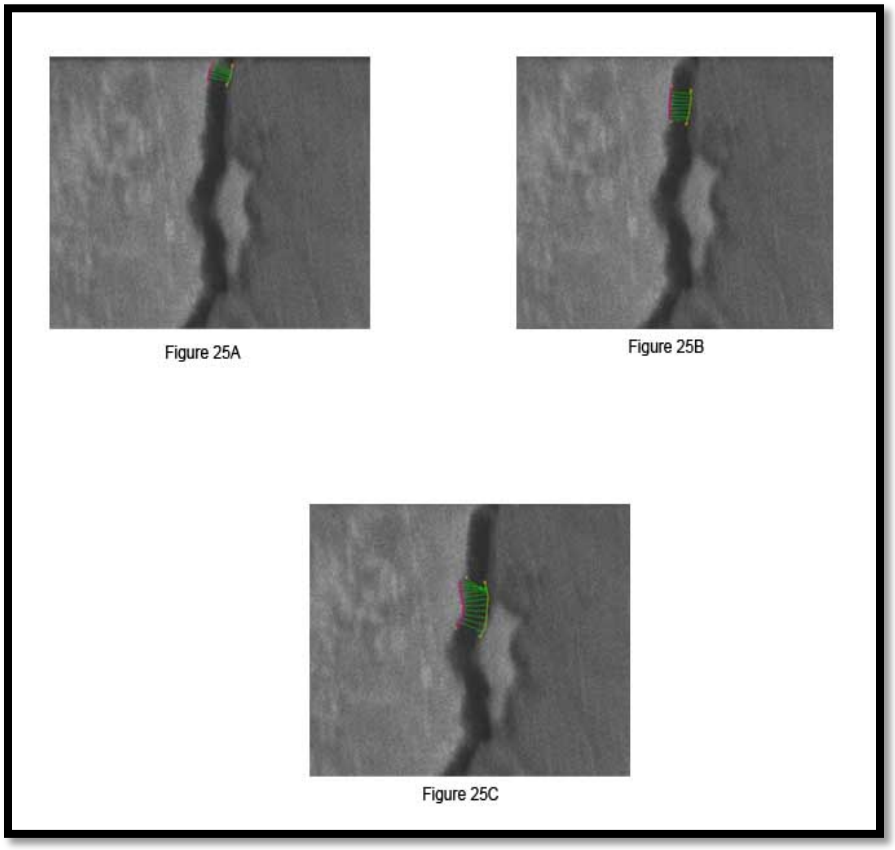


Figure 24-SEM analysis of interface distance in femur



Figures 25A-25C- Measurement of interface distance in tibia

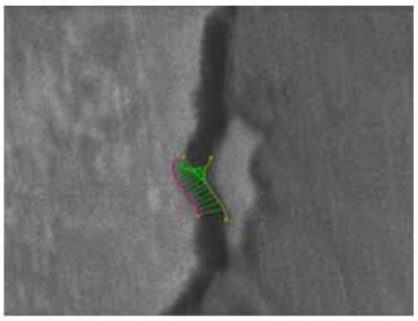


Figure 25D

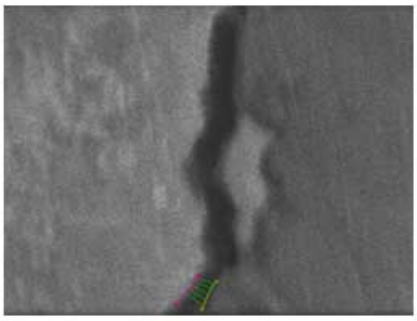


Figure 25E

Figures 25D-25E- Measurement of interface distance in tibia

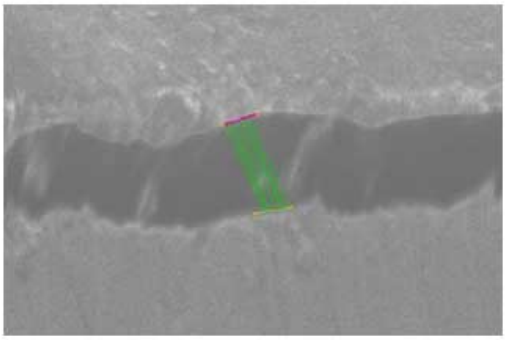


Figure 26A

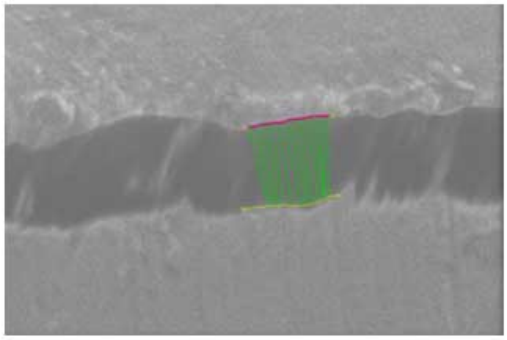


Figure 26B

Figures 26A-26B- Measurement of interface distance in femur

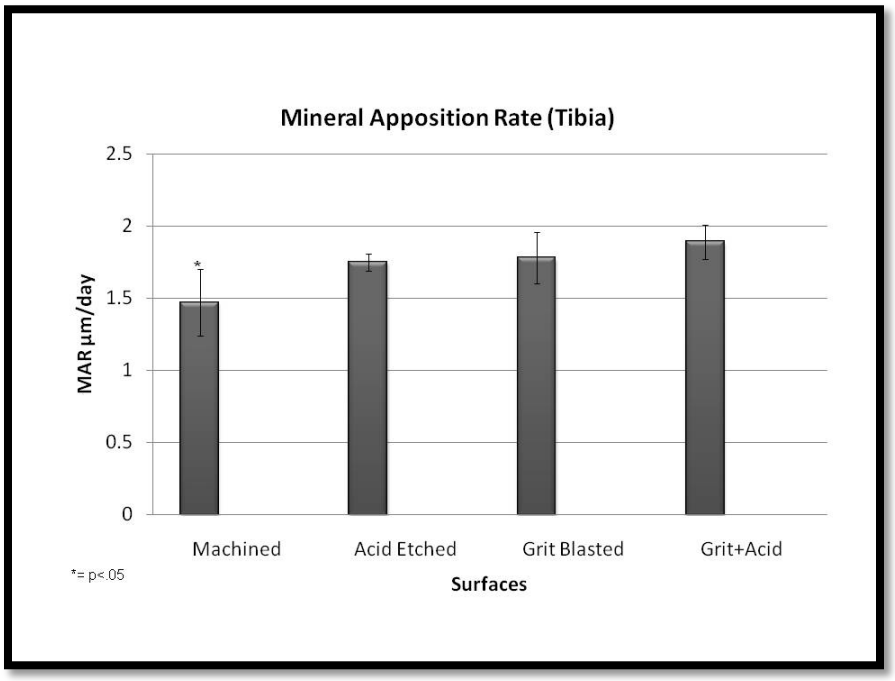


Figure 27- Mineral apposition rate in tibia

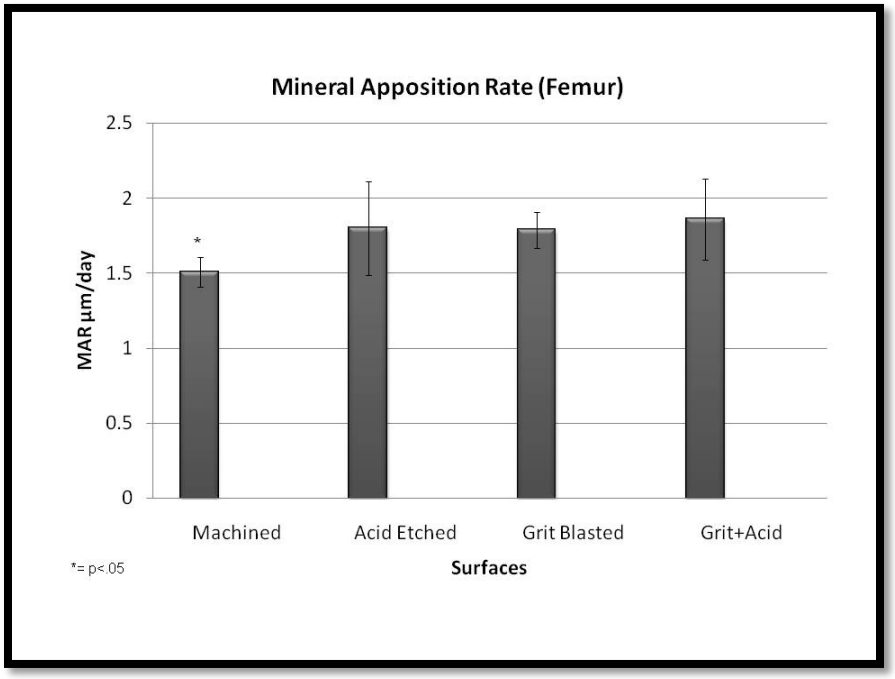


Figure 28- Mineral apposition rate in femur

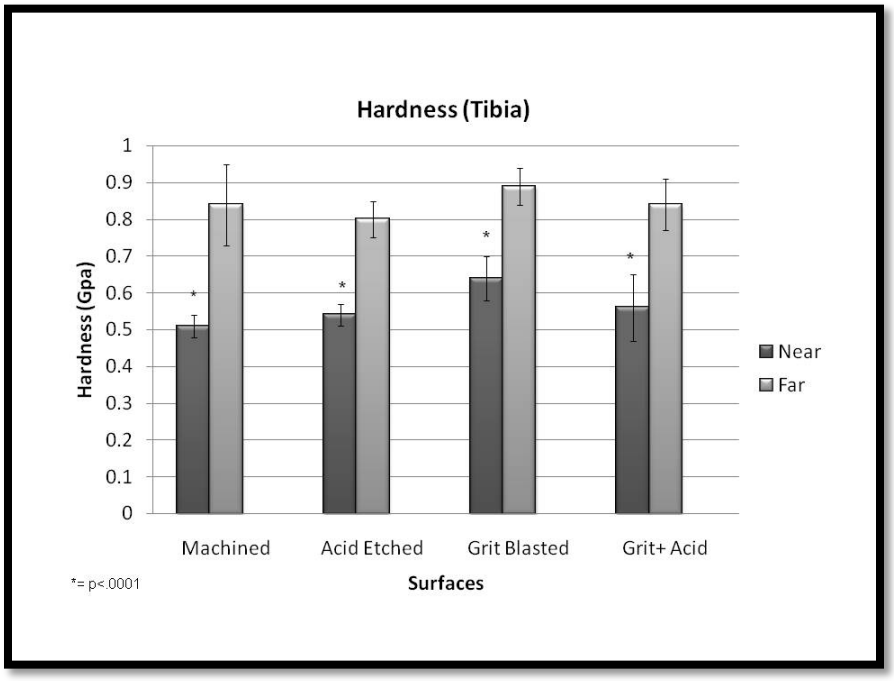


Figure 29- Micro hardness of bone in tibia

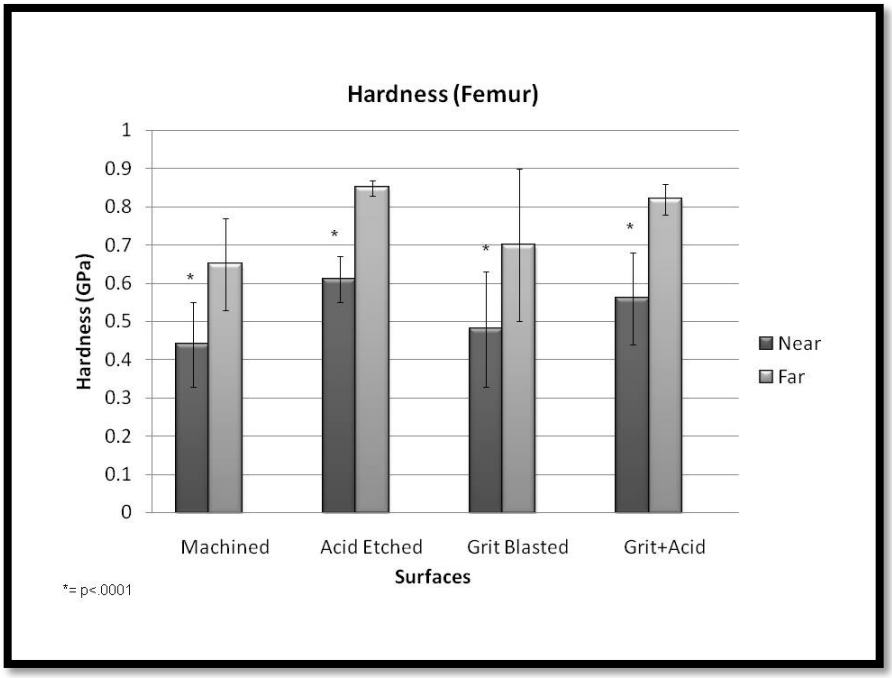


Figure 30- Micro hardness of bone in femur

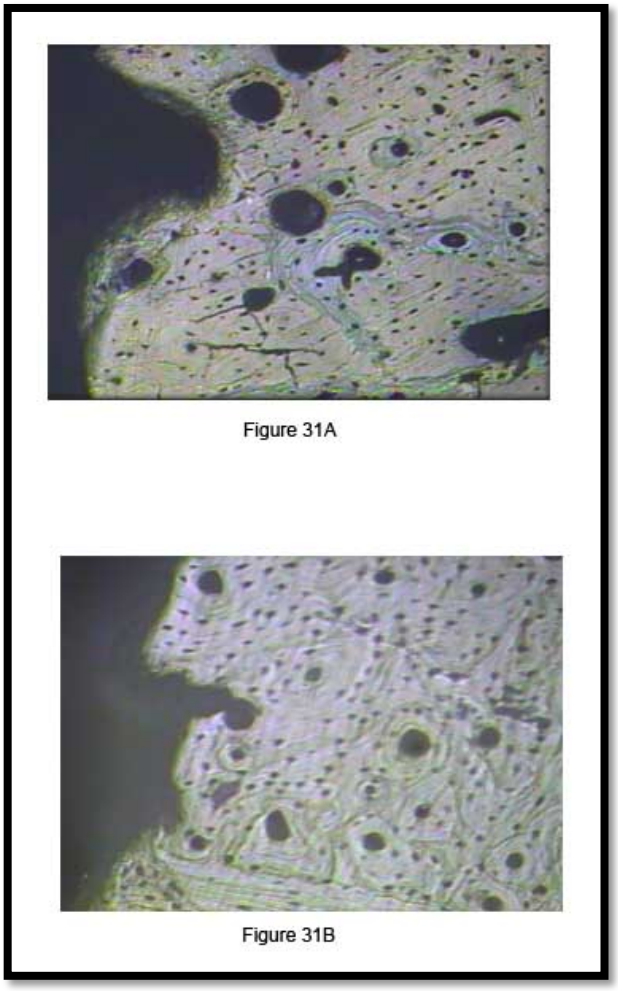


Figure 31A

Figure 31B

Figures 31A-31B- Bone adjacent to four different implant surfaces in tibia

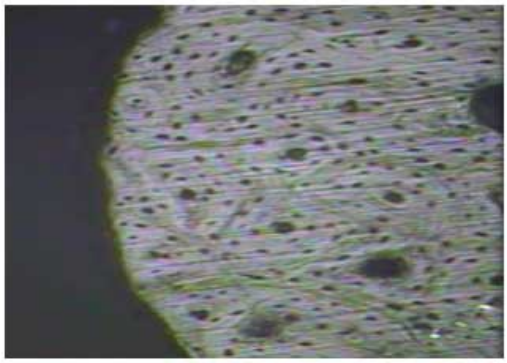


Figure 31C

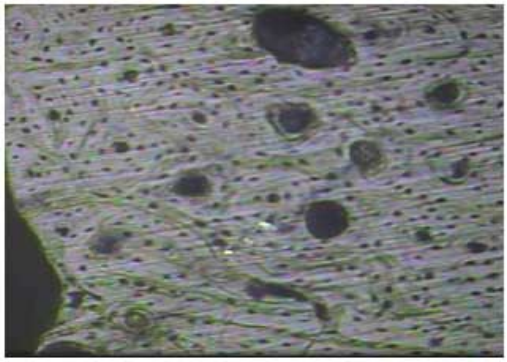


Figure 31D

Figures 31C-31D- Bone adjacent to four different implant surfaces in tibia

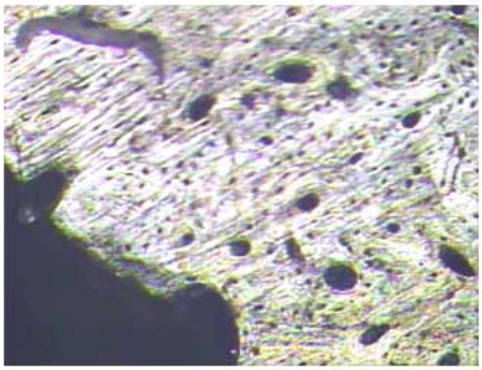


Figure 32A

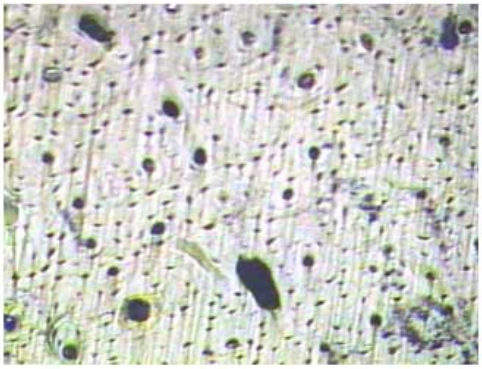


Figure 32B

Figures 32A-32B- Bone adjacent to four different implant surfaces in femur

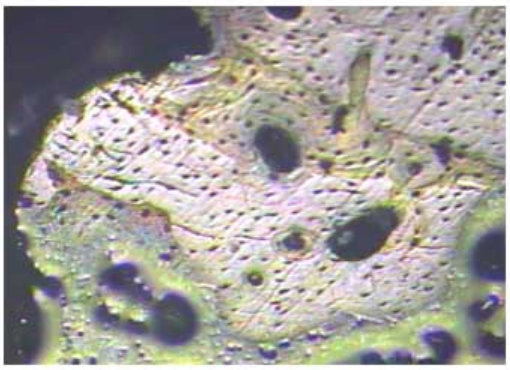


Figure 32C

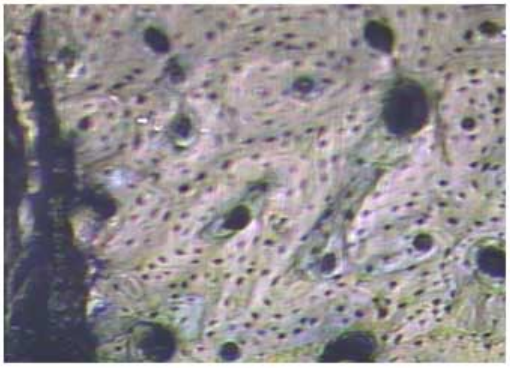


Figure 32D

Figures 32C-32D- Bone adjacent to four different implant surfaces in femur

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Curriculum Vitae

Sumit Yadav

Education

1997-02	Bachelor of Dental Surgery (B.D.S), Manipal University, India
2003-06	Mater of Dental Surgery (M.D.S), Ragiv Ganhi University, India
2007-11	Doctor of Philosophy (Ph.D), Indiana University, Indiana

Positions

09/13/2010	Clinical Instructor in Residence, Division of Orthodontics, University of Connecticut Health Center, CT

Additional Education Training

2001-02	Fellowship in General Education, Manipal University, India
2008-10	Preparing Future Faculty Certificate Program, Indiana University

Awards

2008	Oscar Orthodontic Award
2010	Dentaurum Travel Award
2010	American Orthodontics Travel Award
2010	Maynard K. Hine Award
2010	Charley Schultz Award

Memberships

1997-	Member, Indian Dental Association
2003-	Member, Indian Orthodontic Society
2007-	Member, American Association of Orthodontics
2007-	Member, World Federation of Orthodontics
2007-	Member, International Association of Dental Research
2007-	Member, American Association of Dental Research
2007-	Member, Indiana Bone Club
2007-	Member, Indiana Dental Association

Editorial Review Board

2008-	American Journal of Orthodontics
2008-	Angle Orthodontist
2009-	European Journal of Orthodontics
2009-	Orthodontics and Craniofacial Research
2010-	Journal of Dental Research
2010-	World Journal of Orthodontics
2010-	Journal of Oral Sciences

Selected Peer-reviewed publications

1. Yadav S, Patil S, Keluskar KM (2005). Canine Distraction: A Review. Journal of Indian Orthodontic Society.
2. Upadhyay M, Yadav S, Keluskar KM Easy to bond lingual retainers. Feb, 2006, Orthodontic Cyber Journal.
3. Nagaraj K, Yadav S, Upadhyay M. A simple and effective way of augmenting anchorage (May, 2006, Orthodontic Cyber Journal).
4. Yadav S, Upadhyay M, Patil S, Nagaraj K (2006). Work better with your FFAs. Journal of Clinical Orthodontics.
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