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Effects of Low-Frequency Ultrasound Treatment of Titanium Surface Roughness on Osteoblast Phenotype and Maturation

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Abstract

Objective—Low-frequency ultrasound is widely used in the treatment of chronically infected wounds. To investigate its feasibility as a method for *in situ* restoration of metal implant surfaces in cases of peri-implantitis, we evaluated how low-frequency ultrasound affected surface properties of and response of human osteoblast-like MG63 cells to titanium (Ti).

Material and Methods—Three Ti surfaces [hydrophobic/smooth (pretreatment, PT); hydrophobic/rough (sandblasted/acid-etched, SLA); and hydrophilic/rough (SLA processed and stored hydrophilicity, mSLA)] were subjected to 25 kHz ultrasound for 10 min/cm². Substrate roughness, chemical composition, and wettability were analyzed before and after ultrasound application. Osteoblastic maturation of cells on sonicated disks was compared to cells on untreated disks.

Results—Ultrasound treatment altered the topography of all surfaces. Contact angles were reduced and chemical compositions were altered by ultrasound on PT and SLA surfaces. Cell response to sonicated PT was comparable to untreated PT. Alkaline phosphatase was increased on sonicated SLA compared to untreated SLA, whereas DNA, osteocalcin, BMP2, osteoprotegerin, and VEGFA were unchanged. Cells produced less osteocalcin and BMP2 on sonicated mSLA than on untreated mSLA, but no other parameters were affected.

Conclusions—These results show that low-frequency ultrasound altered Ti surface properties. Osteoblasts were sensitive to the changes induced by ultrasound treatment. The data suggest that the effect is to delay differentiation, but it is unclear whether this delay will prevent osseointegration. These results suggest that low-frequency ultrasound may be useful for treating

implant surfaces *in situ* leading to successful re-osseointegration of implants affected by peri-implantitis.

Keywords

Titanium implant surface characterization; Ultrasound; Periodontitis; Peri-implantitis

1. INTRODUCTION

Peri-implantitis has a prevalence ranging from 28% to 56% among patients and from 12% to 43% among implant sites, making it one of the biggest problems of dental implant surgery (Zitzmann & Berglund 2008). The early stage, called mucositis, is restricted to the mucosa surrounding the neck of the implant, while later stage peri-implantitis causes loss of the supporting bone (Lindhe & Meyle 2008). Disease patterns and risk factors for peri-implantitis are similar to those of periodontitis (Belibasakis et al. 2015; Heitz-Mayfield & Lang 2010; Papaioannou et al. 1996; Van Dyke & Sheilesh 2005), and patients with a history of periodontitis have a higher risk of developing peri-implantitis (Lindhe & Meyle 2008).

Both periodontitis and peri-implantitis are caused by bacteria capable of forming a biofilm on the surface of a tooth or an implant, which protects the bacteria from antimicrobial agents (Højby et al. 2011). However, the degree to which plaque formation on teeth is comparable to biofilm formation on dental implants is controversial. Similar bacterial species can be found in periodontitis and peri-implantitis (Mombelli & Décaillot 2011; Van Dyke & Sheilesh 2005). In partially edentulous patients, the pathogens present in the peri-implant pocket resemble those of neighboring periodontal pockets (Botero et al. 2005; Hultin et al. 2002). Other studies suggest that the bacterial colonization pattern of teeth and implants differs (Dabdoub et al. 2013; Heuer et al. 2007; Kumar et al. 2012; Listgarten & Lai 1999; Mombelli & Décaillot 2011), possibly due to differences in the surface properties of teeth and implants. Studies have shown that surface roughness, hydrophilicity, and chemistry influence bacterial adhesion and biofilm formation, supporting this hypothesis (Almaguer-Flores et al. 2012; Renvert et al. 2008; Teughels et al. 2006).

Because the bacteria associated with peri-implantitis form a biofilm impenetrable to antibiotics (Proctor & Peters 1998), current treatment requires complete removal of the existing biofilm (Costerton 2005). Chemical, electrophysical, and mechanical methods have been used to clean the implants as part of the surgical protocol, with varying results. Mechanically abrading the implant surface using scalers or abrasives can damage the implant surface (Belibasakis et al. 2015), potentially influencing the regeneration of an optimal bone-implant interface. Thus, there remains a need for cleaning methods that cause minimal damage to the implant surface.

Ultrasonic cleaning is a common mechanical method capable of removing biofilm and necrotic tissues through cavitation. Waves travel through the tissue and cause gas bubbles to oscillate and expand (Bulat 1963), thus mechanically removing biofilms and necrotic tissues. At lower frequencies, gas bubbles can expand more, increasing cleaning ability (Walmsley et al. 1988). Also, the depth of penetration is more profound using low-frequency ultrasound in

contrast to high-frequency ultrasound and avoids development of heat that could cause tissue damage. It has been shown that application of low-frequency ultrasound reduces bacterial counts of infected wounds (Schoenbach & Song 1980; Serena et al. 2009), enhances the effect of antibiotics on infected polyethylene implants in animal studies (Carmen et al. 2004; Qian et al. 1997; Rediske et al. 2000), increases the release of gentamicin from bone cement causing a reduction in bacterial viability (Ensing et al. 2006), and can remove biofilms to some extent (Nishikawa et al. 2010; Xu et al. 2012). After the biofilm has been removed, local antibiotics can be applied to kill newly exposed bacteria, preventing the recurrence of peri-implantitis (Belibasakis et al. 2015; Van Dyke & Sheilesh 2005).

Ultrasonic cleaning is used currently to remove biofilm in the surgical treatment phase of peri-implantitis as well as periodontitis because it is less invasive than mechanical debridement, it is relatively painless, it reduces blood loss, and it is not known to cause any systemic side effects (Breuing et al. 2005). However, it is unknown whether this process can alter the surface properties of a titanium (Ti) dental implant, and if so, whether cell responses to the implant are affected that might impact bone regeneration and osseointegration.

Alterations to Ti implant surface properties have been shown to affect osteoblastic differentiation of mesenchymal stem cells (MSCs) (Olivares-Navarrete et al. 2011; Schwartz et al. 1999; Shen et al. 2015) as well as osteoblast maturation (Lohmann et al. 2000; Zhao et al. 2006). Also, the ability of these cells to produce factors that regulate bone formation and angiogenesis are affected (Gittens et al. 2011) and thereby influence the process of osseointegration. Therefore, in the present study, we investigated the effects of low-frequency ultrasound cleaning on Ti surface properties using three well-characterized surfaces used clinically on dental implants: a smooth/hydrophobic surface (PT), a rough/hydrophobic surface created by sandblasting and acid etching PT (SLA), and a rough/hydrophilic surface prepared by processing SLA under N₂ followed by storage in aqueous solution (mSLA). We then assessed the effects of surface sonication on the responses of human osteoblast-like cells *in vitro*.

2. MATERIAL AND METHODS

2.1 Ti disks

Ti disks were punched from 1mm thick sheets of grade 2 unalloyed Ti (ASTM F67) and supplied by Institut Straumann AG (Basel, Switzerland). The diameter of the disks was 15 mm so that they would fit snugly into the well of a 24-well tissue culture plate. Pre-treatment surfaces (PT) were degreased in acetone, exposed to a solution of 2% ammonium fluoride, 2% hydrofluoric acid, and 10% nitric acid for 30 s at 55°C. To produce SLA surfaces, the PT disks were sandblasted at 5 bar with 0.25 – 0.50 µm corundum grit, further acid-etched in a solution of hydrochloric and sulfuric acids, and heated above 100°C for several minutes (a proprietary process of Institut Straumann AG). Both PT and SLA surfaces were then rinsed in deionized water. To produce a hydrophilic SLA surface (modified SLA, mSLA), sandblasting and acid etching were performed under N₂ protection to prevent exposure to air and directly stored in isotonic NaCl solution in a sealed glass tube. All three types of disks were sterilized for 12 h by gamma irradiation at 25 kGy. The chemistry and

topography of these disks have been well characterized (Gittens et al. 2011; Olivares-Navarrete et al. 2011; Rupp et al. 2006; Singh et al. 2014; Zhao et al. 2005).

2.2 Ultrasound application

Ultrasound treatment of all Ti surfaces was performed under sterile conditions. The ultrasound was generated using the Sonoca 180/185 device (Söring GmbH, Quickborn, Germany) and applied using a handpiece with an ultrasound head (Huf 97-103, working length: 60mm, external caliber of tip: 6mm) following the manufacturer's instructions. The surfaces were subjected to ultrasound treatment for 10 min/cm² at a frequency of 25 kHz with a fluid flow (sterile water) of 10 ml/min. The distance between surface and ultrasound head was 1cm. Afterwards, PTson and SLAson disks were repackaged under sterile conditions; mSLAson disks were immediately placed back into glass tubes containing a sterile NaCl solution. All disks were sterilized under UV light for 12 h before use in cell culture studies.

2.3 Surface characterization

2.3.1 Scanning electron microscopy (SEM)—The surface topography of treated and untreated surfaces (2 disks per group) was examined by using a field-emission-gun electron microscope (Ultra 60 FEG-SEM, Carl Zeiss SMT Ltd., Cambridge, UK). Images (six per disk) were recorded using a 5 kV accelerating voltage and a 30 µm aperture.

2.3.2 Confocal laser microscopy (CLM)—Surface roughness (S_a) was measured by using a confocal laser microscope (Lext, Olympus, Center Valley, PA). Ultrasound treated and untreated surfaces (2 disks per group) were scanned at six randomized locations each using a scan height step of 100 nm, a 20x objective, and a cutoff wavelength of 200 µm. Each measurement was performed over an area of 644 µm × 644 µm. Mean values of surface roughness were calculated from n=12 measurements per disk type using the included LEXT 3D surface analysis software.

2.3.3 Contact angle—Contact angle of ultrasound treated and untreated surfaces was determined using a goniometer (CAM 100, KSV, Helsinki, Finland). This device was equipped with a digital camera and image analysis software. mSLA surfaces were dried under nitrogen before analysis. The surfaces (2 disks per group) were measured three times each at randomized locations (n=6). However, SEM indicated that SLAson surfaces had areas with two different morphologies. Accordingly, we determined contact angles separately for each morphology (n=3 measurements/ type): SLAson surface regions with characteristic sandblasted/acid-etched topography (SLAson/1) and SLAson with flat topography (SLAson/2). Ultra-pure water was used as a wetting liquid with a drop size of 5 µL.

2.3.4 X-ray photoelectron spectroscopy (XPS)—The chemical composition of ultrasound treated and untreated surfaces was analyzed under vacuum by X-ray photoelectron spectroscopy (XPS, Thermo K-Alpha XPS, Thermo Fisher Scientific, Waltham, MA). This device was equipped with a monochromatic Al-K α X-ray source ($h\nu = 1468.6$ eV photons). The surfaces (2 disks per group) were scanned two times each (n=4).

measurements/ disk type). The XPS analysis chamber was evacuated to a pressure of 5×10^{-8} mbar or lower before collecting XPS spectra using an X-ray spot size of 400 μm and pass energy of 100 eV with 1 eV increments at a 55° takeoff angle.

2.4 Cell culture and cell response

2.4.1. Cell culture—MG63 human osteoblast-like cells were obtained from the American Type Culture Collection (Rockville, MD). This cell line was originally derived from a femoral osteosarcoma of a 14-year old Caucasian male (Heremans et al. 1978) and has been well characterized. The cells were cultured in Dulbecco's modified Eagle medium (DMEM), containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂ and 100% humidity. Cells were cultured in a 24-well plate at a density of 10,000 cells/cm² either directly on the tissue culture polystyrene (TCPS) culture wells or on Ti disks that had been ultrasonically treated (PTson, SLAson, mSLAson) or left untreated (PT, SLA, mSLA) (n = 6 independent cultures per group). Media were exchanged 24 h after plating and every 48 h thereafter until confluence was observed on TCPS. At confluence, cells were treated with fresh media for 24 h and harvested for cell response studies. Conditioned media were collected, and cell layers were washed twice with phosphate-buffered saline (PBS) to remove any non-adherent cells. The cell layers were then treated with 500 μL of 0.05% Triton X-100 and sonicated at an amplitude of 60 for 5 s to mechanically lyse the cells.

2.4.2 Cell number—To evaluate cell number, DNA content in the cell layer lysates was measured using a commercially available kit (Quant-iT™ PicoGreen® dsDNA Assay, Thermo Fisher Scientific). Fluorescence was measured using a fluorescent multimode detector (DTX880, Beckman Coulter, Brea, CA) at an excitation wavelength of 485nm and emission wavelength of 538nm and compared to a standard.

2.4.3 Cell maturation—Osteoblastic maturation was determined by measuring alkaline phosphatase specific activity of cell layer lysates as an early marker; osteocalcin content in the conditioned media was measured as a late marker. Alkaline phosphatase specific activity was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate at a pH of 10.2 as described previously (Bretaudiere & Spillman 1984). Results were normalized to protein content of cell lysates that was measured by colorimetric cuprous cations in biuret reaction (BCA Protein Assay Kit, Pierce Biotechnology Inc., Rockford, IL, USA) at 570 nm (Microplate reader, Bio-Rad Laboratories Inc., Hercules, CA, USA). Osteocalcin was measured using a commercially available radioimmunoassay kit (Human Osteocalcin RIA Kit, Biomedical Technologies, Stoughton, MA) as described previously (Bretaudiere & Spillmann 1984). Briefly, 50 μL of conditioned media were mixed with [I-125] osteocalcin tracer and human osteocalcin antiserum (100 μL each) and incubated at 37°C for 2.5 h. Goat anti-rabbit IgG, polyethylene glycol (100 μL each), and 1ml of PBS were added and centrifuged at 15000× g for 15 min at 4°C. The supernatant was decanted, and the pellets were counted for 1 min in an LS1500 gamma counter (Beckman Coulter, Brea, CA). The production of osteocalcin was normalized to DNA content.

2.4.4 Cytokine receptors and growth factors—To evaluate protein levels of cytokine receptors and growth factors, the conditioned media were assayed for the RANK ligand decoy receptor osteoprotegerin (OPG), vascular endothelial growth factor (VEGF), and bone morphogenetic protein 2 (BMP2), using enzyme-linked immunosorbent assay (ELISA) kits (DY805 Osteoprotegerin DuoSet, DY355 BMP-2 DuoSet, and DY293B VEGF DuoSet; R&D Systems, Minneapolis, MN). Protein levels of cytokine receptors and growth factors were normalized to DNA content.

2.5 Statistical analysis

All measurements from the surface characterization experiments are presented as mean $\pm$ standard deviation for the number of indicated measurements in each study. Data from individual cell experiments are shown and presented as mean $\pm$ standard error for $n=6$ independent cultures per variable. All cell experiments were repeated at least twice to ensure the validity of the results. Data were evaluated by a one-way analysis of variance. Significant differences between groups were determined using Bonferroni correction to Student's t-test. p values < 0.05 were considered as statistically significant differences.

3. RESULTS

3.1 Surface characterization

3.1.1 Macroscopic appearance—After ultrasound application, some surface modifications were visible macroscopically (data not shown). PT and mSLA surfaces only showed some superficial scratches, but appeared generally homogeneous and not distinctly altered. SLAson surfaces appeared inhomogeneous with unaltered regions and regions that appeared flattened, the latter affecting approximately 30% of the total surface.

3.1.2 Scanning electron microscopy (SEM)—Surface topography of ultrasound treated and untreated PT surfaces were similar at the micro-scale (Fig. 1a, b). However, on the ultrasound treated PTson surfaces, nano-modifications of the surface structure were detected (Fig. 1b, arrow) that were not seen on PT surfaces (Fig. 1a). SLAson surfaces appeared similar to the untreated SLA disks over 70% of the surface area (Fig. 1c, d). The SLAson surface regions comparable to the SLA surface were termed SLAson/1. In the remaining areas, a change of surface topography was found, termed SLAson/2 (Fig. 1d, arrow). These altered regions appeared to be flattened or melted (Fig. 1e, arrow). No differences were observed between treated and untreated mSLA surfaces (Fig. 1f, g).

3.1.3 Confocal laser microscopy (CLM)—Average surface roughness (S_a) was significantly decreased on ultrasound treated surfaces when compared to untreated surfaces of the same type (Table 1). This effect was greatest on PT and much less pronounced on the SLA and mSLA substrates. Both SLA and mSLA surfaces were significantly rougher than PT surfaces. Similarly, SLAson and mSLAson surfaces were significantly rougher compared to PTson.

3.1.4 Contact angle—Contact angle measurements (Table 2) revealed SLA as the most hydrophobic surface followed by PT. mSLA had a super hydrophilic surface with a contact

angle of 0°. Interestingly, contact angles for PTson and SLAson/1 were lower than for the untreated surfaces. Moreover, the contact angle in the altered region of SLAson/2 was decreased to 0°. mSLAson had a contact angle of 0°, which was identical to mSLA.

3.1.5 X-ray photoelectron spectroscopy (XPS)—The chemical composition of the sonicated surfaces was composed mainly of titanium, oxygen, and carbon (Table 3). Decreased carbon and increased oxygen content were observed on the mSLA and mSLAson surfaces compared to the other surfaces. No major differences were seen regarding the distribution of the three primary elements between treated and untreated surfaces of the same type. Interestingly, traces of silicon, aluminum, chlorine, calcium, sodium, and potassium were detected on PTson and SLAson. However, these other elements were absent from the mSLAson.

3.2 Cell response

3.2.1 Cell proliferation—DNA content varied as a function of surface roughness and hydrophilicity, but sonication did not alter the surface effects on DNA content (Fig. 2a). DNA content was significantly lower on SLAson, mSLA, and mSLAson compared to TCPS, PT, and PTson. Similar levels of DNA content were detected on mSLA and mSLAson, which were lower compared to SLAson.

3.2.2 Cell maturation—Alkaline phosphatase specific activity (Fig. 2b) was significantly lower on all surfaces except PT compared to TCPS. However, similar levels of activity were detected on PT, PTson, and SLAson. The lowest levels of alkaline phosphatase activity were detected on SLA, mSLA, and mSLAson.

No difference in MG63 osteocalcin (Fig. 2c) production was detected among TCPS, PT, PTson, and mSLAson. SLA and SLAson had similar levels of osteocalcin and were significantly higher compared to PT and PTson. The highest content of osteocalcin was measured on mSLA with significant differences compared to all other surfaces. Accordingly, osteocalcin production was significantly reduced on mSLAson compared to the untreated surface.

BMP2 production was sensitive to ultrasound treatment of the surface (Fig. 2d). Compared to TCPS there was no difference in BMP2 produced by cultures on either PT surface. In contrast, cultures on SLA and mSLA surfaces showed a significant increase in BMP2 compared to TCPS and both PT surfaces. This increase was greater on mSLA compared to SLA. After ultrasound treatment, however, production of BMP2 on SLAson and mSLAson was reduced to levels seen in cultures grown on TCPS. This reduction was only significant for mSLAson.

3.2.3 Cytokine receptors and growth factors—Levels of OPG (Fig. 3a) were lowest on TCPS, both PT surfaces and SLA with no significant differences. A significant increase was seen on SLAson compared to TCPS and PT. Cells on both mSLA surfaces produced increased OPG compared to TCPS, both PT surfaces, and SLA. Cells on mSLA produced a further increase in OPG compared to SLAson. No significant differences were seen between treated and untreated surfaces of each type.

VEGF (Fig. 3b) production was comparable in cultures grown on TCPS, both PT, and both SLA surfaces. VEGF levels were increased on both mSLA surfaces. This effect was significant on mSLA significant compared to all other surfaces, but on mSLAson, the increase was only significant compared to TCPS and PT. No significant differences were seen between treated and untreated surfaces of each type.

4. DISCUSSION

This study is the first demonstration of the effects of low-frequency ultrasound on Ti implant surfaces used clinically. In general, the average roughness of all sonicated surfaces was lower than the untreated surfaces, particularly on PT, indicating that some ablation of the surface occurred. SEM indicated that, for the most part, the original topography was maintained and was similar to previously published results (Gittens et al. 2011; Olivares-Navarrete et al. 2011; Rupp et al. 2006; Singh et al. 2014; Zhao et al. 2005). However, defects that covered about 30% of the surface were seen on SLAson. Why these defects were only present on SLAson is not clear, since all sonicated surfaces received the same ultrasound treatment. SLA had the highest contact angle suggesting higher organic contamination, which may have affected the abrasion resulting from the ultrasound. This is supported by the observation that treatment with ultrasound appeared to reduce surface carbon contamination; as a result, the contact angle on sonicated PT was reduced by 30% and on SLAson was reduced by 50-100%.

Another possibility is that production of the original PT surface before sandblasting and acid etching may have caused some marks of the metal on the surface. This typically leaves brush marks, and studies have shown that they are no longer detectable after application of low-frequency ultrasound on CoCr-knee prostheses (Singh et al. 2014). Whether a similar mechanism was involved in the present study is not clear.

Sonication had the greatest effect on PT, reducing S_a by 40%, although a reduction in microscale roughness was evident on all surfaces. Analysis of surface morphology was limited to average surface roughness, which may have obscured small differences that other analytic parameters might have detected. Our results show that surface chemistry was altered by sonication. Both PTson and SLAson exhibited deposits of Si, Al, Cl, Ca and Na and SLAson also had K on the surface following sonication. In contrast, the fully hydrated mSLAson surface was not altered in this manner, suggesting that the contaminants came from the sonication solution and were not deposited on mSLA due to the existing hydration.

The response of the cells to implants depends on the adsorption of proteins from the serum to the material's surface. However, cell responses to the sonicated surfaces were similar to those reported previously for PT, SLA, and mSLA, with some notable differences. DNA content was reduced on SLAson compared to PTson, but it was not different from SLA. Alkaline phosphatase specific activity was reduced on SLA and mSLA compared to PT, whereas osteocalcin was increased, indicating a later stage of maturation (Lian & Stein 1993). Alkaline phosphatase was reduced on SLA and mSLA compared to PT, whereas osteocalcin was increased showing a later stage of maturation. Alkaline phosphatase was reduced on mSLAson, but not on SLAson, suggesting that cells on sonicated SLA were less

differentiated than cells on the hydrophilic rough surface. Similarly, cells on mSLAson produced less osteocalcin than cells on mSLA, again suggesting a delay in osteoblast maturation on the sonicated surface. This difference in osteoblast maturation is likely due to the lower levels of BMP2 produced by the cells on SLAson and mSLAson compared to SLA and mSLA. In contrast to the differential effects of sonication on osteoblast maturation, sonication had no effect on the production of osteoprotegerin or VEGF, which mediate osteoclast differentiation and angiogenesis, which are important for modulating bone remodeling (Deckers et al. 2000; Walsh & Choi 2014).

5. CONCLUSIONS

The results of this study indicate that low-frequency ultrasound treatment can alter microscale and nanoscale topography of Ti implants. While sonication altered the surface chemistry of hydrophobic implants, it had no effect on the chemistry of fully hydrated Ti surfaces, suggesting that it may not impact the surface chemistry of implants treated in situ under clinical conditions. Osteoblast differentiation and maturation on the sonicated rough-surfaces appeared delayed in comparison with cultured on the unsonicated substrates. Alkaline phosphatase specific activity was higher on SLAson but osteocalcin was not elevated, indicating the cells may have been at an early stage of osteoblast maturation. Osteocalcin levels were lower on mSLAson compared to mSLA whereas alkaline phosphatase was comparable to mSLA, suggesting potential inhibition of maturation. Reduced BMP2 production on mSLAson may have contributed to this effect. In contrast, production of osteoprotegerin and VEGF were not altered by sonication, indicating that pro-osteogenic contributions of surface microtopography to net new vascularized bone formation were not mitigated by treatment. This study did not assess the effects of sonication on production of anti-inflammatory cytokines. However, taken together, this study suggests that low-frequency ultrasound cleaning may provide a useful alternative for treatment of peri-implantitis by eliminating the infection and its products from the defect site and the implant surface, allowing regeneration and re-osseointegration to occur. Although no animal model of peri-implantitis currently exists, the next set of studies will have to confirm its effectiveness for use clinically.

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Figure 1.

Effects of ultrasound treatment on Ti Surfaces. Scanning electron micrographs of untreated smooth/hydrophobic (PT, a), rough/hydrophobic (SLA, c), or rough/hydrophilic (mSLA, f) or disks after ultrasound treatment (b,d,e,g). The ultrasound was applied for 10 min/cm² at a frequency of 25 kHz with a fluid flow of 10ml/min on PT (b), SLA (d,e) and mSLA (g); arrows show examples of qualitative changes seen in surface topography. Scale bar indicates 1 µm.

Figure 2.

Effects of ultrasound treated Ti surfaces on osteoblast proliferation and differentiation. The ultrasound was applied to Ti disks for 10 min/cm² at a frequency of 25 kHz with a fluid flow of 10ml/min. MG63 cells were plated on non-treated or sonicated PT, SLA and mSLA surfaces. Cells were grown to confluence on a TCPs control. DNA content (a), alkaline phosphatase specific activity (b), secreted osteocalcin (c), and secreted BMP2 (d) were measured. Data are presented as mean ± SE of six independent cultures per disk type. * p<0.05 vs. TCPs, #p<0.05 vs. PT, ^p<0.05 vs. PTson, \$p<0.05 vs. SLA, &p<0.05 vs. SLAson, %p<0.05 vs. mSLA. TCPs is included for reference.

Figure 3.

Effects of ultrasound treated surfaces of Ti disks on protein levels of growth factors. The ultrasound was applied to Ti disks for 10 min/cm² at a frequency of 25 kHz with a fluid flow of 10ml/min. MG63 cells were plated on non-treated or sonicated PT, SLA, and mSLA surfaces. Cells were grown to confluence on a TCPS control. Osteoprotegerin (a) and VEGF (b) levels were measured in the conditioned media. Data are represented as mean $\pm$ SE of six independent cultures per disk type. * $p < 0.05$ vs. TCPS, # $p < 0.05$ vs. PT, ^ $p < 0.05$ vs. PTson, \$ $p < 0.05$ vs. SLA, & $p < 0.05$ vs. SLAson, % $p < 0.05$ vs. mSLA. TCPS is included for reference.

Table 1

Effects of ultrasound treatment on surface roughness (S_a). Mean values $\pm$ one standard deviation (SD) of $n=12$ measurements per variable. Differences between sonicated (son) and untouched samples were examined by t-test.

Surface	S_a [μm] $\pm$ SD	p-value
PT	1.1 ± 0.01	<0.0001
PTson	0.57 ± 0.03	
SLA	3.38 ± 0.01	<0.0001
SLAson	3.03 ± 0.49	
mSLA	3.52 ± 0.03	0.004
mSLAson	3.22 ± 0.09	

Table 2

Effects of ultrasound treatment on contact angle. Mean values $\pm$ one standard deviation (SD) of n=6 measurements per variable (n=3 each for SLAson/1 and /2). Differences between sonicated (son) and untouched samples were examined by t-test.

Surface	Contact angle [$^{\circ}$] $\pm$ SD	p-value
PT	93.0 $\pm$ 0.4	<0.0001
PTson	64.9 $\pm$ 10.4	
SLA	127.0 $\pm$ 3.7	<0.0001 vs SLAson/1 <0.0001 vs SLAson/2
SLAson/1	61.6 $\pm$ 9.1	<0.0001 vs SLAson/2
SLAson/2	0	-
mSLA	0	
mSLAson	0	

Table 3

Effects of ultrasound treatment on surface chemical composition [%] measured with x-ray photoelectron spectroscopy (XPS). Mean values $\pm$ one standard deviation (SD) of n=4 measurements per disk type. Differences in Ti, O, and C were compared between sonicated and unsonicated values using t-tests and calculated p-values are given under each comparison.

Element	Surface					
	PT	PTson	SLA	SLAson	mSLA	
Ti	17.9 $\pm$ 0.6	14.8 $\pm$ 2.6	15.0 $\pm$ 0.4	9.3 $\pm$ 3.0	22.0 $\pm$ 0.3	23.8 $\pm$ 0.9
	p=0.0478		p=0.0001		p=0.4271	
O	47.7 $\pm$ 0.4	48.9 $\pm$ 4.2	48.0 $\pm$ 0.5	38.8 $\pm$ 2.5	60.0 $\pm$ 1.4	60.4 $\pm$ 0.9
	p=0.8671		p<0.0001		p=0.5033	
C	31.0 $\pm$ 0.5	26.2 $\pm$ 5.1	35.8 $\pm$ 0.6	33.1 $\pm$ 3.4	17.0 $\pm$ 0.3	15.8 $\pm$ 1.7
	p=0.0170		p=0.1715		p=0.0693	
Si	-	2.8 $\pm$ 2.1	-	1.3 $\pm$ 1.5	-	-
Al	-	3.0 $\pm$ 2.2	-	2.7 $\pm$ 2.2	-	-
Cl	-	2.1 $\pm$ 1.2	-	6.3 $\pm$ 3.0	-	-
Ca	-	0.7 $\pm$ 0.8	-	1.2 $\pm$ 1.9	-	-
Na	-	1.6 $\pm$ 3.5	-	6.9 $\pm$ 5.5	-	-
K	-	-	-	0.3 $\pm$ 0.5	-	-