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Variation in Osteoblast Retention Ability of Titanium Surfaces with Different Topographies

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Abstract

The ability of titanium surfaces to retain osteogenic cells is critical for osseointegration. In particular, the cell-retention ability under exogenous force holds a key to successful osseointegration in immediately or early loaded implants. However, it is unknown which surfaces are better than the others in terms of their cell-retention ability, how much is the potential difference among different surfaces, and what topographical variables determine the ability. Rat bone marrow- derived osteoblasts were cultured on titanium disks with four different surface topographies: machined, sandblasted, acid-etched, and sandblasted + acid-etched surfaces. After a 24-h incubation, cells were detached using vibrational force combined with enzymatic protein degradation (trypsinization). The cell-retention ability was greater in the order of sandblasted + acid-etched surface, sandblasted surface, acid-etched surface, and machined surface. There was a significant linear correlation between the cell-retention ability and the degree of surface roughness. Sa (average roughness) and Sdr (developed interfacial area ratio) were highly correlated with the cell-retention ability. However, the number of cells recruited during a 24-h incubation was negatively correlated with the degree of surface roughness, with the sandblasted + acid-etched surface recruiting the least number of cells. In conclusion, the rougher the titanium surfaces, the stronger their cell-retention ability. Sdr was the most effective topographical determinant to predict both cell-retention and recruitment ability.

Keywords: Sdr (Developed interfacial area ratio); Cell-Recruitment ability; Cell-Retention ability; Mechanical detachment test

Abbreviations

ANOVA: One-Way Analysis of Variance; μm: Micrometer; N: Newton; SEM: Scanning Electron Microscopy

Introduction

The ability of titanium surfaces to retain osteogenic cells is critical for osseointegration. In particular, the cell-retention ability under exogenous force is key to successful osseointegration in immediately or early loaded implants [1,2]. Immediate/early implant loading minimizes the functional limitations and psychological stress of missing teeth, especially in the esthetic zone [1,2]. However, some researchers have reported reduced implant survival with immediate loading [3-9]. A randomized controlled study reported a survival rate of 100% with conventional loading protocols and 75% with immediate loading protocols [4]. A recent systematic review concluded that the risk of implant failure increases significantly with the use of immediate loading protocols [7]. In order to improve the osteoconductivity of titanium implants, various methods of surface modification have been developed to roughen titanium surfaces. Surface modifications consist of mechanical, chemical, and physicochemical treatments, as well as other coating-based methods, including machining, sandblasting, acid-etching, anodization, plasma spraying, laser treatment, apatite-coating, or a combination thereof [10,11]. Surface topography and roughness influence the biological responses of osteoblast cells [12]. For improved osseointegration of implants, researchers have assessed the impact of surface roughness at the micro-scale [13]. Immediate loading requires consideration of the cell-retention ability of the titanium surface at an early stage. However, it is unknown which surfaces are better than the others in terms of their cell-retention ability, how much is the potential difference among different surfaces, and what topographical variables determine the

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Table 1: Surface roughness parameters analyzed in this study.

Classification	Symbol	Name	Definition
Amplitude/height parameters	Sa	Average roughness	The arithmetical mean of the absolute values of height.
	Sz	Peak-to-valley roughness	The sum of the maximum peak height value and the maximum pit depth value.
	Sku	Kurtosis	A measure of the sharpness of the roughness profile. The higher the value, the sharper the height profile.
	Ssk	Skewness	The degrees of profile skew and express the symmetry of peaks and valleys using the average line as the center. Ssk=0: Symmetrical about the average line. Ssk>0: Skewed downward relative to the average line.
Spatial parameters	Str	Texture aspect ratio	A measure of uniformity of the surface texture, a range from 0 to 1. Str>0.5 indicates strong isotropy, while Str<0.3 indicates strong anisotropy.
Hybrid parameters	Sdr	Developed interfacial area ratio	The percentage of the definition area's additional surface area contributed by the texture as compared to the projected, planar definition area.
	Sdq	Root mean square gradient	A root mean square of slopes at all points in the definition area.

ability. Therefore, the objectives if this study were to examine the cell-retention ability of titanium discs with different surface topographies during the initial stage of cell culture, to examine the correlation between surface roughness and cell-retention ability, and to identify topographical factors that contribute most to cell-retention ability.

Materials and Methods

Titanium sample preparation

Disks (diameter: 20 mm, thickness: 2 mm) of grade 2 commercially pure titanium were prepared by machining. Sandblasted titanium surfaces were created by alumina sandblasting of the machined surfaces. An acid-etched surface was prepared by regular acid-etching with 67% (w/w) H_2SO_4 (Sigma-Aldrich, St. Louis, MO, USA) at 110°C for 75s. Finally, sandblasted + acid-etched titanium surfaces were created by alumina sandblasting, regular acid-etching with 67% (w/w) H_2SO_4 (Sigma-Aldrich, St. Louis, MO, USA) at 110°C for 75s. These surface preparations are characteristic of those used in dental implants [14].

Titanium surface characterization

The surface morphologies of the machined, sandblasted, acid-etched and sandblasted + acid-etched surfaces were examined by SEM (Nova 230 Nano SEM, FEI, Hillsboro, OR, USA). In order to identify potential measurable differences in surface morphology among the four surfaces, quantitative assessments of three-dimensional profiles were performed. The amplitude/height parameters were Sa (average roughness), Sz (peak-to-valley roughness), Sku (kurtosis), and Ssk (skewness). The spatial parameter was Str (the aspect ratio of the surface texture), and the hybrid parameters were Sdr (developed interfacial area ratio) and Sdq (root mean square gradient). All samples were measured using three-dimensional profiles (Nanto Co., Ltd.) (n=6) (Table 1).

Osteoblastic cell culture

Bone-marrow-derived osteoblastic cells were isolated from the femurs of eight-week-old male Sprague-Dawley rats and placed into alpha-modified Eagle's medium supplemented with 15% fetal bovine serum, 50 mg/mL ascorbic acid, 10 mM Na-β-glycerophosphate, 10^{-8} M dexamethasone, and antibiotic–antimycotic solution containing 10,000 units/mL penicillin G sodium, 10,000 mg/mL streptomycin sulfate, and 25 mg/mL amphotericin B, as previously described [14]. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. At 80% confluency, cells were detached using 0.25% trypsin-1 mM EDTA-4Na and were seeded onto titanium disks placed in a 12-well culture dish at a density of 3×10^4 cells/cm^2. The culture medium was renewed every three days. All experiments were performed following protocols approved by The Chancellor's Animal Research Committee at the University of California at Los Angeles (ARC #2005-175-41E, approved on 30 January 2018), the PHS Policy for the Humane Care and Use of Laboratory Animals, and the UCLA Animal Care and Use Training Manual guidelines.

Cell-retention ability

The cell-retention ability of titanium surfaces was evaluated by a mechanical detachment assay, as established previously [15]. After culturing for 24 hours, cells were gently rinsed once with PBS and a mechanical detachment test was performed by vibrating the culture dish (amplitude: 10 mm, frequency: 30 Hz) at 37°C for 20 min. A hematocytometer was used to count the number of detached cells. The cells remaining on the surfaces were then completely detached using 300 μL of 0.25% trypsin-1 mmol/L EDTA-4Na for 5 min at 37°C and counted. The cell-retention ability was calculated according to the following equation:

(Number of remaining cells)/(Number of detached cells + Number of remaining cells) × 100[16].

Cell-recruitment ability

The number of cell attachments was determined by measuring the number of cells attached to titanium disks after 24 hours of incubation. After the mechanical detachment test, a hematocytometer was used to count the number of detached cells, and the remaining cells on the surfaces were completely detached using 0.25% trypsin-1 mmol/L EDTA-4Na for 5 min at 37°C and counted. The number of cells is the sum of these values.

Statistical analysis

Data on surface roughness parameters were collected from six sites on four different disks (n=6). All culture studies were performed in quadruple (n=4). This sample size is consistent with those from similar previous studies [16]. Statistical analyses were carried out using the SPSS (Version 22.0; SPSS Inc., Chicago, IL, USA) statistics program. All statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by Bonferroni test to assess differences among groups. All data are expressed as the group means ± standard deviation. Results with a probability level of 0.05 or less were considered to be significant. In addition, a correlation analysis between the surface roughness and the cell-retention ability was performed, and a correlation analysis between the surface roughness and the cell-recruitment ability on the titanium discs was also performed.

Results

Surface morphology of titanium

The four differently prepared titanium surfaces each exhibited

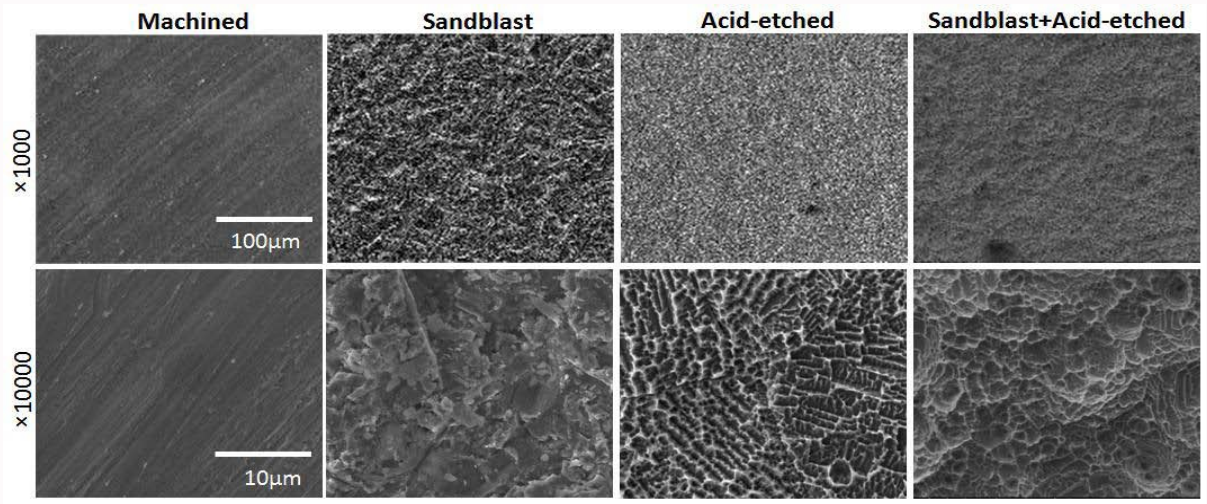

Figure 1: Surface morphology of titanium surfaces used in the present study. Scanning electron micrographs of the machined, sandblasted, acid-etched, and sandblasted + acid-etched surfaces.

different surface morphologies (Figure 1). As shown in low-magnification SEM images, the machined surface showed parallel traces formed during the concentric machining process, whereas sandblasted surfaces exhibited typical roughness and irregularities, caused by the sandblasting, at a micron scale. The sandblasted + acid-etched surface showed undefined irregularities. High-magnification images of the machined surface showed that parallel traces formed. The sandblasted surfaces were confirmed to have microroughness and irregularities. The created roughness was relatively irregular in shape and larger in scale compared to that on the acid-etched surface. The acid-etched surface exhibited a typical micro-roughened morphology, consisting of microscale pits with a peak-to-peak distance of 1-5 μm. The sandblasted + acid-etched surface was shown to have a typical micro-roughened morphology and a relatively irregular roughness.

Quantitative topographical evaluations of titanium surfaces

The results showed that roughness parameters, such as Sa, Sdr and Sdq, were significantly greater on the acid-etched surface than on the machined surface, were significantly greater on the sandblasted surface than on the acid-etched surface, and were significantly greater on the sandblasted + acid-etched surface than on the sandblasted surface (Figure 2). The values of Sz and Str were significantly greater on the acid-etched surface than on the machined surface, and were significantly greater on the sandblasted surface than on the acid-etched surface, whereas there was no significant difference between the sandblasted surface and sandblasted + acid-etched surface. The value of Sku was significantly greater on the sandblasted surface than on the sandblasted + acid-etched surface. The value of Ssk was significantly higher on both the machined surface and the acid-etched surface than on the sandblasted + acid-etched surface.

Cell-retention ability of different titanium surfaces under mechanical detachment

The cell-retention ability was greater in the order of sandblasted + acid-etched surface (71%), sandblasted surface (64%), acid-etched surface (56%), and machined surface (47%). The cell-retention ability was 1.36 times greater for the sandblasted surface 1.19 times greater for the acid-etched surface, and 1.51 times greater for the sandblasted

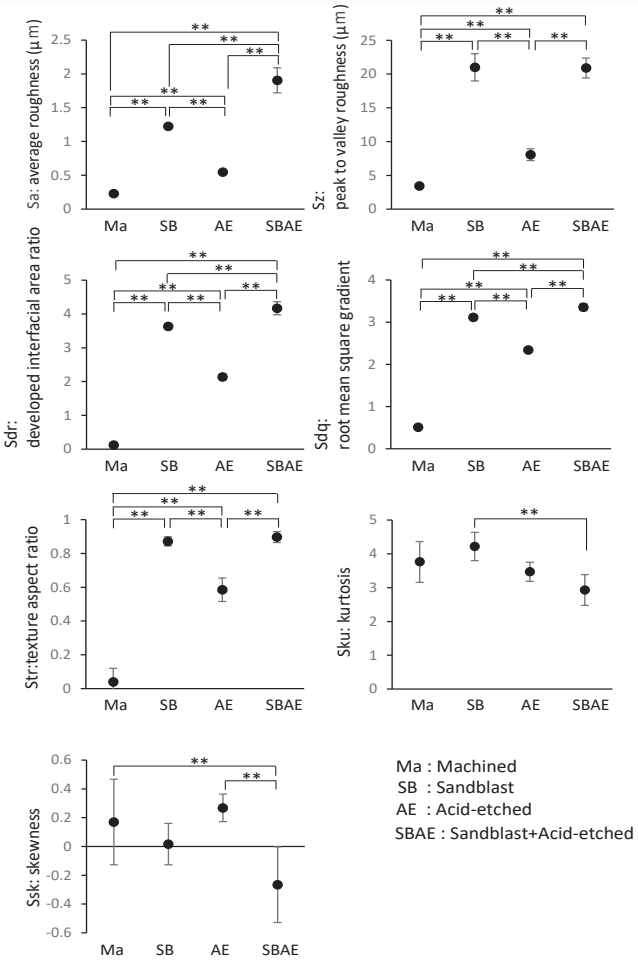

Figure 2: Quantitative measurement of surface roughness for the machined, sandblasted, acid-etched, sandblasted + acid-etched titanium surfaces. The average roughness (Sa), peak-to-valley roughness (Sz), developed interfacial area ratio (Sdr), root-mean-square roughness (Sdq), aspect ratio of the surface texture (Str), kurtosis (Sku), and skewness (Ssk) are indicated. Each value represents the mean ± standard deviation of six sites on the four different surfaces (n=6).
**p<0.001, one-way ANOVA followed by a Bonferroni test

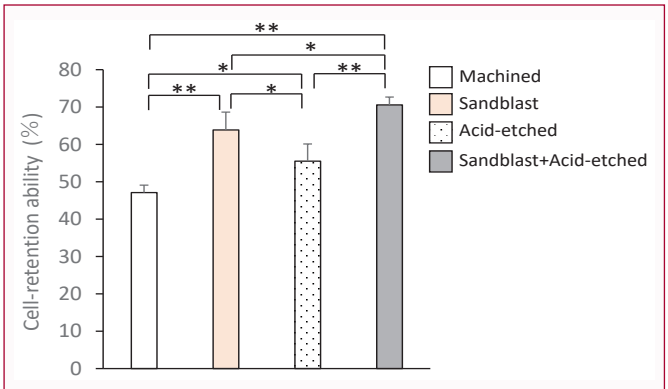

Figure 3: A detachment test was performed using cells incubated for 24 hours in order to determine the cell-titanium surface attachment strength. The cell-retention ability on four different titanium surfaces is shown. Each value represents the mean ± standard deviation for four experiments (n=4). **p<0.001, *p<0.05, one-way ANOVA followed by a Bonferroni test

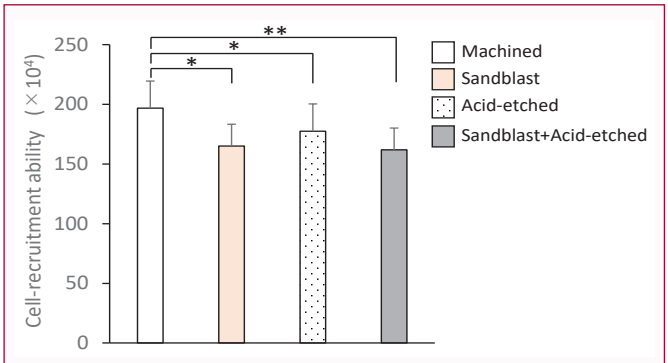

Figure 4: Results for the cell-recruitment ability to four different titanium surfaces. The cell-recruitment ability to four different titanium surfaces during 24 hours incubation. Each value represents the mean ± standard deviation for four experiments (n = 4).
**p<0.001, *p<0.05, one-way ANOVA followed by a Bonferroni test

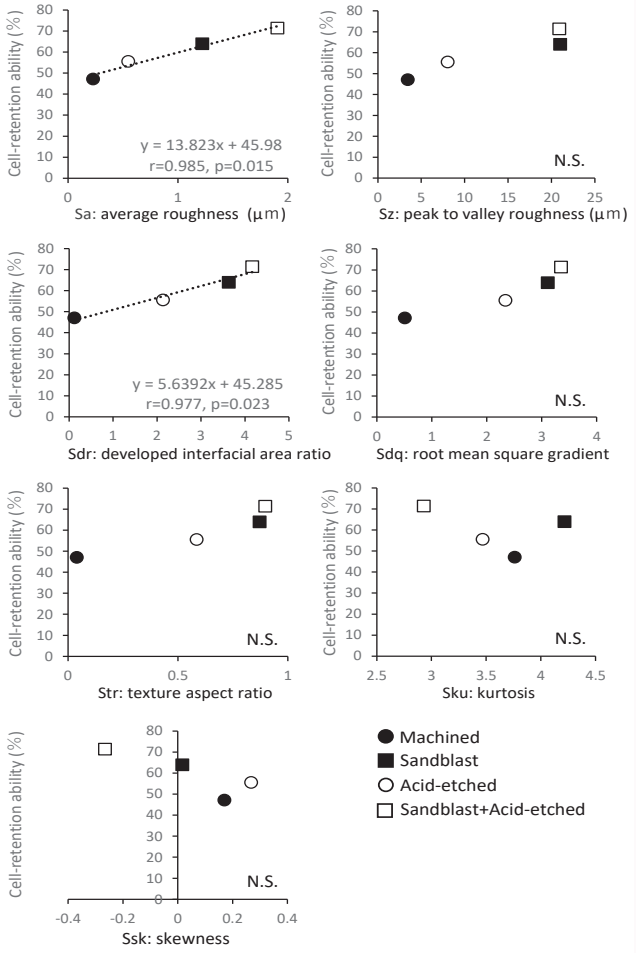

Figure 5: Linear equations and correlation coefficient between the cell-retention ability and the surface roughness (Sa, Sz, Sdr, Sdq, Str, Sku, and Ssk). N.S. Statistically non-significant correlation.

+ acid-etched surface as compared to the machined surface (Figure 3).

Cell-recruitment ability on four different titanium surfaces

The number of cells attached to titanium surfaces for 24 hours, with the sandblasted + acid-etched surface developing the least cell-recruitment ability, was 197 × 10^4 cells on the machined titanium surfaces, 165 × 10^4 cells on the sandblasted titanium surfaces, 178 × 10^4 cells on the acid-etched titanium surfaces, and 162 × 10^4 cells on the sandblasted + acid-etched titanium surfaces (Figure 4). Compared to the sandblasted + acid etched surface, the cell-recruitment ability on the machined titanium surface was more than 1.2 times greater. In addition, compared to the acid-etched surface, the cell-recruitment ability on the machined titanium surface was more than 1.1 times greater. The machined surface showed significantly greater values than the sandblasted and acid-etched surfaces or the sandblasted + acid-etched surface.

Correlation coefficient between cell-retention ability and surface roughness

There was a significant linear correlation between the cell-retention ability and the degree of surface roughness. Sa (average roughness) (R=0.985, p<0.0151) and Sdr (developed interfacial area ratio) (R=0.977, p<0.0226) were highly correlated with the cell-retention ability (Figure 5).

Correlation coefficient between cell-recruitment ability and surface roughness

The cell-recruitment ability during 24 hours incubation was negatively correlated with the degree of surface roughness. The cell-recruitment ability and Sdr (developed interfacial area ratio) (R= -0.998, p<0.0020), Sdq (root-mean-square roughness) (R= -0.995, p<0.0048), and Str (aspect ratio of the surface texture) (R= -0.996, p<0.0039) were significantly negatively correlated (Figure 6).

Discussion

In the present study, using a mechanical detachment test, we examined how the cell-retention ability changes at the titanium interface for four different surface morphologies. In addition, the relationship between the surface roughness and cell-retention ability was examined for four different surface roughnesses. The performance of the detachment assay on cultured osteoblasts revealed that the cell-retention ability was substantially increased on the rough surface as compared to the machined surface. The cell-recruitment ability revealed that cell proliferation was substantially reduced on the rough surface as compared to the machined surface. The obtained results indicate that the rough surface effectively promotes the cell-retention ability of titanium but does not effectively promote the cell-recruitment ability.

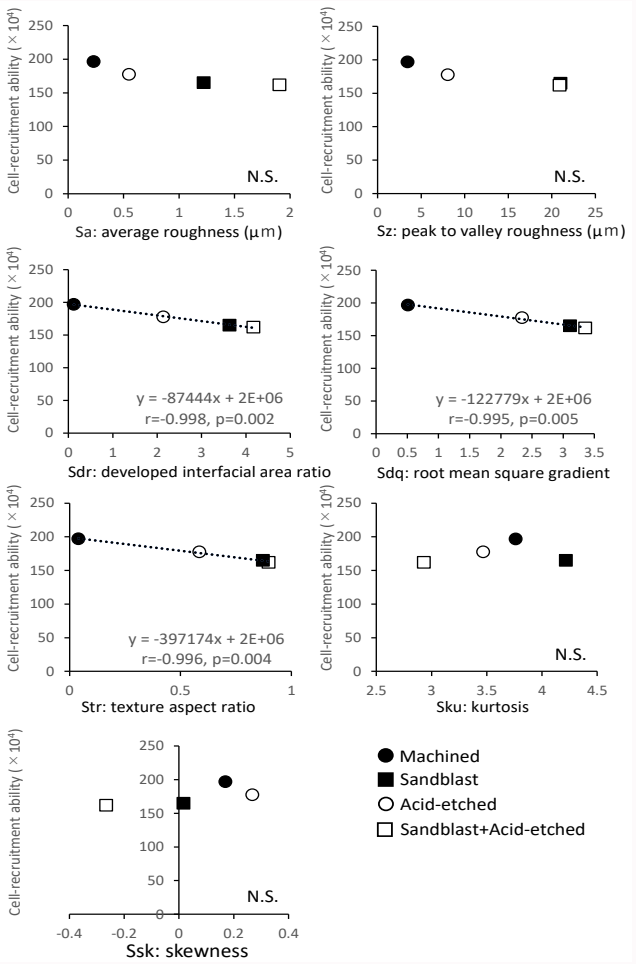

Figure 6: Linear equations and correlation coefficient between the cell-recruitment ability and the surface roughness (Sa, Sz, Sdr, Sdq, Str, Sku, and Ssk). N.S. :Statistically non-significant correlation.

We examined the cell-retention ability and cell-recruitment ability in the present study. The rat bone marrow cells used in the experiment of the present study are known to differentiate towards an osteoblast-like phenotype when supplemented with dexamethasone and β-glycerophosphate [17-20]. Osteoblast differentiation methods have been reported to use many kinds of reagents, including 1,25-dihydroxyvitamin D3, [21], hormones, [22,23] growth factors, [24,25] bone morphogenetic proteins [26,27], Aluminum Chloride (AlCl3) [28], Sodium Fluoride (NaF) [24], prostaglandins [29,30], β-glycerophosphate [31], and ascorbic acid [32]. There are also numerous reports on the timing of administering differentiation-inducing reagents to these cells [33-35]. Cells harvested from rat bone marrow were differentiated in the present study using an osteogenic induction medium containing ascorbic acid, β-glycerophosphate, and dexamethasone. Machined titanium surfaces are known to increase the cell-recruitment ability and the rate of cell proliferation in the early stages of cell culture, as compared to rough titanium surfaces [36,37]. This was observed in the present study, which is consistent with previous reports. The machined surface increased the cell-recruitment ability more than 1.22-fold as compared with those of the sandblasted + acid-etched micro-rough surface.

The results of the present *in vitro* study demonstrate a known principle of osteoblasts i.e., the inverted relationship between proliferation and differentiation for a rough surface [16]. The rate of proliferation is reduced on rougher biomaterial surfaces, although rougher surfaces have an advantage in that they promote cellular differentiation [36,38]. Similarly, roughened titanium surfaces promote osteoblastic differentiation better than machined, smooth surfaces and result in faster bone formation [36,38]. However, bone volume is ultimately reduced compared to that achieved with a machined surface owing to the reduction in osteoblastic proliferation. Consistent with this, several in vitro studies have shown that cell density and proliferative activity are reduced on rough titanium surfaces as compared with those on relatively smoother surfaces [39,40].

In the present study, a mechanical detachment test was performed on the assumption of implant vibration due to immediate loading of implants. The general consensus is that the rougher the surface, the stronger the osseointegration [39,40], which leaves the important question as to whether the roughness and the cell-retention ability are proportional, and, if so, what is the proportional relationship.

Assessing surface morphology using multiple parameters is always necessary in the field of material science, and, in fact, only Sa expresses the vertical profile of the surfaces. Since the acid-etched surface and the sandblasted surface exhibit irregular shapes compared to the machined surface, it is considered necessary to use Sa in combination with other parameters. The cell-retention ability and Sa and Sdr were nearly perfectly significantly positively correlated. Not only the vertical profile of the surface of Sa, but also the hybrid profile of the surface, i.e. Sdr, exhibit a high correlation. Sdr is a hybrid parameter that provides information about the number and height of peaks on an implant surface taking horizontal and spatial aspects of the surface roughness into consideration [41]. The cell-recruitment ability is significantly negatively correlated with the spatial parameter (Str) and the hybrid parameters (Sdr and Sdq). The value of Str, which ranges from 0 to 1, is a measure of the uniformity of the surface texture. Here, Str>0.5 indicates strong isotropy, whereas Str<0.3 indicates strong anisotropy. If Str is close to 1, the surface is direction independent. In sandblasted and sandblasted + acid-etched samples, Str was strongly isotropic, and the surface was direction independent. Moreover, Sdr is the percentage of the defined additional surface area contributed by the texture as compared to the projected, planar definition area. In addition, Sdq is the percentage of the root mean square of the slopes at all points in the defined area. These two parameters can take into account vertical, horizontal, and diagonal parameters. The increase in the cell-recruitment ability and the increase in the surface roughness had an inverse relationship. The spatial parameter i.e., Str, and the hybrid parameters i.e., Sdr and Sdq, were most involved in negative correlation. The machined surface and the acid-etched surface (Ssk>0) have numerous fine peaks, and the sandblasted surface (Ssk≈0) is symmetric with respect to the average line. The sandblasted + acid-etched surface (Ssk<0) has numerous fine valleys. The Sku>3 surface has many sharp peaks and valleys, and the Sku<3 surface is flat. For the sandblasted surface, Sku>3, and for the sandblasted + acid-etched surface, Sku<3. The sandblasted surface had many sharp peaks and valleys, whereas the sandblasted + acid-etched surface had sharp peaks produced by sandblasting and was melted and rounded by acid treatment.

Conclusion

Our findings indicate that the rougher the titanium surfaces, the stronger their cell-retention ability. The sandblasted + acid-

etched surface showed the greatest ability among the surfaces tested. However, the cell-recruitment ability was negatively correlated with surface roughness and was the smallest for sandblasted + acid-etched surfaces. Sdr was the most effective topographical determinant to predict both cell retention and recruitment abilities.

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