In vitro evaluation of NaOCl-mediated functionalization of biologically aged titanium surfaces

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The aim of this study was to evaluate the NaOCl-mediated biofunctionalization of titanium surfaces. Titanium disks stored for 2 weeks were immersed in 5% NaOCl solution for 24 h. A disk immersed in distilled water for 24 h was used as a control. X-ray photoelectron spectrometer assay of the titanium surface after NaOCl treatment demonstrated that organic contaminants containing carbon and nitrogen were removed and the number of hydroxyl groups increased. The NaOCl treatment substantially converted the titanium surface to superhydrophilic status (θ<5°), which resulted in an increased number of attached cells and enhanced cell spreading on the NaOCl-treated surfaces. These results indicate that biofunctionalization of the biologically degraded titanium surfaces can be achieved by chemical surface treatment with 5% NaOCl. The mechanism for desorption of strongly adsorbed organic molecules with polar groups such as amino and aldehyde groups from titanium surfaces by ClO− was elucidated.

Keywords: Titanium, NaOCl treatment, Chemical surface treatment, Biological aging

INTRODUCTION

Dental implant therapy is now widely accepted globally. A dental implant body made of pure titanium or titanium alloy integrates with the surrounding bone and thus supports the dental prosthesis. The formation of a direct interface between the implant and bone without intervening soft tissue is termed osseointegration⁵. However, the total implant area covered by bone (bone–titanium contact percentage) has been reported to be 45±16%⁶, or 50–65%⁷, far below the ideal of 100%. A potential reason for this has been recently proposed that biological aging degrades the biocompatibility of the titanium surface over time after processing⁸. Biological aging is associated with an inevitable occurrence of progressive contamination of the titanium surfaces by organic compounds, such as polycarbonyls and hydrocarbons quickly adsorbed on the titanium implant surface from the atmosphere, water, and cleaning solutions⁹,⁰. The aging of titanium reduces the initial behavior and response of osteoblasts, such as cell migration, attachment, and proliferation⁹. Additionally, the age-induced reduction of these in vitro cell behaviors has been observed on various surface topographies, not only acid-etch-created microtopography, but also machined and sandblasted surfaces⁹,⁰.

There is therefore a need to improve the bioactivity of titanium for therapeutic and experimental purposes. Various surface treatment methods have been suggested to achieve this; for instance, ultraviolet light treatment⁹, gamma ray treatment¹¹, alkali and heat treatment¹¹, atmospheric pressure plasma jet treatment¹², and cold-plasma techniques¹³. Most of these surface treatments, however, are costly and required special devices. To overcome these shortcomings, we have proposed a new approach to biological aging with 5% sodium hypochlorite solution (NaOCl)¹⁴. Kono et al.¹⁵ performed a series of animal studies and reported that with NaOCl treatment at the early-stage of healing (2 weeks), the strength of bone–titanium integration for polished and acid-etched surfaces increased 1.3 times and 1.4 times over those of untreated polished and untreated acid-etched titanium, respectively, when evaluated with push-in tests. Moreover, histological images of peri-implant tissue at 2 weeks showed that bone formation occurred more extensively around NaOCl-treated implants than around untreated implants. These results demonstrate that NaOCl pretreatment enhanced the osseointegration capability of titanium, indicating successful NaOCl-mediated biofunctionalization of the titanium implant surface. Thus, the aim of the present study was to further evaluate the NaOCl-mediated biofunctionalization of the titanium surface by surface characterization using an X-ray photoelectron spectrometer (XPS) and an automatic contact angle measuring device. In vitro protein adsorption assays and cell culture experiments were also undertaken. Additionally, the underlying mechanisms of NaOCl-mediated biofunctionalization of titanium were also elucidated considering the interactions of ClO− with organic contaminants adsorbed.

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on the titanium surface as well as the time-dependent biological degradation process of titanium.

MATERIALS AND METHODS

Titanium samples and chemical surface treatment
Commercially pure grade-2 titanium disks (JIS, Japan Industrial Specification H 4600, 99.9 mass% Ti; GC, Tokyo, Japan) with a diameter of 6 and 15 mm were purchased. The disks were polished mechanically to a mirror finish using colloidal silica. The 6-mm-diameter titanium disks were used for XPS analysis and the 15-mm-diameter titanium disks were used for the other experiments. The polished disks were cleaned ultrasonically in distilled deionized water for 5 min and then dried with oil-free compressed air. These titanium disks were stored in the dark under ambient temperatures for 2 weeks since 2 weeks seems to be a critical time period for degrading the wettability and cytocompatibility of the titanium surfaces according to the results reported by Att et al.

The samples were divided into three groups: untreated titanium, NaOCl-treated titanium, and H2O-treated titanium. NaOCl-treated titanium and H2O-treated titanium were prepared by immersing samples in 5% NaOCl solution or distilled deionized water for 24 h. These specimens were then washed gently three times with distilled deionized water. The processes for titanium specimen preparation are schematically illustrated in Fig. 1.

Surface characterization of the titanium samples
The surface of the titanium samples was examined by X-ray photoelectron spectroscopy (XPS) to determine the composition. T 2p, O 1s, C 1s and N 1s spectra were obtained using an XPS (ESCA-850, Shimadzu, Japan) with nonmonochromatic Mg K source operated at a 7 kV accelerating voltage and 30 mA current under a vacuum of $1 \times 10^{-6}$ Pa.

Water wettability of the titanium surfaces was examined by measuring the contact angle of 4 µL of distilled deionized water on the titanium disks using an automatic contact angle measuring device (Phoenix Alpha, Surface Electro Optics, Suwon, Korea).

To confirm adsorption of collagen I on the titanium surfaces, samples were incubated with collagen I-FITC conjugate (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After three washes with phosphate-buffered saline (PBS), samples were observed by confocal laser scanning microscope operated with EZ-C1 system software (Confocal laser scanning microscope, ECLIPSE TE-2000, Nikon, Tokyo, Japan). The adsorption of collagen I was evaluated by measuring the mean gray value of five randomly chosen images from three specimens of each group as the fluorescence intensity using ImageJ software (NIH, Bethesda, ML, USA).

Initial cell attachment of human bone marrow mesenchymal stem cells (hBM-MSCs)

hBM-MSCs (Poietics™, Lonza, Switzerland) were cultured on 10 cm tissue culture dishes (Falcon BD, Franklin Lakes, NJ, USA) using mesenchymal stem cell basal medium with mesenchymal stem cell growth supplement (MSCGM; Lonza) containing fetal bovine serum (FBS), L-glutamine, and GA-1000 (Gentamicin/Amphotericin-B). The cells were cultured at 37°C in humid 5% CO2 in air.

Cell attachment on each titanium disk was evaluated by counting the number of attached cells. The titanium disks were placed into a 24-well plate and hBM-MSCs were seeded at a density of $6.0 \times 10^4$ cells/cm². After 4 h of incubation, the titanium disks were rinsed with PBS to remove unattached cells. Adherent cells were then detached from the disks using 0.05% trypsin-0.53 mM EDTA-4 Na (Invitrogen, Paisley, UK) at 37°C for 5 min. The cells were pelleted to remove the trypsin and then resuspended in fresh growth medium. The cells in the solution were counted with a hemocytometer.

The density of the cells attached to the titanium surface was examined by scanning electron microscopy (SEM). The disks were placed in 24-well plates, using one per well. The hBM-MSCs were seeded at a density of 5,700 cells/cm² and incubated for 4 h. After incubation, samples were washed twice with PBS and fixed in 2.5% glutaraldehyde buffered in PBS. The samples were washed with PBS six times for 5 min each, dehydrated in ethanol/water mixtures of 50, 70, and 80% for 5 min each, 90% for 10 min and 100% for 20 min. The samples were then critical-point dried with CO2 and sputter-
coated with gold. The samples were examined through a scanning electron microscope (SSX-550, Shimadzu) operated at an accelerating voltage of 15 kV.

Confocal laser scanning microscopy was used to examine cell morphology and cytoskeletal arrangement in the hBM-MSCs seeded onto the titanium surface. The disks were placed into a 24-well plate and seeded at a density of 5,700 cells/cm². After 4 h of culture, the cells were stained using the fluorescent dye rhodamine phalloidin (Molecular Probes, Eugene, OR, USA), a mouse anti-vinculin monoclonal antibody (Abcam, Cambridge, MA, USA) and then by an FITC-conjugated anti-mouse secondary antibody (Abcam). The area, perimeter, Feret’s diameter, and circularity of the cells were quantified using ImageJ software.

Treatment-time-dependent changes for water wettability and the number of initial cell attachments
The titanium disks were prepared by immersion in 5% NaOCl solution or distilled deionized water for 15 min, 6 h, 12 h or 24 h. The water wettability and the number of initial cells attached on the titanium surfaces were evaluated as described above.

Statistical analysis
Statistical differences were evaluated using analysis of variance (ANOVA) with Tukey’s tests and a paired t test. A p value<0.05 was considered statistically significant.

RESULTS
Characteristics of titanium surfaces by XPS
Figure 2 shows Ti 2p (A), C 1s (B), and N 1s (C) spectra obtained from the untreated and H2O-treated surface. The intensity of the Ti 2p peak at 454.0 eV which corresponds to the titanium in a metallic state was slightly lower for the H2O-treated than the untreated surface, suggesting that the thickness of the surface oxide film increased during the water immersion treatment (Fig. 2A). The small Ti 2p peak at 450.5 eV was one of the satellite peaks associated with the main Ti 2p₃/₂ peak at 458.9 eV due to TiO₂. There was no significant change in the spectral intensity of the C 1s peak at 284.5 eV for aliphatic hydrocarbons between the untreated and H2O-treated surface. The intensity of the C 1s peak at 288.0 eV for the carboxyl or carbonyl group was slightly lower for the H2O-treated surface than for the untreated surface (Fig. 2B). The N 1s peak at 400.0 eV was attributed to organic contaminants adsorbed on the titanium surface during storage in the dark for 2 weeks¹⁴. No noticeable change in the intensity of the N 1s peak was observed before or after the water immersion

![Fig. 2](image)

Ti 2p, C 1s and N 1s spectra obtained from the same specimens before and after immersion in deionized water.

| Group         | OH(a) + H₂O | OH(b) |
|---------------|-------------|-------|
| Untreated     | 13.87%      | 5.18% |
| H₂O-treated   | 17.67%      | 5.68% |

Fig. 3 The percentage area of hydroxyl groups in O 1s spectra before and after immersion in deionized water.
treatment. Figure 3 shows O 1s spectra obtained from the untreated and H2O-treated surface. The main peak at 530.0 eV corresponded to the bulk oxygen of TiO2. Two types of hydroxyl groups: an acidic hydroxyl group (a) and a basic hydroxyl group (b) were present on the surface oxide film of titanium. The subpeak at 531.2 eV was assigned to both acidic hydroxyl group and physisorbed H2O, and the peak at 532.5 eV to basic hydroxyl group. The percentage area of the two subpeaks increased by 3.8% for the acidic hydroxyl group and physisorbed H2O, and by 0.5% for the basic hydroxyl group after water immersion treatment for 24 h.

Figure 4 shows Ti 2p, C 1s, and N 1s spectra obtained from the untreated and the NaOCl-treated groups. The intensity of the Ti 2p peak at 454.0 eV was attributed to the fact that the titanium in a metallic state was much lower for the NaOCl-treated surface than for the untreated surface (Fig. 4A), suggesting that the thickness of the oxide film on the titanium disk increased during the NaOCl treatment. From the relative intensity of the peak at 454.0 eV with respect to the peak at 458.7 eV attributed to TiO2 shown in Figs. 2A and 4A, the thickness of the oxide film was greater for the NaOCl-treated than for H2O-treated surface. Unlike the results for the water immersion treatment, the intensity of the C 1s peak at 288.0 eV and the N 1s peak at 400.0 eV decreased substantially after NaOCl treatment (Figs. 4B and C). Both percentage areas of the O 1s subpeaks increased by 4.8% for the acidic hydroxyl group and physisorbed H2O, and by 0.6% for the basic hydroxyl group after 5% NaOCl treatment (Fig. 5), suggesting that the number of both types of hydroxyl groups and physisorbed H2O on the oxide film was higher after the NaOCl treatment than after the water immersion treatment (Figs. 3 and 5).

Water wettability of titanium surfaces
Figure 6 shows side-view images of 4 µL water droplets placed on titanium disks (A) along with the measured contact angle (B). The water contact angle of the untreated titanium was 59.4±2.1°, indicating that the surface was hydrophobic. With the water immersion treatment or NaOCl treatment, the contact angle substantially decreased (7.9±2.9° and 3.8±0.6° respectively), showing that the hydrophobic surface of the aged titanium was converted to a hydrophilic surface.

Collagen I adsorption capacity of titanium
Figure 7 shows the fluorescence intensity of collagen I-FITC conjugate attached on the untreated titanium, H2O-treated titanium and NaOCl-treated titanium obtained by measuring the mean gray value of confocal laser scanning microscopy images. The fluorescence intensity of NaOCl-treated surfaces was significantly higher than that of the other groups (p<0.05). In addition, the fluorescence intensity of H2O-treated surfaces was significantly higher than that of the untreated surfaces.
Fig. 6  (A) Image of the 4 µL water droplets. (B) The water contact angle was obtained from image analysis of the 4 µL water droplets. Data are shown as mean±SD (n=3). *: p<0.05 for significance

Fig. 7  The amount of adsorbed collagen I on each titanium surface. Fluorescence intensity was obtained by measuring the mean gray value of confocal laser scanning microscopy images using ImageJ software. Data are shown as the mean±SD (n=3). *: p<0.05 for significance

**Number of cells attached to titanium disks after incubation for 4 h**

Figure 8A shows the relative number of cells attached to the titanium surfaces, as measured with a hemocytometer. The number of cells attached to the NaOCl-treated and H₂O-treated surfaces was twice that of untreated surfaces (p<0.05). There were no significant differences in the number of attached cells between the NaOCl-treated and H₂O-treated surfaces. SEM images also showed that more cells were attached to the H₂O-treated and NaOCl-treated surfaces than to untreated surfaces (Fig. 8B).

**Spreading and vinculin expression of hBM-MSCs on untreated, H₂O-treated, and NaOCl-treated titanium surfaces**

Figure 9A shows fluorescent images of hBM-MSCs on the titanium surfaces. Confocal laser scanning
microscopic images of the hBM-MSCs after staining with rhodamine phalloidin showed that after incubation for 4 h, the cells on the NaOCl-treated titanium were clearly larger than those on the untreated and H₂O-treated titanium surfaces. Cells on the NaOCl-treated titanium surfaces were enlarged with a clear stretch of lamellipodia-like actin projections and stressed fibers within their cytoplasm. Cytomorphometric evaluation
Fig. 10 (A) Water contact angle obtained from image analysis of the 4 µL water droplets. Data are shown as mean±SD (n=3). (B) Variation in the relative number of cells attached to the titanium surface according to the treatment time. Data are shown as mean±SD (n=4). *: p<0.05 for significance of the area, perimeter and Feret’s diameter of the cells on the NaOCl-treated titanium surfaces showed greater values for these parameters than those on the untreated titanium surfaces (Fig. 9B). In particular, the actin area of the cells on the NaOCl-treated titanium surfaces was significantly larger than that of the untreated and H₂O-treated titanium surfaces (p<0.05). The hBM-MSCs on the all titanium surfaces expressed vinculin in their cytoplasm at 4 h incubation. A more extensive expression of vinculin at the tip of cellular projections, however, was observed on the NaOCl-treated titanium surfaces than on the H₂O-treated and untreated titanium surfaces.

DISCUSSION

Adsorption of volatile organic compounds (VOCs) in air and its relationship to biological aging of titanium

Several studies have demonstrated that titanium undergoes time-dependent degradation and its capability to integrate with surrounding bone reduces with time. This is known as biological degradation. The adsorption of VOCs from the air onto the titanium surface, which changes the hydrophilic titanium surface to a hydrophobic surface, is considered to be responsible for this biological degradation of titanium. It is well known that these VOCs adsorb instantaneously on most solid substances including titanium. This physical adsorption of VOCs from the air onto the titanium surface is inevitable and very fast. Consequently, when the surface is analyzed by XPS, a strong C 1s peak due to saturated hydrocarbons is always observed at 284.5–285.0 eV when samples have been processed in air before being introduced to the high vacuum chamber of XPS. In contrast, the biological aging of titanium reported in literature is rather a slow process. Most titanium surfaces immediately after polishing in air show superhydrophilic status (θ<5°). These facts suggest that VOCs initially adsorbed onto the titanium surface may not affect the hydrophilicity of titanium and are not responsible for the biological degradation of titanium.

Physical adsorption of VOCs onto the titanium surface is instantaneous as mentioned above. In the low-pressure region of the adsorption isotherm where Henry’s law is valid, the VOC level is proportional to the partial pressure (or concentration) of each VOC in air:

\[ X = K_H P \]  

where \( X \) is the surface coverage, \( P \) the partial pressure, and \( K_H \) the Henry’s adsorption constant. Among VOCs in air, the average concentration of alkanes (aliphatic saturated hydrocarbons) such as methane, ethane and propane is about 2 ppm which is approximately three orders of magnitudes higher than other VOCs. Thus, it can easily be deduced from this fact that the initially adsorbed contaminants on the titanium surface would be mostly these low molecular weight alkanes. Physical adsorption of these non-polar hydrocarbons is caused by van der Waals force, a distance-dependent weak interaction between atoms or molecules. This suggests that these adsorbed hydrocarbons can be instantaneously
replaced by water molecules which strongly interact with the surface hydroxyl groups of titanium by hydrogen bonds when a water droplet is placed on the surface to measure the contact angle (Fig. 11A). This is why these adsorbed non-polar hydrocarbons do not affect the hydrophilicity of the titanium surface.

Att et al. 4) showed that the initial hydrophilic surface of titanium gradually changed to become hydrophobic, and biological degradation of titanium progressed in a time-dependent manner. In this study, we also demonstrated that the water contact angle of the titanium was about 60° (Fig. 6). These results suggest the presence of hydrophobic organic contaminants which interacted more strongly with the titanium surface than aliphatic saturated hydrocarbons. The atmospheric gas phase contains a variety of VOCs with polar groups, such as amines, aldehydes, alcohols, and carboxylic acids23). Although the concentration of these molecules in the atmosphere is extremely low, at ppb level21), they can interact with the surface hydroxyl groups of titanium by hydrogen bonding, as shown in Fig. 11.

With aging in air, the initially adsorbed non-polar hydrocarbons could be gradually replaced by these more preferentially adsorbed polar VOCs and consequently, the hydrophilic titanium surface would be changed to a hydrophobic surface.

Desorption of VOCs from the aged titanium surface with water immersion treatment

With water immersion treatment for 24 h, the contact angle of water droplets significantly decreased from 59.4° to 7.9° (Fig. 6). In addition to VOCs physically adsorbed by van der Waals force, VOCs with polar groups adsorbed by hydrogen bonds were largely subjected to hydrolysis and removed from the titanium surface. As shown in Fig. 2B, the C 1s peak at around 288.0 eV due to carboxyl and/or carbonyl groups decreased after water immersion treatment for 24 h. Water immersion treatment was thus useful for removing many VOCs with and without polar groups, but its effect is limited. For treatment time, at least 6 h was needed to remove the polar hydrocarbon with carboxyl and/or carbonyl groups and change the titanium surface from hydrophobic to hydrophilic (Fig. 10A). Additionally, no significant difference was observed in the spectral intensity of the N 1s peaks before and after the water immersion treatment for 24 h, suggesting that few atmospheric organic-nitrogen compounds such as amines and amides were removed from the surface.

Using some typical bond energy values24-26) for hydrogen bonds (about 25 kJ/mol for O−H···O, 23 for O−H···N, 10 for N−H···O, 8 for C−H···O) and van der Waals force (less than 2 kJ/mol for the induced force), the replacement of VOCs from the titanium surface can be evaluated as follows. When a non-polar alkane type...
VOC is replaced by a water molecule (Fig. 11A), the system would be stabilized by as much as \((-2)\cdot(-25\times2)=48\ \text{kJ/mol}\). In the case of replacement of a VOC of an amine type by a water molecule (Fig. 11B), the stabilization energy amounts to \([-10]+(-23)-(-25\times2)=17\ \text{kJ/mol}\). Similarly, for the aldehyde type VOC (Fig. 11C), energy stabilization with replacement by a water molecule may become \([-10]+(-25)-(-25\times2)=15\ \text{kJ/mol}\). Although this is semi-quantitative and a rough estimation, it can still explain why nonpolar VOCs (hydrocarbons) are more easily replaced by water than those with polar functional groups.

**Effects of sodium hypochlorite on removal of adsorbed VOCs with polar groups from the aged titanium surface**

Sodium hypochlorite is widely used as an oxidizing agent and a disinfectant\(^{27,28}\). This chlorine compound has been reported to react with a number of organic compounds including biological molecules at physiological pH conditions\(^{29}\). With NaOCl treatment for 24 h, the contact angle of a water droplet significantly decreased from 59.4° to 3.8°, which was much lower than that with water immersion treatment (Fig. 6). Moreover, NaOCl treatment for 15 min is effective in converting the surface to a hydrophilic state in a short time in comparison with water immersion treatment (Fig. 10). The XPS spectral intensity of both the C 1s peak at 288 eV and the N 1s peak at 400 eV due to VOCs with polar groups also decreased significantly with NaOCl treatment. In addition to hydrocarbons with carboxyl and/or carbonyl groups, the organic nitrogen compounds which remained after water immersion treatment were obviously removed from the titanium surface by NaOCl treatment. Since ClO\(^-\) was reported to react readily with amines\(^{29}\), the adsorbed amines reacted with ClO\(^-\) to yield chloramines (reactions 2 and 3) and probably desorbed from the titanium surface as shown in Fig. 11B.

\[
\begin{align*}
R-\text{NH}_2+\text{ClO}^- & \rightarrow R-\text{NHCl}+\text{OH}^- & (2) \\
R-\text{NH}_2+2\text{ClO}^- & \rightarrow R-\text{NCl}_2+2\text{OH}^- & (3)
\end{align*}
\]

Amides can also react with NaOCl to yield chloramides (reaction 4)\(^{29}\), and also desorb from the titanium surface.

\[
\begin{align*}
R-\text{CONH}_2+\text{ClO}^- & \rightarrow R-\text{CONHCl}+\text{OH}^- & (4)
\end{align*}
\]

Due to these strong oxidation effects of NaOCl, it was possible to readily detach the organic nitrogen compounds with polar groups that could not be removed by water immersion treatment. As shown in Fig. 11C, the adsorbed aldehydes with a carbonyl group can also react with NaOCl to yield acyl chlorides. The adsorbed aldehydes were found to be more readily desorbed in 5% NaOCl solution than in water (Fig. 10 A).

**Enhanced initial cytocompatibility of the aged titanium surface with NaOCl treatment**

After implantation, the implant surfaces are in contact with body fluids and interact with a number of proteins and different cell types\(^{30}\). Hence, the initial cell behavior on the implant surface is a key factor in determining the osteoconductive capacity of implants during the early stage of osseointegration. Feng et al.\(^{31}\) also showed that the number of surface hydroxyl groups significantly influenced the initial behavior of osteoblasts on the titanium surface. In the present study, it was clearly demonstrated that the number of both acidic and basic hydroxyl groups on the titanium surface increased after NaOCl treatment and water immersion treatment with the detachment of organic contaminants from the surface, as shown in Figs. 3 and 5. Consequently, the aged titanium surface changed from a hydrophobic to a hydrophilic state (Fig. 6) and the amount of adsorbed collagen I on both the NaOCl-treated and the H\(_2\text{O}\)-treated titanium surfaces was significantly higher than that on the titanium (\(p<0.05\), Fig. 7). With the increased number of surface hydroxyl groups and adsorbed cell adhesive proteins such as collagen I, the number of attached hBM-MSCs after incubation for 4 h was also significantly increased on both the NaOCl-treated and H\(_2\text{O}\)-treated titanium, in comparison with that on the titanium. These results are consistent with those obtained by previous studies with surface treatments of aged titanium by UV-irradiation\(^{20}\) and radio frequency glow-discharge\(^{22}\). Since the number of osteogenic cells attached to the titanium directly affects the volume of peri-implant bone formation\(^7\), these hydrophilic treatments for aged titanium implants are expected to enhance the osteoconductivity of titanium implants during the early stage of osseointegration. Among these hydrophilic treatments of aged titanium, both NaOCl treatment and water immersion treatment are simple chemical surface treatments in a wet process, so that no special devices are required for the treatment, and they can be effectively applied to dental and surgical implants with porous surface structures.

In our previous study using a rat model, we found that the biomechanical strength of bone–implant integration for NaOCl-treated titanium at 2 weeks after implantation into the femur was 1.3-fold greater than that for H\(_2\text{O}\)-treated titanium\(^{40}\). The reason for this can be attributed to the fact that NaOCl treatment is more effective than water immersion treatment in reducing the amount of adsorbed hydrocarbons with polar groups including amino groups which strongly interact with surface hydroxyl group (Figs. 2 and 4). As a result, the adherent cells on the NaOCl-treated titanium were markedly enlarged with a clear stretch of lamellipodia-like actin projections, stressed fibers and more extensive expression of vinculin within their cytoplasm compared with cells on the untreated and H\(_2\text{O}\)-treated titanium surfaces. This enhanced cytocompatibility of the aged titanium with NaOCl-treatment is one of the reasons for the excellent bone–implant integration of NaOCl-treated titanium implants we demonstrated previously\(^{14}\).

**CONCLUSIONS**

Due to the strong oxidation effects of ClO\(^-\), NaOCl...
treatment for the aged titanium surface contributed to a decrease in the amount of adsorbed hydrocarbon contaminants with polar groups which strongly interact with surface OH groups in a short time. The increase in the number of surface OH groups after NaOCl treatment resulted in significant enhancement of the adsorption of collagen I as well as the adhesion and spreading of hBM-MSCs. NaOCl-mediated biofunctionalization of titanium implants is a simple chemical chair-side pretreatment in a wet process, which may be beneficial for enhancing osseointegration, especially for dental and surgical implants with irregularly shaped surfaces and porous surface structures.

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CONFLICTS OF INTERESTS
No potential conflicts of interest are disclosed.

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