Surface properties and early murine pre-osteoblastic cell responses of anodized TiO₂ surfaces

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ABSTRACT

The noted anodic oxidation of titanium surfaces by the utilization of an electrochemical method is one of the procedures that can be used for the enhancement of osseointegration. This study evaluated the surface characteristics and the cell response of titanium samples that were modified by different treatment conditions. The samples were subsequently divided into 4 groups. In this study, Group I was noted as an anodized TiO₂ film using a constant voltage, 270 V for 30 sec. Further, Group II was noted as using an anodized TiO₂ film using a constant voltage, 270 V for 60 sec. Also, Group III was an anodized TiO₂ film using a constant voltage, 270 V for 90 sec. The control was a non-treated machined titanium surface. The results were as follows: the XRD analysis showed that the crystallinity of anodic oxide film was composed of anatase and rutile. The study continued with the procedure of increasing the time for anodization, whereby it was noted that the intensity of the TiO₂ peaks for anatase phase decreased, while the TiO₂ peak for the rutile phase was seen to have increased. In the MTT assay, it was noted that there was no significant difference in the response of fetal rat calvarial cells to the anodized titanium surfaces with the different treatment conditions. Similarly, the Group II and III showed higher ALP activity levels compared with the control and Group I (p<0.01). In the RT-PCR analysis, it is noted that the bone sialoprotein mRNA expression in Group II and III increased approximately 1.7-fold and 1.5-fold respectively, compared with the control, which was cp-Ti. The osteocalcin mRNA expression in Group II and III also increased approximately 1.6-fold and 1.7-fold respectively, compared with the control. Fundamentally, these results suggest that the anodized TiO₂ surfaces treated at 60 and 90 sec, should promote cellular activity of the noted and reviewed osteoblasts, as compared with the machined Ti surface.

KEY WORDS: Alkaline phosphatase level, Anodized TiO₂ surface, Osteoblast, Surface modification

Introduction

Titanium (Ti) is the most popular materials for dental and orthopedic implants due to their biocompatibility and mechanical properties which lead to successful clinical performance in implant dentistry [1]. However, it was also reported that bone response to implant surfaces was dependent on the chemical and physical properties of titanium surfaces, thereby affecting implant success [2]. Thus, many studies have been focused on the surface treatment

of the Ti implant.

Several techniques have been used to produce micro-rough Ti surfaces for promoting bone ingrowth and fixation between implants and bone. Among them surface blasting, acid-etching and combination of both are widely used methods to modify surface topography [3]. In addition to surface topography, surface chemistry is also important for peri-implant bone apposition.

Thin native oxide films formed on Ti surface spontaneously (1.5-10 nm), and titanium dioxide (TiO₂) forms a direct bond to bone tissues. However, the layer of naturally formed film is too thin to prohibit toxic metal ions such as aluminum and vanadium being released in the human body and inducing possible cytotoxic effect and neurological disorders [4,5]. Surface modifications are, therefore, indispensable for titanium and its alloys to form a thick surface oxide layer for enhanced corrosion and a

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bioactive layer for apposition and growth of bone cells.

Anodic oxidation of titanium surfaces by electrochemical method is one of the methods for solving above problems [6]. After anodic oxidation, titanium-based metals form bone-like apatite in simulated body fluid (SBF) which has ion concentrations nearly equal to human body fluid. This phenomenon also occurs on the surfaces of bioactive glass and glass ceramics [7,8]. Research showed that high degree of bone contact and bone formation was achieved with anodized titanium surface [9-11]. Saldana *et al.* reported that the ability of human osteoblasts to differentiate when cultured on thermally oxidized titanium alloy [12].

Osteoblasts are pivotal in their control of bone remodeling and a biological response would need to be demonstrated to clarify the action of anodized titanium *in vivo* and *in vitro*.

The characteristics of anodized Ti surface varied according to the anodizing conditions such as duration time and electrolytic compositions used [13]. And anodized Ti surface characteristics, such as surface morphology and thickness, may affect many cellular responses such as cell adhesion, morphology, proliferation, and differentiation. However, there is no comprehensive study with regard to interaction of osteoblasts with anodized titanium with different treatment conditions.

Thus, the purpose of this study was to evaluate the surface characteristics and cell response of titanium samples modified by different treatment conditions.

Material and Methods

Fabrication of anodized titanium surfaces

All specimens were kindly provided by the school of Materials Science and Engineering, Chonnam National University. Briefly, all commercially pure titanium (grade II, cp-Ti) disks were formed into disks 12 or 25 mm diameter and 1 mm thickness. Cp-Ti disks were ground with 240 grit silicon carbide papers. These disks were ultrasonically degreased in acetone and ethanol for 10 min each, with deionized water rinsing between applications of each solvent. The samples were then divided into 4 groups. Group I was anodized TiO₂ film using a constant voltage, 270 V for 30 sec. The disks were anodized using pulse power (650 Hz). The electrolyte solution contained 0.15 M calcium acetate and 0.02 M calcium glycerophos-

phate. Group II was anodized TiO₂ film using a constant voltage, 270 V for 60 sec. Group III was anodized TiO₂ film using a constant voltage, 270 V for 90 sec. The control was non-treated machined titanium surface. The control and test groups were examined with atomic force microscopy (AFM; Nano ScopeIIIa, DK Digital Instrument, Santa Barbara, USA) to provide surface roughness information on test surfaces. The surface morphology of anodized Ti disks and their cross-sections were observed by scanning electron microscopy (SEM; S-4700, Hitachi, Tyoko, Japan). The surfaces of anodized Ti disks were examined with x-ray diffractometer (XRD; DMAX/1200, Rigaku, Tyoko, Japan).

Evaluation of corrosion resistance

The samples for corrosion test were embedded in a room temperature curing epoxy resin leaving an exposure area of 10 × 1 mm². The control and test groups were exposed to the electrolyte. The electrolyte used was a phosphate buffered saline (PBS) at a room temperature. A three-electrode cell set-up was used with a saturated calomel electrode (SCE), a platinum wire as reference, and a counter electrode. A potentiodynamic polarization scan using a frequency response analyzer (Gamry model EIS 300, Warminster, PA, USA) coupled to a potentiostat PCI4/300, was acquired following 7 days of immersion in PBS.

Sample preparation for evaluation of biologic response

All disks were placed under aseptic conditions in the bottom of 12- or 6-well culture dishes, and then rinsed 3 times in 70% ethanol, exposed to UV light for 1 hr and air dried in a laminar flow.

Cell culture of fetal rat calvarial cells

Osteoblast-enriched cell preparations were obtained from Sprague-Dawley 21 day fetal calvaria by sequential collagenase digestion. The periosteum from newborn calvaria was removed and bone tissue was cut into small pieces with scissors. The pieces of calvarial bone were then digested with the mixture of enzyme containing of 0.5% type II collagenase (Type II; Invitrogen, USA) in phosphate buffered saline at 37°C. During sequential digestion period of 15 min, the cells from the 3rd to the 5th digestion were pooled and filtered with 200 µm plastic meshed screen and plated in 75 mm tissue culture plastic. Cells were cultured in BGJb media (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inacti-

vated fetal bovine serum (FBS), 100 mg/ml penicillin, and 100 mg/ml streptomycin at 37°C humidified atmosphere of 5% CO₂-95% air.

Cell viability test

Cells were cultured on machined Ti and three anodized TiO₂ surfaces in 12-well plates at a density of 1×10^5 cells/ml with BGJb medium. The control was cultured on tissue culture plate. After 24 hr, the medium was changed and the cells were cultured for additional 2 days. Following incubation, cell proliferation was assessed by MTT assay (CellTiter 96 AQueous, Promega, Madison, WI, USA). In these experiments, the amount of reduced formazan product was directly proportional to the number of viable cells. Formazan accumulation was quantified by absorbance at 490 nm by an enzyme-linked immunosorbent assay (ELISA) plate reader (microplate manager, BioRad, Richmond, CA, USA) and analyzed. All experiments were carried out in triplicate.

Alkaline phosphatase (ALP) activity

Determination of ALP activity was performed at day 7. For this purpose, fetal rat calvarial cells were seeded on machined Ti and three anodized TiO₂ surfaces in 12-well plates at a density of 1×10^5 cells/ml with BGJb medium, containing 10% FBS. To induce differentiation into osteoblasts of fetal rat calvarial cells, 40 µg/ml ascorbate and 20 µg/ml β-glycerol phosphate were added to the BGJb media. Briefly, cells were lysed in Triton 0.1% (Triton X-100) in PBS, then frozen at -20°C and thawed. One hundred microliter of cell lysates was mixed with 200 µl of 10 mM p-nitrophenol phosphate and 100 µl of 1.5 M 2-amino-2-methyl-1-propanol buffer, and then incubated for 30 min at 37°C. ALP activity was measured by absorbance reading at 405 nm with a spectrophotometer (SmartSpecTM,

Hercules, CA, USA). All experiments were carried out in triplicate.

Reverse transcription polymerase chain reaction (RT-PCR) gene expression analysis

Gene expression of mineralized matrix markers was evaluated by RT-PCR. All sample disks were placed under aseptic conditions in the 6-well tissue culture dishes. Then 2×10^5 cells/ml in BGJb medium containing 10% FBS were seeded into each well and incubated for 1 day. After an initial attachment period, the media was switched to mineralizing media containing 10% FBS, 40 µg/ml ascorbate and 20 µg/ml β-glycerol phosphate for 6 days and was changed every 3 days.

Total RNA was isolated at day 7 using the methodology described by the manufacturer. First-strand cDNA synthesis was carried out in an AmpliTron II thermocycler using SuperScript II (Invitrogen). PCR was subsequently performed for 30 cycles using amplification primer sets (Sigma-Genosys, USA) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), bone sialoproteins (BSP), collagen type-I (COL-I), and osteocalcin (OCN) genes as listed in Table 1.

RT-PCR products were separated and analyzed by agarose gel electrophoresis. Resulting images were captured using a Gel-Doc imaging system (BioRad) equipped with UV light and a gel scanner. Semiquantitative comparison GAPDH, an unregulated house keeping gene, was performed to assess changes in gene expression among all tested samples. All experiments were performed in duplicate.

Statistical analysis

An analysis of variance followed by Duncan's test was used to assess the data regarding surface roughness, cell proliferation and ALP activity. Statistical significance was

Table 1. Amplification primer sets and conditions used in polymerase chain reaction

Primer	Expected base pairs	Sequence (5'-3')
GAPDH-sense (+)	418	CACCATGGAGAAGCCGGGG
GAPDH-antisense (-)		GACGGACACATTGGGGGTAG
BSP-sense (+)	1068	AACAATCCGTGCCACTCA
BSP-antisense (-)		GGAGGGGGCTTCACTGAT
COL 1-sense (+)	250	TCTCCACTCTTCTAGGTTCCCT
COL-1-antisense (-)		TTGGGTCATTTCACATGC
OCN-sense (+)	198	TCTGACAAACCTTCATGTCC
OCN-antisense (-)		AAATAGTGATACCGTAGATGCC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BSP, bone sialoprotein; COL I, type 1 collagen; OCN, Osteocalcin.

defined as $P < 0.01$ (SPSS 2.0 SPCC Inc., Chicago, IL, USA).

Results

Surface characterization and roughness test

Fig. 1 showed SEM and AFM images of anodized Ti and control surfaces. Roughness measurements of the

Fig. 1. Overview and cross-sectional view of SEM images of anodized TiO_2 films with different treatment conditions. (A) TiO_2 films anodized for 30 sec under 270 V. (B) TiO_2 films anodized for 60 sec under 270 V. (C) Titanium oxide films anodized for 90 sec under 270V. *Red line: Thickness of anodic oxide film.

Table 2. Summary of atomic force microscopy (AFM) surface roughness of control and anodized Ti surfaces

Groups	RMS* Roughness (μm)
Control	0.27 ± 0.08
I	0.25 ± 0.05
II	0.28 ± 0.04
III	0.34 ± 0.07

Control: non-treated machined titanium surface. Group I: anodized TiO_2 film using a constant voltage, 270 V for 30 sec. Group II: anodized TiO_2 film for 60 sec. Group III: anodized TiO_2 film for 90 sec. *RMS: root mean square.

AFM information yielded root mean square (RMS) values of the surface topographies. The surface roughness had a tendency to increase when the anodizing time was increased (Table 2). In SEM observations, the anodic oxide films showed many overlapping micropores and microprojections. The anodic films were relatively uniform in the thickness. The thickness of anodic oxide films was 1.32 μm , 1.82 μm , and 2.54 μm respectively. The size and thickness of micropores increased as the anodizing time was increased.

Fig. 2 was shown the X-Ray diffraction patterns of anodic TiO_2 films formed in titanium surfaces. XRD analysis showed that the crystallinity of anodic oxide film was composed of anatase and rutile. With increasing time for anodization, the intensity of the TiO_2 peaks for anatase phase decreased, while TiO_2 peak for rutile phase increased.

Fig. 2. X-Ray diffraction patterns of anodized titanium in different treatment conditions.

Fig. 3. Potentiodynamic polarization curves for anodized titanium in different treatment conditions.

#1; The control, #2; Group I, #3; Group II, #4; Group III.

Fig. 4. Morphology of primary rat calvarial cells on Ti surfaces. (A) Primary rat calvarial cells on control surface. (B) Primary rat calvarial cells on titanium oxide films anodized for 30 sec. (C) Primary rat calvarial cells on titanium oxide films anodized for 60 sec. (D) Primary rat calvarial cells on titanium oxide films anodized for 90 sec.

Evaluation of corrosion resistance

In order to evaluate the corrosion protection by anodized TiO₂ films, potentiodynamic polarization test was performed. The polarization curves for anodized TiO₂ films were shown in Fig. 3. The corrosion protection of TiO₂ films was not increased by anodization. By comparison with the anodized TiO₂ films, there was no difference among different treatment conditions.

SEM images of cells on anodized Ti surfaces

Under SEM, cells adhered and grew well on the surfaces of all groups. No difference in cell morphology was observed in anodized Ti surfaces without regard to anodizing time. The cells spreaded extensively and totally flattened on all anodized Ti surfaces. They were in polygonal shapes and individual cells were flat in appearance. On cp-Ti surfaces, cells also spreaded polygonally and cell projections connecting cells were visible (Fig. 4).

Cell viability test

Cell viability was measured by the MTT assay. The cells in all groups proliferated actively within culture

Fig. 5. Cell viability test after 3 days on control, Group I, Group II, and Group III.

period, showing good cell viability. There was no significant difference in the response of fetal rat calvarial cells to anodized titanium surfaces with different treatment conditions. Although not statistically significant, cell proliferations of anodized Ti disks were lowered from 6 to 13% when compared to control (Fig. 5).

Alkaline phosphatase (ALP) activity

After seven days of culture, the cells were provided to measure the ALP activity. The ALP activity was shown in Fig. 6. The group II and III showed higher ALP activity levels compared with control and Group I ($p < 0.01$, Fig. 6). Group I had higher ALP activity level compared with control whereas, there was no significant difference between Group I and control.

RT-PCR gene expression analysis

After 7 days culture, the expression of COL-I, BSP, and OCN mRNA from cells were evaluated by RT-PCR. For the type I collagen gene (COL-I), the expression level of COL-I of control and test groups were consistent. In

Fig. 6. Alkaline phosphatase activity of primary rat calvarial cells on control.

Group I, Group II, and Group III at day 7 (U/ 100,000 cells).

*Significantly different compared to control ($p < 0.01$).

Fig. 7. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of (A) bone sialoprotein (BSP), (B) collagen type-I (COL-I), (C) osteocalcin (OCN), and (D) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in rat calvarial cells.

Group II and III, bone sialoprotein mRNA expression increased approximately 1.7-fold and 1.5-fold respectively, compared with control, cp-Ti. Osteocalcin mRNA expression in Group II and III also increased approximately 1.6-fold and 1.7-fold respectively, compared with control (Fig. 7).

Discussion

After implantation into the bone, oxidized Ti surfaces bind with water and formed $-OH$ sites which have a negative charge at physiological pH [6]. Oxide surfaces are known to spontaneously nucleate calcium phosphate layers (apatite) in contact with simulated body fluid [6,13]. Theoretically, the oxidized layer provides a kinetic barrier that prevents Ti from corrosion and provides bone implant materials that promote calcium phosphate crystal, protein, and cellular bonding [6]. However, TiO_2 surfaces are easily contaminated to organic and inorganic anions at the atmosphere within sec to 1 min, resulting in altered surface chemical composition and decreased surface energy [14].

Many studies have been performed to modify the surface characteristics of titanium implants for improving the osseointegration. One of surface modifications is an anodic oxidation that is a well-established surface modification technique for value metals [8-10, 13]. Anodized TiO_2 films may have different mechanical and chemical characteristics by different treatment conditions. The cellular response of osteoblasts to anodized TiO_2 films might respond differently according to treatment conditions.

Thus, in this study, anodized TiO_2 films were fabricated by different treatment times and cell response of primary rat calvarial cell on anodized TiO_2 films was investigated by scanning electron microscopy (SEM), cell proliferation, ELISA, alkaline phosphatase analysis, and reverse transcription polymerase chain reaction (RT-PCR) method.

Surface roughness can greatly affect the proliferation and protein synthesis of osteoblast cells that are cultured on a metal substrate characteristics in the healing of bone [15]. Many studies have demonstrated that roughness has a great influence on cell responses. According to this respects, the test samples used in this study were controlled that they had a similar roughness after they were polished.

Because the mechanical characteristics of anodized TiO_2 films are depends on its treatment conditions, the anodization time and voltage were very important. Based on the preliminary study, it was established the anodization voltage of 270 V and time for fabrication of anodized TiO_2 films went up to 90 sec. XRD analysis showed that the crystallinity of anodic oxide film was composed of anatase and rutile. Fig. 2 was shown that the intensity of the TiO_2 peaks for anatase phase decreased, while TiO_2 peak for rutile phase increased as increasing time for anodization. Therefore, as the anodizing time was increased, TiO_2 film was composed of rutile phase rather than anatase, and the cell size and diameter of the pore increased.

Although corrosion protection of three test groups was not enhanced by anodization, both the control and test groups formed stable passive layer on titanium surfaces. Thus, the result of corrosion test of titanium substrate proved the protective role of anodized TiO_2 films in this

study.

For each specimen, cell morphology was examined by the SEM. SEM study have provided some insight into the cell response to surface chemistry and morphology. In this study, it was shown that cells had spread extensively and flattened on control (non-treated machined titanium surface) and Group I-III anodized TiO₂ surface. The absence of significant morphological modification with different treatment conditions was indicated that anodized TiO₂ films were cytocompatible. These appearances of cells were in line with those observed in other studies [16,17] titanium and titanium alloys.

A MTT assay is a method for determining the number of viable cells in proliferation. MTS (or Owen's reagents) is bio-reduced by cells into a formazan that is soluble in tissue culture medium. The quantity of formazan product as measured by spectrophotometer is directly proportional to the number of living cells in culture. In this study, it was noted that, after three days incubation, there was no difference among control and test groups. But it was observed that cell proliferation on control was better than on the anodized TiO₂ surfaces. This result was in contrast to other reports [17,18] that anodized TiO₂ surfaces induced a significant increase in growth and proliferations. It was not clear why anodized TiO₂ surfaces slightly reduced cell proliferation than control in this study. A possible explanation of conflicting results was the type cells for studies. In most of studies, permanent cell lines such as MC3T3-E1 or MG63 cells, were used for cell proliferation assay whereas, the primary cells in this study, were used for cell proliferation. Whether primary cell or immortalized cell lines would be used is controversial. In this study, primary osteoblasts were obtained from fetal rat calvaria. This is an excellent source of osteoblasts because cells from young animals proliferate rapidly. Cells from the third, fourth and fifth digests were collected because these later digests provide a more pure culture, containing most cells that expressed an osteoblast-like phenotype [19]. Primary cell strains derived from living tissues are necessary and have been recommended by the ISO for specific testing to simulate the *in vivo* situation [20]. However, further studies are required to clearly understand the reason for the difference in cell proliferation.

Since ALP can mediate bone mineralization by decomposing phosphate compounds and stimulating the combination of phosphate and calcium in extracellular matrix, the ALP activity is used as a biomarker for expressing

osteoblast activity. Cells grown on Group II and III showed 80-90% higher alkaline phosphatase levels compared to those on control ($p < 0.01$, Fig. 6). This result indicates that anodized TiO₂ surfaces seemed to affect alkaline phosphatase activity. Enhancement of alkaline phosphatase activity on anodized TiO₂ surface was due to production of matrix vesicles, indicating a facilitation of osteoblastic differentiation. Matrix vesicles are extracellular organelles enriched in alkaline phosphatase specific activity and are associated with initial calcification *in vivo*. However, increased alkaline phosphatase activity did not present increased cell proliferation onto the chemical modification of Ti substrate because the data showed decreased cell proliferation on TiO₂ nanotubular and anodized TiO₂ surface, although it was statistically insignificant.

To study the possible molecular events that were affected by TiO₂ nanotubular and anodized TiO₂ surface, bone-associated markers (bone sialoprotein and osteocalcin) were evaluated by semiquantitative PCR. In PCR analysis, mRNA expression was analyzed at day seven. Type I collagen expression is an essential component of the extracellular matrix that is required before mineralized matrix formation. In this study, mRNA level of type I collagen on control, TiO₂ nanotubular, and anodized TiO₂ surface was consistent. This result indicated that control and TiO₂ films did not inhibit mineralized matrix expression of fetal rat calvarial cells. It is known that bone sialoprotein and osteocalcin are secreted by osteoblasts and regulate calcification [21]. Cells on anodized TiO₂ surfaces increased bone sialoprotein mRNA and osteocalcin RNA expression than cells on cp-Ti. Studies on the developmental expression of bone sialoprotein have shown that bone sialoprotein mRNA is expressed at high levels by osteoblasts at the onset of bone formation, and under steady-state conditions *in vitro* bone sialoprotein nucleates hydroxyapatite crystal formation indicating a role for this protein in the initial mineralization of bone. This study showed that, in Group II and III, bone sialoprotein and osteocalcin mRNA expression increased approximately 1.5-fold to 1.7-fold, compared with control. Therefore, in this study, increased mRNA expression level of bone sialoprotein and osteocalcin together with increased activity of alkaline phosphatase indicated a more differentiated phenotype of osteoblasts cultured on anodized TiO₂ surface.

In summary, this study was shown that anodized TiO₂ surfaces would promote cellular activity of osteoblasts,

potentially contributing to rapid osseointegration. However, cellular response of osteoblasts was different according to different treatment times, thickness of anodized TiO₂ films. Thus, further studies will be needed to elucidate the relation between thickness of TiO₂ film and cellular response before clinical applications are considered.

Conclusions

The characteristics of anodized Ti surface varied according to the anodizing conditions such as duration time and electrolytic compositions used. And anodized Ti surface characteristics, such as surface morphology and thickness, may affect many cellular responses such as cell adhesion, morphology, proliferation, and differentiation.

Thus, this study was to evaluate the surface characteristics and cell response of titanium samples modified by different treatment conditions. The samples were divided into 4 groups. Group I was anodized TiO₂ film using a constant voltage, 270 V for 30 sec. Group II was anodized TiO₂ film using a constant voltage, 270 V for 60 sec. Group III was anodized TiO₂ film using a constant voltage, 270 V for 90 sec. The control was non-treated machined titanium surface.

The results were as follows;

1. The size and thickness of anodized TiO₂ micropores increased as the anodizing time increased. XRD analysis showed that the crystallinity of anodic oxide film was composed of anatase and rutile. With increasing time for anodization, the intensity of the TiO₂ peaks for anatase phase decreased, while TiO₂ peak for rutile phase increased.

2. The corrosion protection of TiO₂ films was not increased by anodization. By comparison with the anodized TiO₂ films, there was no difference among different treatment conditions.

3. Under SEM, no difference in cell morphology was observed in anodized Ti surfaces without regard to anodizing time.

4. In MTT assay, there was no significant difference in the response of fetal rat calvarial cells to anodized titanium surfaces with different treatment conditions.

5. The Group II and III showed higher ALP activity levels compared with control and Group I ($p < 0.01$).

6. In RT-PCR analysis, bone sialoprotein mRNA expression in Group II and III increased approximately 1.7-fold and 1.5-fold respectively, compared with control, cp-Ti.

Osteocalcin mRNA expression in Group II and III also increased approximately 1.6-fold and 1.7-fold respectively, compared with control.

These results suggest that anodized TiO₂ surfaces treated at 60 and 90 sec should promote cellular activity of osteoblasts compared with treated at 30 sec and machined Ti surface.

Conflict of Interest

The authors declare that they have no competing interests.

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