The Effect of Bio-Conditioning of Titanium Implants for Enhancing Osteogenic Activity

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Early and effective integration of titanium-based materials into bone tissue is of vital importance for long-term stability of implants. Surface modification is commonly used to enhance cell-substrate interactions for improving cell adhesion, proliferation, and activity. Here, the surface of titanium substrates and commercial implants were coated with blood (TiB), fetal bovine serum (TiF), and phosphate-buffered saline (TiP) solution using a spin coating process. Surface roughness and wettability of samples were measured using contact angle measurements and atomic force microscopy. The samples were then exposed to human osteoblast-like MG63 cells in order to evaluate adhesion, growth, differentiation, and morphology on the surface of modified samples. Untreated titanium disks were used as controls. The lowest roughness and wettability values were found in unmodified titanium samples followed by TiP, TiF, and TiB. The percentage of cellular attachment and proliferation for each sample was measured using an MTT (3-[4,5-dimethylthiazol-2yl] 2,5diphenyl-2H-tetrazoliumbromide) assay. Cell adhesion and proliferation were most improved on TiB followed closely by TiF. The results of this study revealed an increased expression of the osteogenic marker protein alkaline phosphatase on TiB and the coated commercial titanium implants. These results suggested that precoating titanium samples with blood may improve cellular response by successfully mimicking a physiological environment that could be beneficial for clinical implant procedures.

Key Words: biological modification, titanium, ossteoinduction, osteoblast-like MG63 cells, dental implant

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INTRODUCTION

Over the decades, titanium and its alloys have been widely used as implant biomaterials for bone tissue engineering applications. Titanium-based implants have been particularly successful due to their proper mechanical strength, corrosion resistance, and biocompatibility. Since there is limited bonding between titanium surfaces and living tissue, improving cell-substrate interactions has remained a challenge for bone biomaterials.[1,2] One approach researchers have taken to solve this problem is by modifying the titanium surface.

Physical, chemical,[1,2] and biological modification[3] of biomaterial surfaces has been shown to lead to accelerated bone formation and higher tissue compatibility.[4] Biological modification, in particular, works toward fulfilling the requirement for enhancing cell adhesion and differentiation in order to achieve faster bone formation. Biological surface modifications include application of proteins.[5,6] growth factors,[7] glycoproteins, glycosaminoglycans,[8] complex media such as platelet-rich plasma,[9,10] peptide sequences,[11,12] fetal bovine serum (FBS),[8,13] and blood.[9,14] Many studies on the biological surface treatment of Ti-based implants have confirmed that osteoblastic cell attachment and proliferation can be improved remarkably in the modified surfaces.[14] Kopf et al[14] found that pre-incubation of titanium substrates with blood could increase surface wettability resulting in enhanced cell migration, attachment, and osseointegration. Himmlova et al[8] used plasma spray to coat 3 blood components, including serum, plasma/platelets, and activated plasma, on the titanium surface. They revealed that the activated plasma coating could provide a uniform surface coating with high osteoblastic proliferation.[10] Auernheimer et al[15] coated titanium surface with an anisidine value–specific cyclic arginylglycylaspartic acid (RGD) peptide. This peptide has the ability to specifically bind to superfamily integrins, including avb3 and avb5. When a novel anchor system was used it was noticed that the attachment of MC3T3-E1 mouse osteoblast cells was increased on the coated materials.[16] Osteoblast cells would selectively bind to RGD peptide sequences through their integrin receptors, leading to increased cell attachment to the material surface. Yoo et al[17] used bone morphogenetic protein 2 and dip coating to modify the titanium surface. Their in vivo study showed improved osseointegration compared with the unmodified samples. Nagai et al[18] demonstrated that collagen coatings could substantially improve initial attachment and activity of human gingival fibroblasts. Although these studies represented the positive effects of biological surface modification on cell

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attachment and proliferation, there is no direct evidence for selection of a material that is more suitable for clinical use.

In the present study, the surface of titanium substrates was coated with blood, FBS, and phosphate-buffered saline (PBS) using spin coating. The surface roughness and topography and the surface wettability were examined by atomic force microscopy (AFM) and contact-angle measurements, respectively. The substrates were then exposed to human osteoblast-like MG63 cells to evaluate their attachment, proliferation, morphology, alkaline phosphatase activity, and total protein content in contact with the coated titanium samples. As the surface of titanium substrates was different from the surface of commercial dental implants commonly used for clinical purposes, the cell adhesion experiment was also performed on the surface of commercial titanium implants (Implant Diffusion International [IDB], Montreuil, France).

Materials and Methods

Materials

The titanium substrates (sheets of Ti–6Al–4V) were commercially available (McMaster-Carr Company, Los Angeles, Calif). Dulbecco's modified eagle medium (DMEM) and tripsin were purchased from Gibco-BRL (Cergy Pontoise, France). MTT (3-[4,5-dimethylthiazol-2yl] 2,5diphenyl-2H-tetrazoliumbromide), FBS, bovine serum albumin, PBS and penicillin/streptomycin were purchased from Sigma-Aldrich (Buchs, Switzerland). Dental implants were obtained from implant diffusion international, Montreuil, France.

Sample coating and preparation

The titanium substrates were cut into discs 13 mm in diameter and 1 mm thick. They were mechanically polished with P1000 silicon carbide paper and subsequently cleaned in an ultrasound bath with ethanol, acetone, isopropanol, and distilled water for 20 minutes each to remove surface contaminants. The samples were autoclaved at 121°C for 30 minutes and dried at 70°C in a conventional oven before coating. The titanium disks were then spin coated with human blood (from a healthy donor), FBS, and PBS for 60 seconds at 2500 rpm. Coated samples will be referred to as TiB, TiF, and TiP, respectively. The coating process was repeated a minimum of 5 times on each sample to obtain a homogeneous and uniform coverage of the coating materials on the samples. All samples were exposed to utraviolet light for 20 minutes to sterilize them before cell culture experiments.

Surface characterization

Surface wettability of coated and uncoated titanium samples was determined by the sessile-drop contact-angle method (OCA 15 plus; Data Physics Corporation, San Jose, Calif) at room temperature. A small deionized water droplet (0.4 μL) was placed on the surface of each sample, and the angle between the droplet and surface was measured for 5 seconds. This process was repeated with 5 more droplets. Surface roughness of the samples was analyzed by AFM (Veeco Instruments Inc, Woodbury, NY). The AFM images were processed using NanoScope Analysis software (version 1.40r1, Bruker, Mass) to provide quantitative 3-dimensional topographical structure. The dimensional roughness parameters were then calculated.

Cell culture

Human osteoblast-like MG63 cells were used for this study and cultured in DMEM (Gibco-BRL) containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL penicillin streptomycin (Gibco-BRL). Cell suspension was plated in a cell culture dish and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was refreshed every 2 days. Cultured cells were detached by trypsinization, suspended in new culture medium, and used for designed experiments.

Cell attachment and proliferation

An MTT assay was used to determine the number of viable adherent cells and the rate of cell proliferation on the surface of samples. MG63 cells were seeded (15 000 cells/mL for proliferation, 50 000 cell/mL for attachment) on each titanium sample in 24-well tissue culture polystyrene plates. After each time point, the samples were transferred to new cell culture plates and washed with PBS (pH 7.4) to remove the non-adherent cells. One milliliter of fresh medium and 0.5 mg/ mL MTT were added to each well, and the plates were incubated for 3 hours to form formazan. After 24 hours of incubation the formazan crystals were dissolved in solubilizing solution and transferred to a 96-well plate. Absorbance of each solution was read at a wavelength of 570 nm with subtraction of 650 nm background using a ultraviolet-visible spectrophotometer. The viable adherent cells and cell proliferation rate on each sample was compared with the control (uncoated titanium sample).

Cell morphology on titanium samples

The samples were placed into a 24-well plate, and cells were seeded onto their surface with a final density of 10 000 cells per well. After culturing for 3 days, media was removed, and samples were washed with PBS. They were subsequently fixed with 2.5% glutaraldehyde (weight/volume) for 24 hours at 4°C. After 3 rinses with PBS, the samples were dehydrated in a sequential series of ethanol solutions (30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) for 15 min each and air-dried at room temperature for 24 hours. The samples were then sputter-coated with gold, and the cell morphology was examined using scanning electron microscopy (SEM; EM3200, Madell Technology, Riverside, Calif).

Alkaline phosphatase activity and total protein content

The osteogenic activity of osteoblasts was evaluated by alkaline phosphatase (ALP) activity after 7, 14, and 21 days of incubation on the surface of samples. The ALP activity was assessed using ALP assay kit (Colorimetric; Abcam, Cambridge, UK) according to manufacturer's protocol. Briefly, cells were grown on 24-well culture plates with a density of 10^4 cells/well. The cells were washed with PBS and scraped off the surfaces by adding lysis buffer. After sonication and centrifugation, aliquots of cell lysis solution were collected for analysis of ALP activity and total protein level. The ALP activity was determined with respect to the release of p-nitrophenol from p-nitrophenylphosphate substrate.

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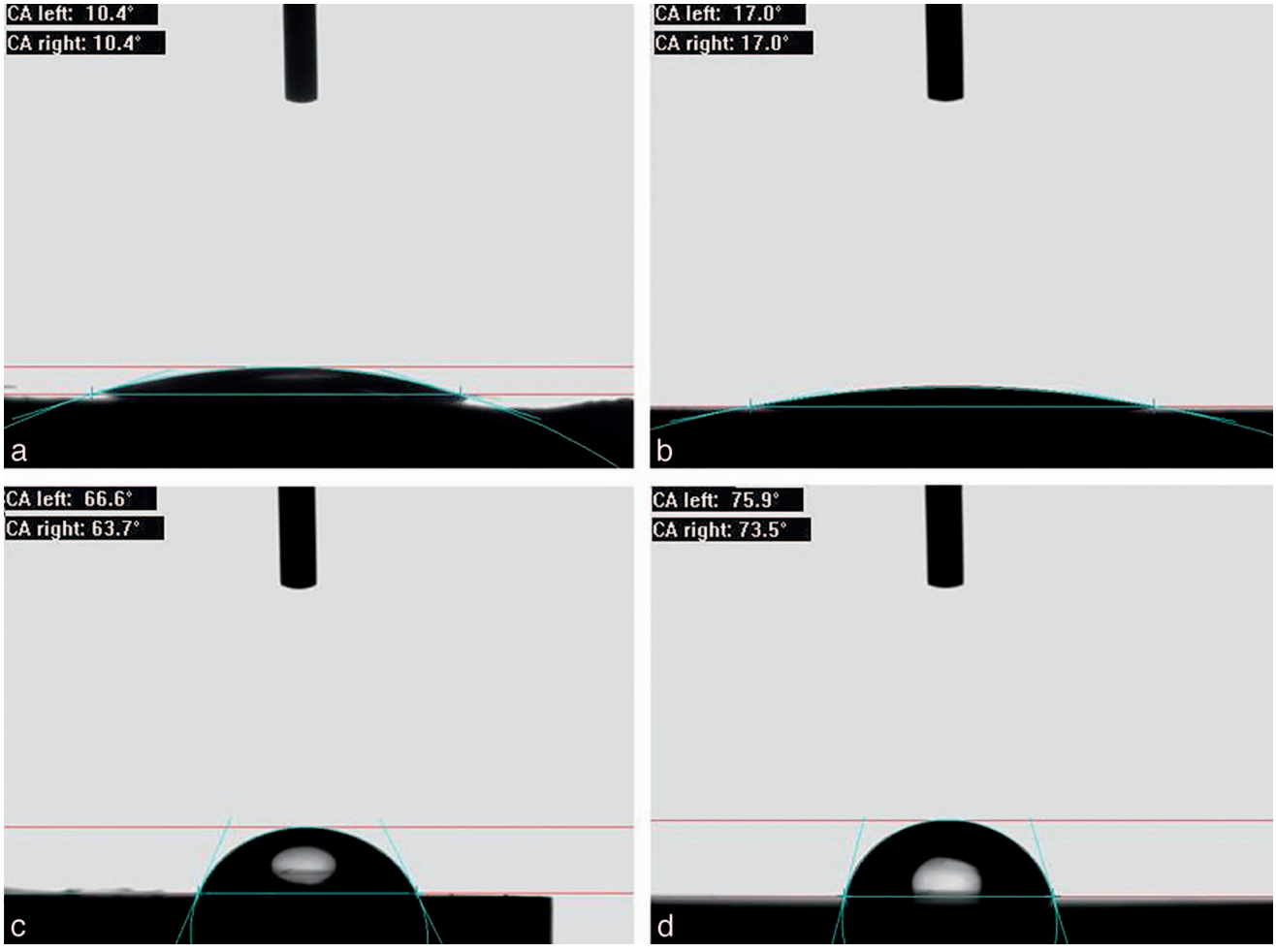

Figure 1. Water contact angle measurements of (a) blood precoated titanium, (b) fetal bovine serum precoated titanium, (c) phosphate-buffered saline precoated titanium, and (d) uncoated titanium (control) samples.

Each reaction was initiated by adding 50 μL of p-nitrophenyl-phosphate to the cell lysis solution. The reaction was stopped after 60 minutes by adding stop solution. Optical density was measured at 405 nm to quantify the amount of p-nitrophenol produced. The ALP activity values were normalized by the total protein content, which was obtained from the same cell lysate. Total protein content was determined using the bicinchoninic acid protein assay kit (EMD Millipore Company, Darmstadt, Germany). Absorbance was measured at 562 nm using Thermo Scientific Multiskan GO Microplate spectrophotometer (Thermo Scientific, Pittsfield, Mass) and the protein content values were obtained from a standard curve. After each time point the number of cells was also measured and used for normalization.

Cell morphology on dental implant

As mentioned earlier, the surface treatment, surface topography and roughness of commercial implants commonly used in clinics are vastly different from materials that are used for laboratory experiments. Therefore, after in vitro experiments with coated titanium substrates, commercial titanium implants were coated in a similar fashion with blood, FBS, and PBS to be used in cell culture experiment. At harvest, the culture media was removed and the samples were rinsed 3 times with PBS, fixed with 2.5% glutaraldehyde, dehydrated in a sequential series of ethanol solutions, coated with gold, and examined with SEM. The cellular morphology on the coated implants was compared with uncoated implant used as control.

Statistical analysis

Each experiment was performed at least in triplicate. All results are summarized as means ± standard deviation; statistical differences were determined by analysis of variance followed by independent sample *t*-test using SPSS software version 21 (SPSS Inc, Chicago, Ill). Results were considered statistically significant when $P < .05$. The methodology was reviewed by an independent statistician.

Results

Surface roughness and wettability

The hydrophilicity of coated and uncoated titanium samples was investigated by contact-angle measurement as shown in Figure 1. The contact angles of the samples were significantly

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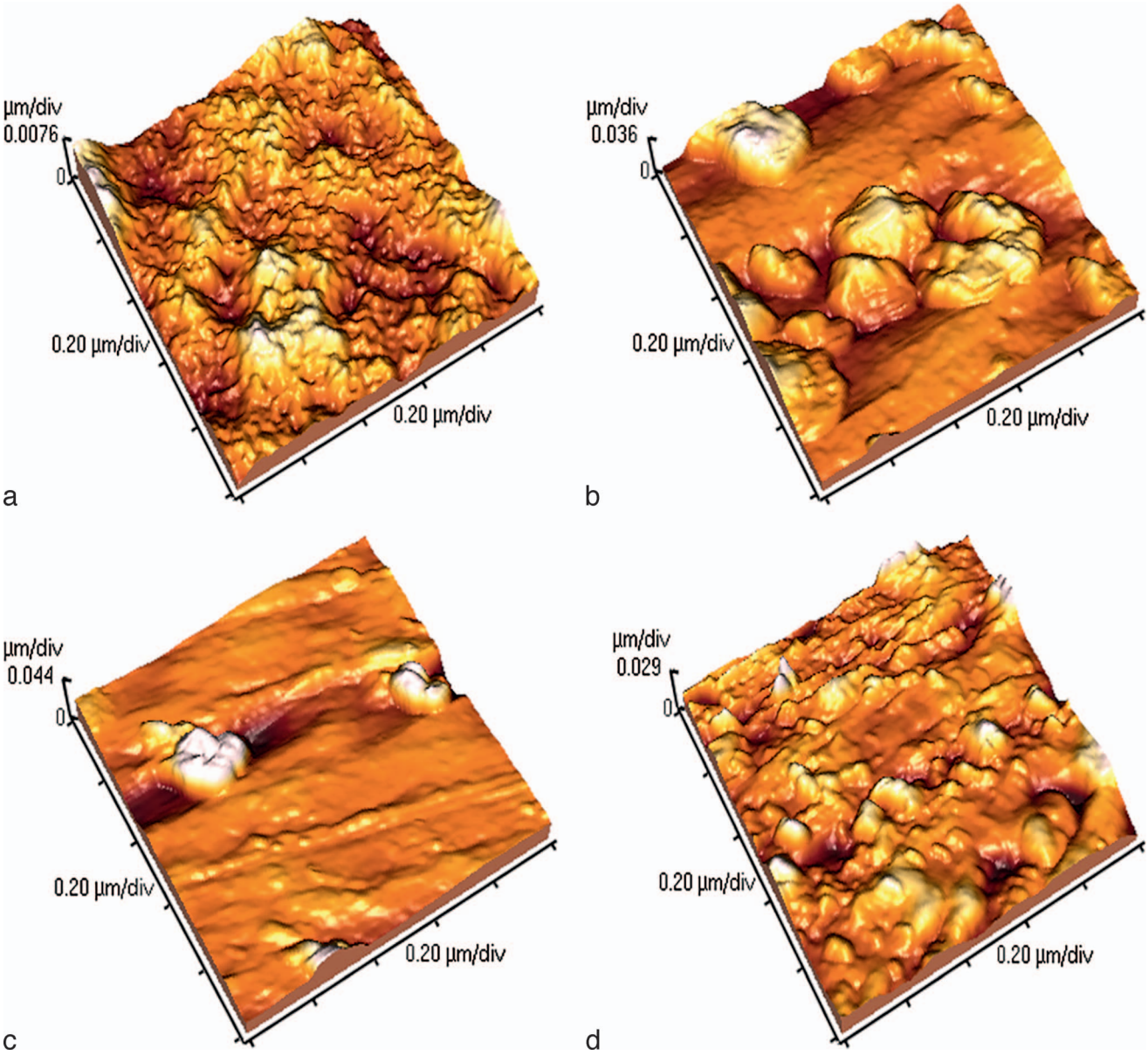

Figure 2. Atomic force microscopy images and root mean square roughness values of (a) fetal bovine serum precoated titanium, (b) blood precoated titanium, (c) phosphate-buffered saline precoated titanium, and (d) control samples.

Table 1

Roughness (means and standard deviations), and wettability values (means and standard deviations) of different samples and control†

Sample	Contact Angle° (Mean)	Contact Angle° (SD)	R_a (µm) (Mean)	R_a (µm) (SD)
TiB	10.4*	0.747	5.87 ± 0.12** nm	0.724
TiF	17.0	0.886	4.18 ± 0.14 nm	0.933
TiP	65.2	2.565	3.88 ± 0.21 nm	1.169
Control	74.7	3.683	3.38 ± 0.11 nm	1.341

*$P < .05$ compared with control, TiP, TiF, and control.

**$P < .05$ compared with TiP, TiF, and control.

†TiB indicates blood precoated titanium; TiF, fetal bovine serum precoated titanium; TiP, phosphate-buffered saline precoated titanium.

decreased on the samples coated with blood (TiB). The control sample surface had a contact angle of 74.7°± 0.12 compared with 10.4° ± 0.15 for the TiB surface (Table 1). The surface topography of different samples was evaluated via AFM, and the images are shown in Figure 2 and values for R_a are reported in Table 1. The roughness increment was found to be 5.87 ± 0.12 nm for TiB, 4.18 ± 0.14 nm for TiF, 3.88 ± 0.21 nm for TiP, and 3.38 ± 0.11 nm for control. The highest roughness was observed on the surface of TiB sample.

Cell attachment and proliferation

The MTT assay involves a reduction reaction that reduces MTT reagent to a blue formazan product when incubated with viable cells, and the absorbance of formazan indirectly reflects

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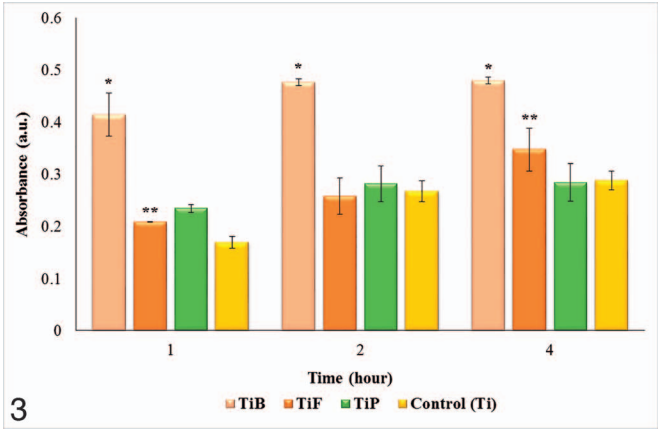

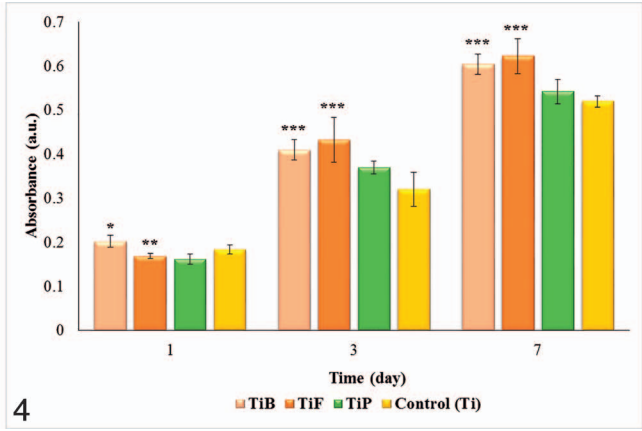

Figures 3 and 4. Figure 3. The MG63 osteoblast cell adhesion on titanium surfaces after 1, 2, and 4 hours. Data are presented as mean ± standard deviation; n = 3. *$P < .05$ compared with control, phosphate-buffered saline precoated titanium (TiP), and fetal bovine serum precoated titanium (TiF). **$P < .05$ compared with control. **Figure 4.** Cell proliferation of MG63 cells after 1, 3, and 7 days on titanium surfaces was measured with MTT assay. Each bar represents the mean of cell proliferation ± standard deviation (n = 3). *$P < .05$ compared with control, phosphate-buffered saline (PBS), and fetal bovine serum (FBS). **$P < .05$ compared with control. ***$P < .05$ compared with control and PBS. MTT indicates 3-(4,5-dimethylthiazol-2yl) 2,5diphenyl-2H-tetrazoliumbromide; TiB, blood precoated titanium.

the level of cell viability in the culture. Figure 3 shows the number of viable MG63 cells that adhere to the sample surface. After 1 hour of culture, cell adhesion to the TiB surface was significantly increased compared with the other samples. After 4 hours, the cell adhesion to TiB and TiF surfaces was significantly higher than that of uncoated samples. At all time points, cell adhesion on the surface of TiB was higher than all the other samples ($P < .05$). The mean value and standard deviation of each samples after 1, 3, and 7 days of culture are reported in Table 2.

Cell proliferation on the surface of samples was also evaluated via MTT assay after 1, 3, and 7 days of cell culture. Titanium samples without any coating were used as controls. After 1 day of culture, the number of cells that were grown on the surface of TiB was higher than that of other samples except the control (Figure 4). After 3 days, the number of cells on TiB and TiF samples was higher than that of other sample and the control. After 7 days, the TiB and TiF surfaces displayed significantly higher numbers of grown cells ($P < .05$). Table 3 demonstrates the mean value and standard deviation of each sample. As mentioned earlier, the fibrin networks and the existing proteins in FBS and blood would improve surface hydrophilicity and surface roughness, which provides more suitable condition for cells to adhere and proliferate on the TiB and TiF samples.

Cell morphology

Morphology of MG63 cells after culturing for 2 days on different titanium samples was evaluated via SEM as shown in Figure 5. The images indicate better interaction of cells with the surface of TiB and TiF as a large number of prominent filopodia and lamellipodia extensions were observed.

Alkaline phosphatase activity

The identification and characterization of osteoblast-like cells is based on the assay of specific metabolites. The activity of ALP, a membrane enzyme, is commonly used in in vitro studies as a relative marker of osteoblastic differentiation. Osteoblastic cell differentiation was assessed by measuring ALP activity, which was normalized to total protein content of the cells (Figure 6). According to the results, the ALP activity of MG63 cells cultured on TiP did not show any significant changes between day 7 and 14 compared with the control. In contrast, TiB showed a higher ALP activity on day 7 which increased further by day 14. The results also showed a significant difference in ALP activity between TiB and TiF, after 14 and 21 days of cell culture. ($P <$

Table 2

Means and standard deviations of MG63 osteoblast cells adhesion on coated samples and control after 1, 2, and 4 hours*

Sample	1 Day (Mean ± SD)	3 Days (Mean ± SD)	7 Days (Mean ± SD)
TiB	0.202 ± 0.0132	0.409 ± 0.0233	0.604 ± 0.0229
TiF	0.168 ± 0.0376	0.424 ± 0.0571	0.622 ± 0.0394
TiP	0.161 ± 0.0111	0.369 ± 0.0138	0.542 ± 0.0282
Control	0.183 ± 0.0105	0.321 ± 0.0387	0.520 ± 0.0129

*TiB indicates blood precoated titanium; TiF, fetal bovine serum precoated titanium; TiP, phosphate-buffered saline precoated titanium.

Table 3

Means and standard deviations of MG63 cell proliferation on coated samples and control after 1, 3, and 7 days*

Sample	1 Hour (Mean ± SD)	2 Hours (Mean ± SD)	4 Hours (Mean ± SD)
TiB	0.414 ± 0.0415	0.477 ± 0.0063	0.480 ± 0.0068
TiF	0.209 ± 0.0005	0.258 ± 0.0346	0.347 ± 0.0410
TiP	0.235 ± 0.0075	0.282 ± 0.0342	0.284 ± 0.0358
Control	0.169 ± 0.0115	0.268 ± 0.0203	0.288 ± 0.0181

*TiB indicates blood precoated titanium; TiF, fetal bovine serum precoated titanium; TiP, phosphate-buffered saline precoated titanium.

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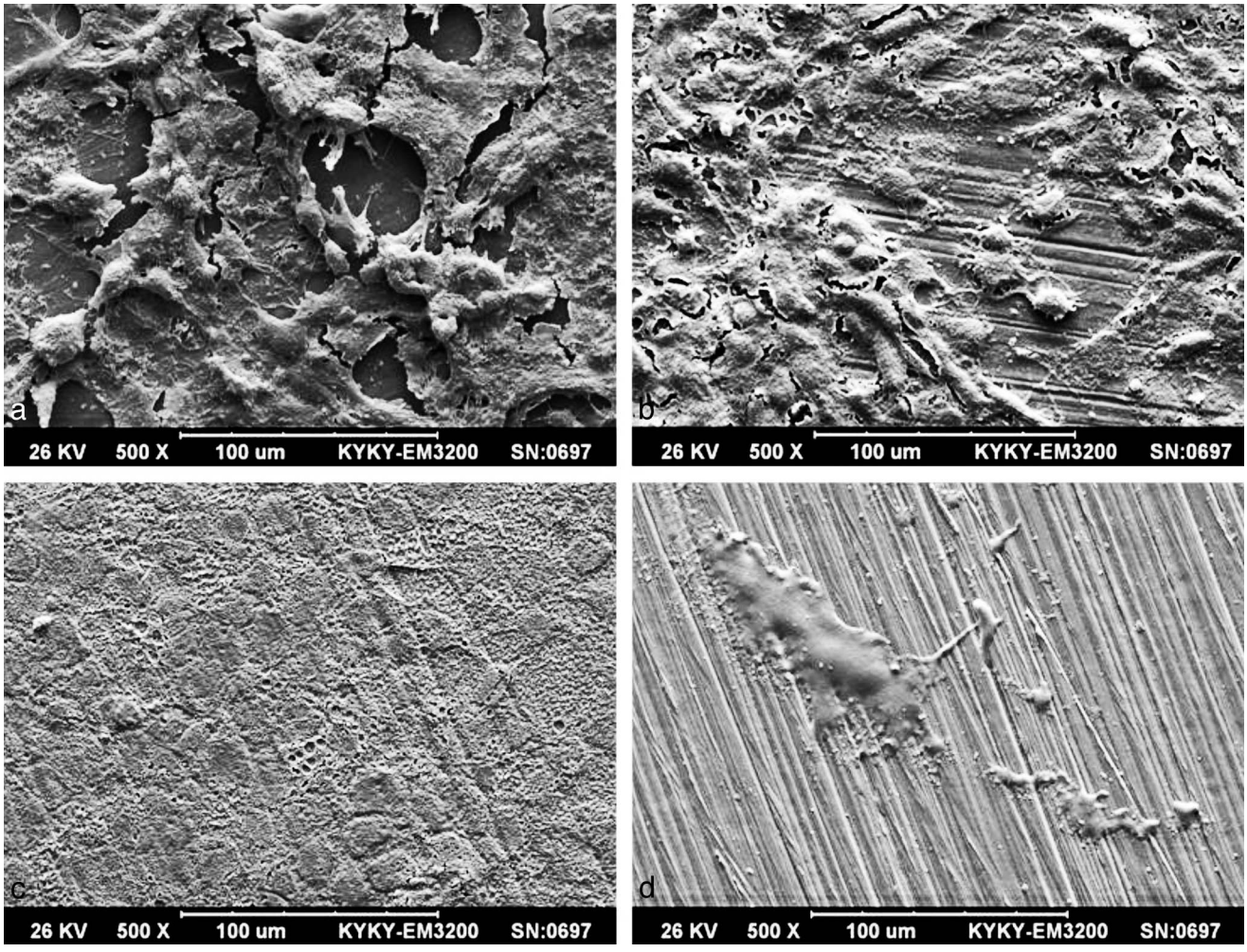

Figure 5. Scanning electron microscopy images of MG63 cells cultured on titanium samples after 2 days of incubation: (a) blood precoated titanium, (b) fetal bovine serum precoated titanium, (c) phosphate-buffered saline precoated titanium, and (d) control.

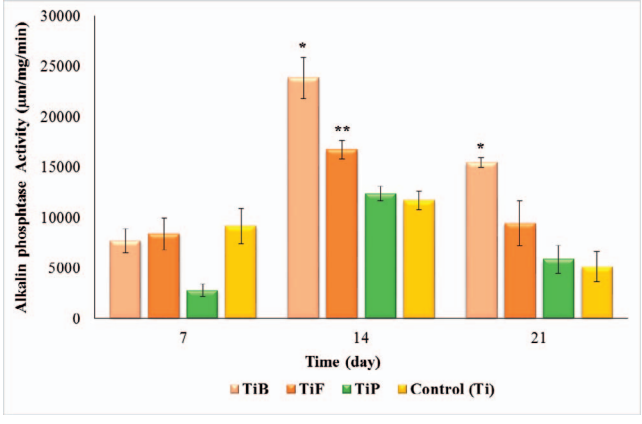

Figure 6. Alkaline phosphatase activity (normalized to total protein contents) of MG63 osteoblasts on different titanium samples after 7, 14, and 21 days. *$P < .05$ compared with control, phosphate-buffered saline precoated titanium (TiP), and fetal bovine serum precoated titanium (TiF). **$P < .05$ compared with control and TiP. TiB indicates blood precoated titanium.

.05). The descriptive statistics of coated samples and control are shown in Table 4.

Cell morphology on dental implants

The surface treatments of commercial dental implants are different from the treatments that are performed in laboratories, which could affect the surface topography and roughness of commercial implants. Therefore, after in vitro experiments with coated titanium substrates, commercial titanium implants (IDB) were used and coated with the same coating materials (blood, FBS, and PBS) and examined for cell-substrates interactions by SEM. The morphology of MG63 cells after culturing for 2 days on the IDB implants is shown in Figure 7. All results were reviewed by an independent statistician.

Discussion

Titanium implants generally provide good biofunctionality and biocompatibility in order to replace and restore functions of diseased and damaged tissues. Many studies have focused on improving the interaction between osteoblastic cells and a

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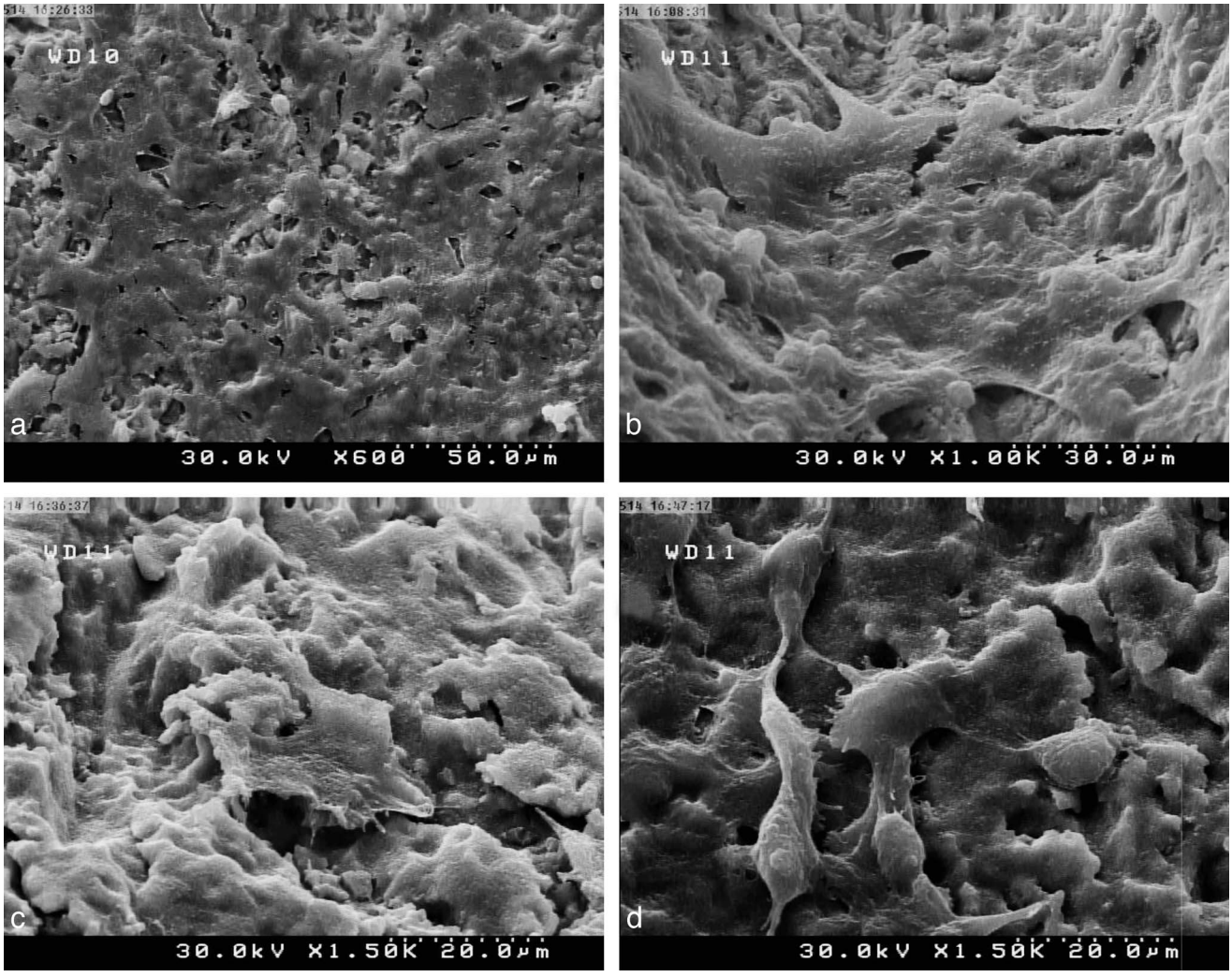

Figure 7. Scanning electron microscopy images of MG63 cells cultured on commercial DBI titanium implants after 2 days of incubation, (a) indicates blood precoated titanium, (b) fetal bovine serum precoated titanium, (c) phosphate-buffered saline precoated titanium, and (d) implants without coating (control).

biomaterial via surface modification. However, the interactions between blood and FBS precoated titanium surface is relatively unexplored. In this study, therefore, after analyzing the properties of the titanium surfaces, we intended to evaluate the effect of Mg63 cell attachment, morphology, proliferation, and differentiation on titanium disks precoated with blood, FBS, PBS, and pristine surfaces. The adhesion of Mg63 cell on the implant surfaces without/with blood, FBS, and PBS coatings was monitored as well. To do this, the basic cellular model coating of a biological layer on the titanium and implant surface was used to mimic the environment immediately upon implantation.[16,19]

According to the AFM analysis, the highest roughness was observed on the surface of the TiB sample. The enhanced surface hydrophilicity and roughness on the surface of the TiB could be due to the blood fibrin network on the surface of this

Table 4

Mean values and standard deviations of alkaline phosphatase activity of MG63 osteoblasts on coated samples and control after 7, 14, and 21 days*

Sample	7 Days (Mean ± SD)	14 Days (Mean ± SD)	21 Days (Mean ± SD)
TiB	7725 ± 1203.32	23818.75 ± 2020.41	15450 ± 447.958
TiF	8368.75 ± 1580.71	16737.5 ± 904.64	9437.5 ± 2236.300
TiP	2800 ± 626.09	12381.25 ± 730.58	5881.25 ± 1379.164
Control	9162.5 ± 1744.32	11716.25 ± 906.35	5150 ± 1511.9524

*TiB indicates blood precoated titanium; TiF, fetal bovine serum precoated titanium; TiP, phosphate-buffered saline precoated titanium.

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sample, which influences the absorption of proteins involved in osteoblastic cells attachment.

The reported increased adhesion to rougher surfaces has been explained with the increase of available surface area for adhesion. It is known that shortly after a surgical procedure the implant surface will contact with blood. This leads to build up of a layer consisting of plasma proteins, such as vitronectin, immunoglobulins, fibrinogen, and fibronectin.[16,20] The protein layer also contains important cell-adhesion promoting and cell-activating ligands, such as fibrinogen, which is a precursor molecule of fibrin. It has been demonstrated that fibrin plays an important role in blood clotting, cellular and matrix interaction, and inflammatory responses.[19,20] In fact, the fibrin network could serve as a scaffold for bone regeneration.[20] Additionally, the results of cell proliferation studies showed that the TiB and TiF surfaces displayed significantly higher numbers of grown cells, after 7 days. As mentioned earlier, the fibrin networks and the existing proteins in FBS and blood would improve surface hydrophilicity and surface roughness, which provide more suitable condition for cells to adhere and proliferate on the TiB and TiF samples.

Our SEM images showed that MG63 cells completely covered the surface of TiB, and a higher cell contact was formed on this sample compared with other samples. Surface roughness and wettability have been shown to influence cell behavior and enhance cell adhesion, proliferation, and differentiation on titanium surfaces.[21] At a molecular level, it has been proven that surface roughness can modulate the local regulatory factors produced by osteoblast-like MG-63 cells, such as prostaglandin E2 and transforming growth factor β1 (TGF-β1).[17] Moreover, the enhanced cell interactions on the surface of TiB could be because of the higher secretion levels of osteocalcin, TGF β1, and prostaglandin E2.[16] Increased secretion of these factors is known to have a positive influence on the morphology and maturity of cells.[19]

The highest ALP protein expression level on day 14 was observed on the TiB sample. The enhanced ALP expression and activity on TiB could be due to the higher surface wettability and roughness, which are known to improve osteoblastic cell growth.

In agreement with the results of cell culture experiments, the interaction of cells on the surface of IDB implants coated with blood was better as indicated by a large number of prominent filopodia and lamellipodia extensions. We believe surface treating implants with blood before implantation could be more favorable for interactions between osteoblastic cells and dental implants

Conclusions

This study showed that precoating titanium surfaces with patient blood would lead to an increased number of attached cells, proliferation, and enhanced ALP activity. The MG63 cells were attached to the surface of blood-coated samples and displayed strong interactions with the fibrin scaffold, which led to enhanced osteogenic differentiation compared with other biologically modified surfaces. It is possible that precoating titanium implants with blood may mimic a physiological environment in which a better clinical outcome may be achieved.

Abbreviations

AFM: atomic force microscopy
ALP: alkaline phosphatase
DMEM: Dulbecco's modified eagle medium
FBS: fetal bovine serum
MTT: 3-(4,5-dimethylthiazol-2yl) 2,5diphenyl-2H-tetrazoliumbromide
PBS: phosphate-buffered saline
RGD: arginylglycylaspartic acid
TGF- β1: transforming growth factor β1
TiB: blood precoated titanium
TiF: FBS precoated titanium
TiP: PBS precoated titanium

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Note

The authors declare that there is no conflict of interest in this study.

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