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Research Article

Osseointegration of Implants Surface-Treated with Various Diameters of TiO_2 Nanotubes in Rabbit

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The aim of this study was to evaluate the osseointegration of implants which were surface-treated with various diameters of TiO_2 nanotubes (30 nm, 70 nm, and 100 nm) in rabbit. Resorbable blast media (RBM) surfaced implants (Osstem, Busan, Korea) 3.5 mm in diameter and 8.5 mm in length were designated as the control group and the implants surface-treated with various diameters of nanotubes (30 nm, 70 nm, and 100 nm) with the same shapes were designated as the experimental groups. The implants were maintained unloaded for 4 and 12 weeks. After this period, the animals were sacrificed and micro-CT analysis, histomorphometric analysis (bone to implant contact (BIC), bone volume (BV)), and removal torque test were performed. Micro-CT analysis, histomorphometric analysis, and removal torque test results all showed the similar pattern, showing that 70 nm experimental group had the highest value at 4 weeks while 30 nm experimental group had the highest value at 12 weeks. Therefore, on the basis of the results above, it can be concluded that 30 nm and 70 nm TiO_2 nanotubes may have positive effects on osteogenesis and osseointegration depending on the healing time.

1. Introduction

Titanium and its alloys have long been used as implantable biomaterials because of their high-quality mechanical properties, resistance to corrosion, and biocompatibility [1–3]. Although the resistance to corrosion and biocompatibility come from inactivity of TiO_2 oxide layer, the osseointegration of implant may also be delayed due to TiO_2 oxide layer. Therefore, various research groups have been trying to modify the TiO_2 surface to promote even earlier and better osseointegration [2, 3].

Schwartz et al. [4, 5] reported that the harmony of surface roughness, surface energy, surface composition, and surface topography are necessary for optimal osseointegration of the implant, and these surface conditions play the important role in adhesion and proliferation of the cell, as well as the adsorption of protein during the early state of healing process. According to studies on the surface roughness of implants, rough-surfaced implants have earlier and stronger osseointegration clinically compared to smooth-surfaced implants since rough-surfaced implants are easier to obtain initial mechanical fixation, which is more advantageous for osteoblast attachment and differentiation [6–10]. The ideal surface roughness for optimal osseointegration with the highest rate is known as 1-2 μm [2, 11].

In recent years, nanoscale surface modification has been attracting more attention ever since several investigators had revealed that nanoscale topography influences cell adhesion and osteoblast differentiation [12–14]. Above all, vertically aligned and laterally spaced TiO_2 nanotubes created by

electrochemical anodization have become increasingly popular for achieving superior osteoblast cell growth and directed osteogenic differentiation of mesenchymal stem cells (MSCs) [15–17]. TiO_2 nanotubes are hydrophilic, which increases the surface area, and may provide increased channeling for proper fluid exchange [15].

In the previous study, cell behavior on the surface of TiO_2 nanotube varied depending on the nanotube diameter. Oh et al. reported that, on the 70 nm TiO_2 nanotube, the adhesion of proteins, osteoblasts, and MSCs showed the highest elongation and cellular activity of osteoblasts and MSCs were obtained on large-sized (70, 100 nm) TiO_2 nanotube [16–19]. On the other hand, Park et al. [20, 21] reported that a spacing of 15 nm provided the optimum length scale for integrin clustering and focal contact formation, inducing osteoblasts, MSCs, and osteoclasts proliferation and differentiation.

While there are many *in vitro* studies about TiO_2 nanotube, there are not many animal studies reporting the effect of various nanotube diameters on osseointegration of titanium implants. Moreover, the optimum TiO_2 nanotube size is still controversial. Therefore, in this study we measured and compared the bone area near the implants and implant removal torques in rabbit to evaluate the osseointegration of implants surface with various diameters of TiO_2 nanotubes histomorphometrically and biomechanically.

2. Materials and Methods

2.1. Implants and TiO_2 Nanotube Fabrication

2.1.1. Implants. Twenty RBM surfaced implants (Osstem, Busan, Korea) 3.5 mm in diameter and 8.5 mm in length were designated as the control group and sixty machined surface implants with the same shape were manufactured (Adtech, Seoul, Korea) for the experimental group.

2.1.2. TiO_2 Nanotube Fabrication. TiO_2 nanotube surfaces were processed on sixty machined surface implants. The nanotubes were prepared in a 1 : 7 volumetric ratio of acetic acid to hydrofluoric acid in water at 5, 15, and 20 V. The samples were then heat-treated at 500°C for 2 h in order to crystallize the amorphous structure into an anatase structure. Implants treated with various diameters (30 nm, 70 nm, and 100 nm) of nanotube were designated as experimental groups (Figure 1). Every group was divided into two categories according to healing period (4 weeks, 12 weeks) (Table 1).

2.2. Experimental Animals and Surgical Procedure

2.2.1. Experimental Animals. Twenty rabbits (New Zealand white), 6 weeks old, weighing approximately 3.5 kg each, were used in this study. Animal selection, management, surgical protocol, and procedures for this study were reviewed and approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

2.2.2. Surgical Procedure. All surgical procedures were performed under general anesthesia. The animals were anesthetized with intravenously administered mixture of 30 mg/kg of Zolazepam (Zoletil Virback Korea Co., Seoul, Korea) and 10 mg/kg of Xylazine HCI (Rumpun, Bayer Korea, Seoul, Korea). After ten minutes, the site of surgery was shaved and sterilized with povidone-iodine and then further anesthetized with 2% lidocaine HCl with epinephrine 1 : 80000 by infiltration. Implants were placed in the right femur of rabbit. The site of implantation was in the middle of the femur where the quality of bone was poor in order to observe the surface characteristic of the implant. After 8 weeks, implants of the same group were placed in the left femur of rabbit (Figure 2). 4 weeks after the second implantation animals were sacrificed by 2% paraformaldehyde injection to heart under a general anesthetic. Then the block sections including implants were preserved and fixed in 10% neutral buffered formalin for 2 weeks. Half of the samples in each group were analyzed radiographically and histomorphometrically while the remaining samples in each group were analyzed biomechanically.

Table 1: Experimental groups classified by nanotube surface treatment.

Week	RBM surface	30 nm	70 nm	100 nm
4	10	10	10	10
12	10	10	10	10

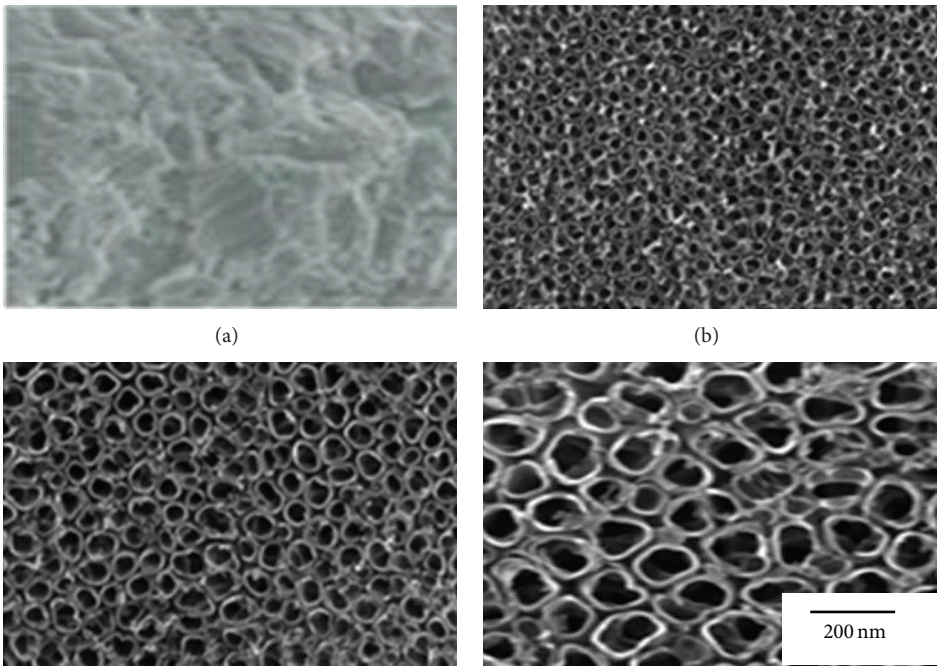
RBM: resorbable blast media.

2.3. Evaluation Method

2.3.1. Micro-Computed Tomography (Micro-CT) Analysis. The mean bone volume within 400 μm of implant surface was measured by micro-CT (Skyscan 1076, Aartselaar, Belgium) at 18 μm pixel, 50 Kv, and 30 μA (Figure 3).

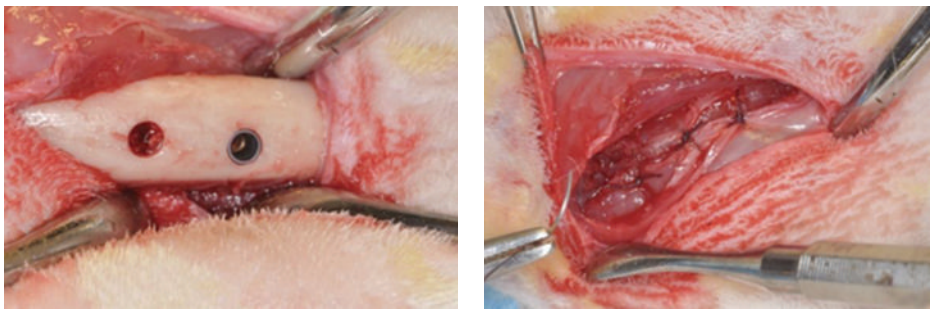
2.3.2. Histologic and Histomorphometric Analysis. The specimens were dehydrated through graded alcohols of 70%, 80%, 95%, and 100% at 2 h intervals for 1 week. The specimens were then embedded in Technovit 7200 (Heraeus Kulzer, Dormagen, Germany) and alcohols (1 : 3, 1 : 1, and 3 : 1 ratio) and sectioned in the buccolingual plane using a diamond saw (Exakt 300, Kulzer, Norderstedt, Germany). From each implant site, the central section was reduced to a final thickness of about 15 μm by microgrinding and polished with a cutting-grinding device (Exakt 400CS, Exakt Apparatebau, Norderstedt, Germany) and finally stained with hematoxylin and eosin.

The stained specimens were scanned and captured using light microscope (Leica DM 2500, Leica Microsystems, Wetzlar, Germany) at ×12.5, ×50 magnification. The bone to implant contact ratio (BIC) was measured in the microthreads and bone volume was measured at both the microthreads (micro-BV) and three consecutive macrothreads (macro-BV) using imaging analyses system (Image-Pro Plus 4.5 Media Cybernetics Inc., Silver Springs, MD, USA) (Figure 4).



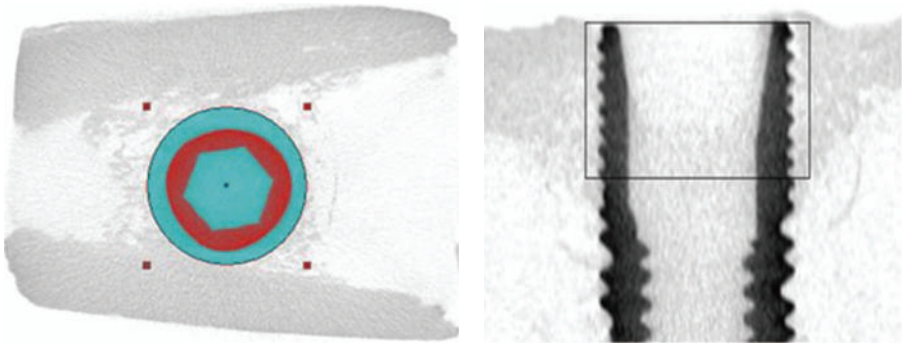
(a) (b) (c) (d)

Figure 1: SEM images of a resorbable blast media surface used as control group (a) and TiO_2 nanotubes with various diameters, 30 (b), 70 (c), and 100 nm (d), processed by controlling anodizing potentials ranging from 5 to 20 V (scale bar, 200 μm). Photo courtesy of Professor Seunghan Oh from Wonkwang University.



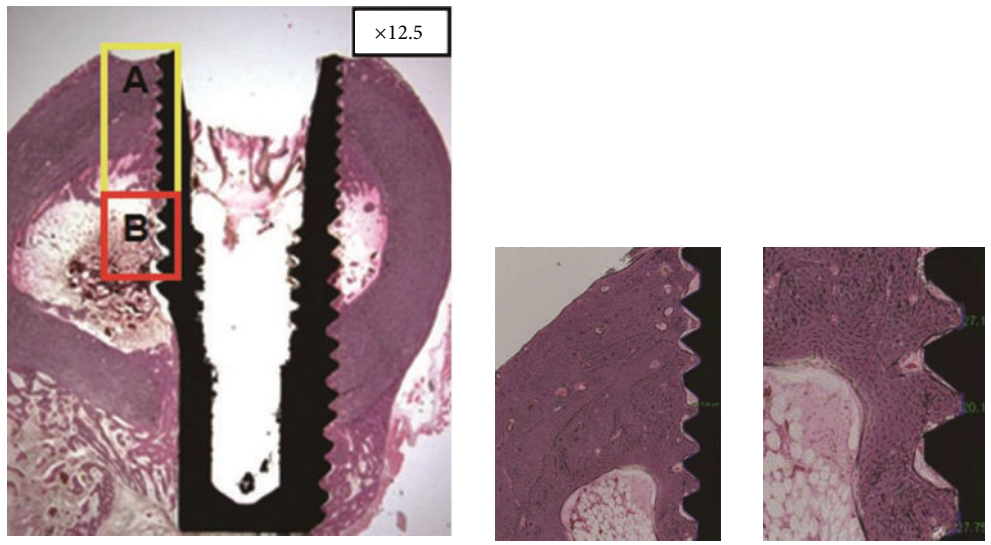
(a) (b)

Figure 2: Surgical procedure and site of implants in the rabbit femur.



(a) (b)

Figure 3: The defined area for measurement of new bone (diameter 4.4 mm × height 2.5 mm).



(a) Microthread (b) Macrothread

Figure 4: Histologic feature of the specimen (H&E stain; 12.5 magnifications). (A) Microthreads area. (B) Three consecutive macrothreads area.

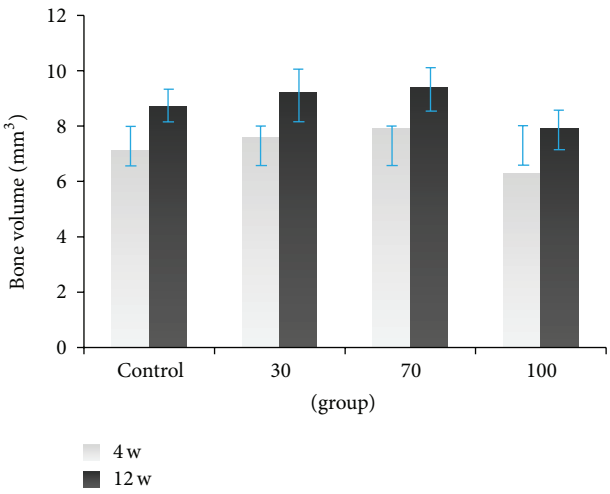


Figure 5: Measurement of micro-CT bone volume (mm^3) at 4 weeks and 12 weeks.

2.3.3. Removal Torque Value (RTV) Analysis. To biomechanically evaluate the osseointegration of implants, removal torque value analysis was performed immediately after sacrifice. Samples with implants were connected to removal torque test apparatus (Mark-10, MGT12, New York, USA) with the long axis of implant parallel to the long axis of apparatus. Screw driver was turned in counterclockwise direction until the implant bone interface was destroyed.

2.3.4. Statistical Analysis. Statistical analysis was performed using the SAS V 9.2 (SAS Institute, Cary, NC). All results were expressed as mean and standard deviation. Kruskal-Wallis test was used in comparing differences among the groups at 4 and 12 weeks to test for relationships between micro-CT BV/BIC/micro-BV/macro-BV and RTV analysis. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Clinical Finding. Among twenty rabbits, four rabbits died from femur fracture or postsurgery stress. In addition, we could not use 16 samples due to femur fracture at the site of implantation. Finally, out of the forty-eight samples acquired, thirty samples were used for micro-CT and histomorphometric analysis and eighteen samples were used for removal torque analysis.

3.2. Micro-CT Scan. Regardless of nanotube diameter, the micro-CT bone volume results at 12 weeks showed significantly higher value than at 4 weeks ($P < 0.05$). At 4 and 12 weeks, 30 nm and 70 nm experimental groups had the highest bone volume, but there were no statistical significant differences between 4 weeks group and 12 weeks group ($P > 0.05$) (Figure 5). From the micro-CT images, implants were found to be well positioned in the middle of femur (Figure 6).

3.3. Histologic and Histomorphometric Analysis. The BIC results at 12 weeks showed significantly higher value than at 4 weeks ($P < 0.05$). At 4 weeks, 70 nm experimental group had the highest BIC results and, at 12 weeks, 30 nm experimental group had the highest BIC results. But there were no statistical significant differences among experimental groups ($P > 0.05$) (Figure 7).

The BV results at 12 weeks showed significantly higher value than at 4 weeks ($P < 0.05$). At 4 weeks, 70 nm experimental group had the highest BV value, but there was no statistical significant differences ($P > 0.05$). At 12 weeks, 30 nm experimental group had significantly higher BV value than 100 nm experimental group ($P < 0.05$) (Figure 8).

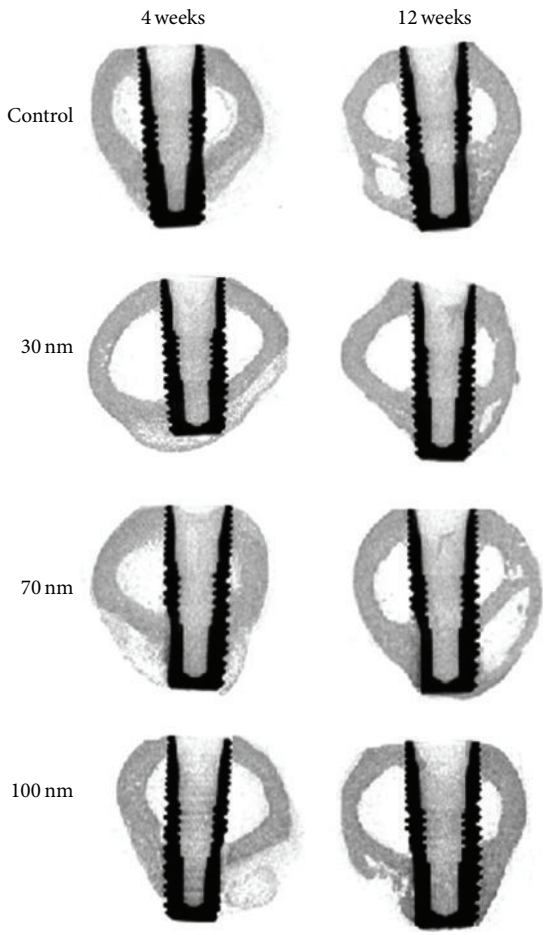


Figure 6: Micro-CT images of representative sample of each group. All the implants were placed favorably in femur.

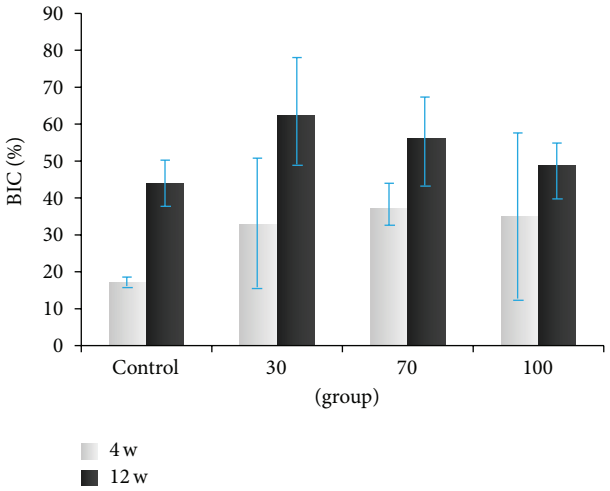


Figure 7: Measurement of BIC (%) at 4 weeks and 12 weeks in defined area which is designated in microthreads.

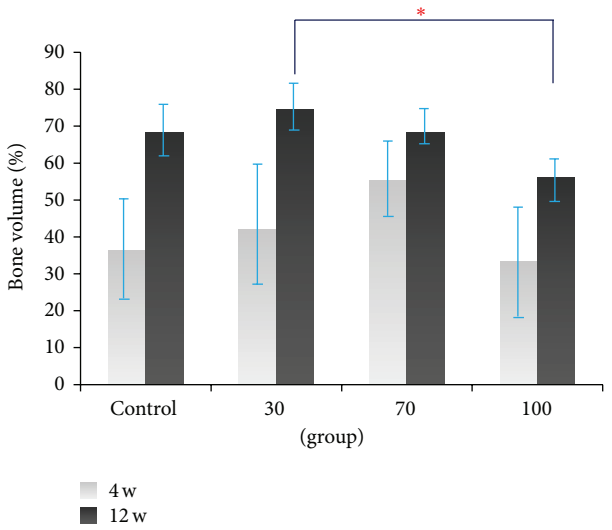


Figure 8: Measurement of bone volume (%) at 4 weeks and 12 weeks in defined area which is designated in microthreads.

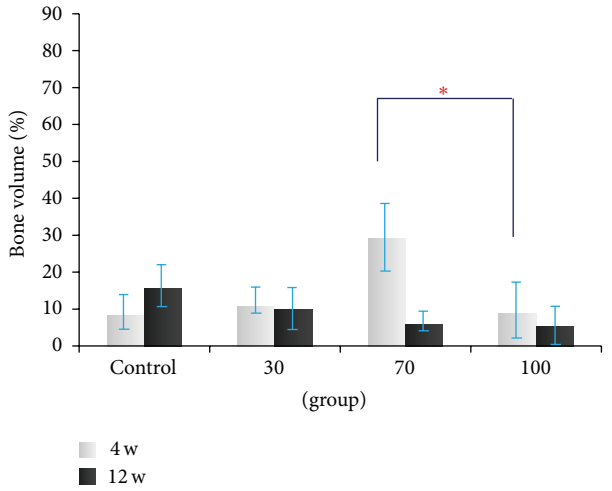


Figure 9: Measurement of bone volume (%) in defined area which is designated in three consecutive macrothreads at 4 weeks and 12 weeks.

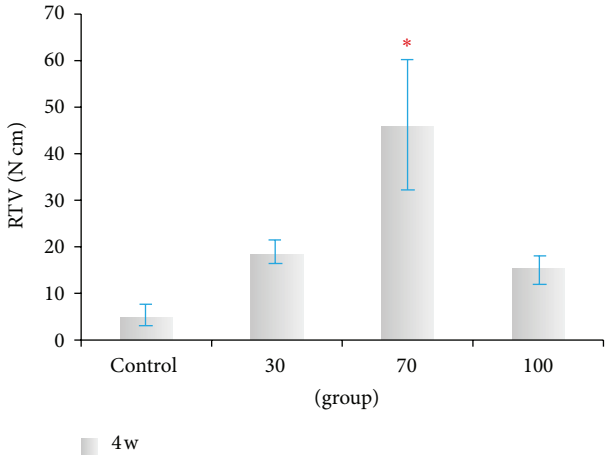


Figure 10: The mean of removal torque values at 4 weeks after implantation.

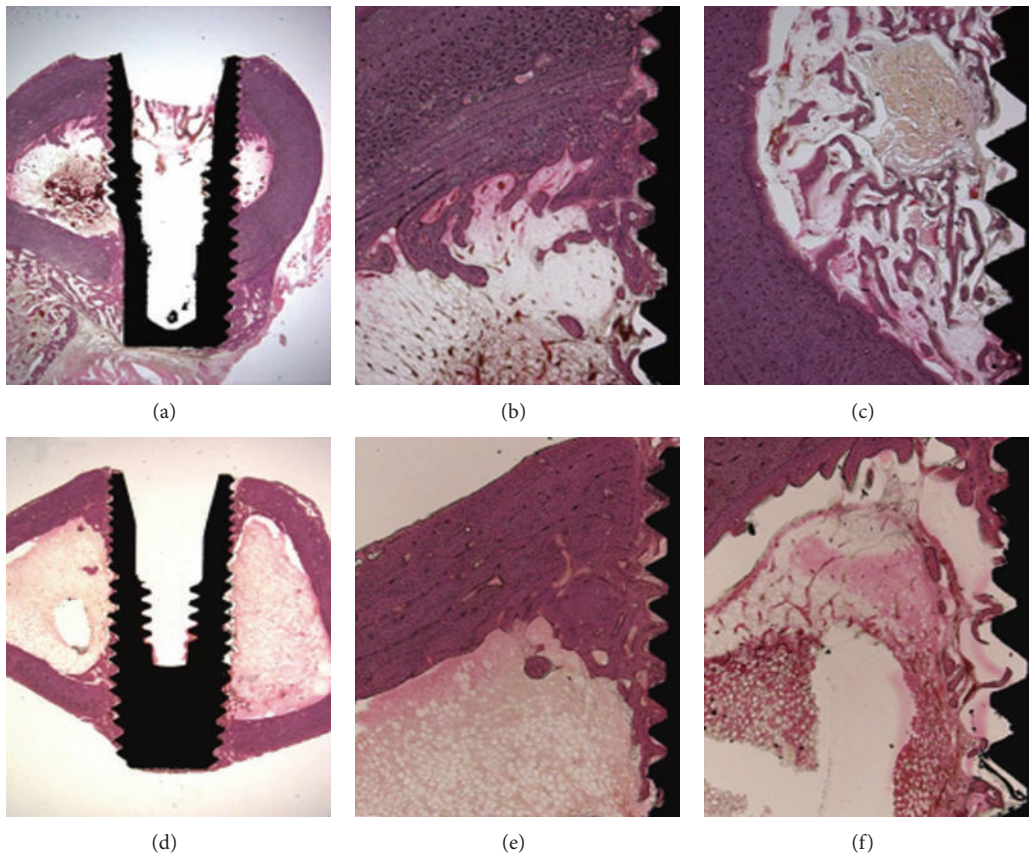


Figure 11: Histologic images of control ((a), (b), and (c)) and 30 nm ((d), (e), and (f)) groups at 4 weeks after implantation. ((a), (d)) H&E stained images at lower magnification (×12.5), ((b), (e)) H&E stained images of microthreads in the A&D (×50), and ((c), (f)) H&E stained images of macrothreads in the A&D (×50).

The bone volume measured at the three consecutive macrothreads (macro-BV) at 4 weeks showed higher values than at 12 weeks. However, there were no statistical significant differences between 4 weeks group and 12 weeks group ($P > 0.05$). At 4 weeks, 70 nm experimental group had significantly higher macro-BV value than 100 nm experimental group ($P < 0.05$). At 12 weeks, control group had the highest macro-BV value, but there were no statistical significant differences ($P > 0.05$) (Figure 9).

3.4. Removal Torque Measurement. At 4 weeks, 70 nm experimental group had higher removal torque value than the other groups ($P < 0.05$) (Figure 10).

4. Discussion

In the installation of implants, adhesion and differentiation of cells on the implant surface are critical factors for a successful osseointegration between the implant and the bone. Therefore, in order to enhance cell adhesion and osteogenesis of cells on the implant surface, there have been many studies on modifying the TiO_2 surface by processing nanostructures on the oxide surface [13]. Vertically aligned and laterally spaced TiO_2 nanotubes created by electrochemical anodization are hydrophilic, which means TiO_2 nanotubes could increase the surface area and provide increased channeling for the proper fluid exchange. In addition, the advantage of TiO_2 nanotube includes simple, low cost, flexible manufacturing and the possibility of its usage as a drug or growth factor delivery system [15]. Therefore, it is important to perform *in vitro* study using small-, medium-, or large-sized animals to compare the osseointegration of the surface-treated implants with various diameters of TiO_2 nanotube. This study evaluated the osseointegration of implants, which is surface-treated with various diameters of TiO_2 nanotube in accordance with the healing time in rabbit.

Previous *in vivo* studies to investigate the effect of TiO_2 nanotube on osseointegration were mostly done in rat model. In the present study, in order to investigate the osseointegration in larger animal, rabbits (New Zealand white) were used. The rabbit is one of the most commonly used animals for medical research, being used in approximately 35% of musculoskeletal research studies, due to its compact size and ease of handling [22]. We installed 4 implants in the rabbit to compare the osseointegration processes between modeling stage (4 weeks) and remodeling stage (12 weeks) in the same animal [23]. And we installed the implants in the middle of the rabbit femur where the quality of bone was poor in order to observe the surface characteristic of the implant. For the

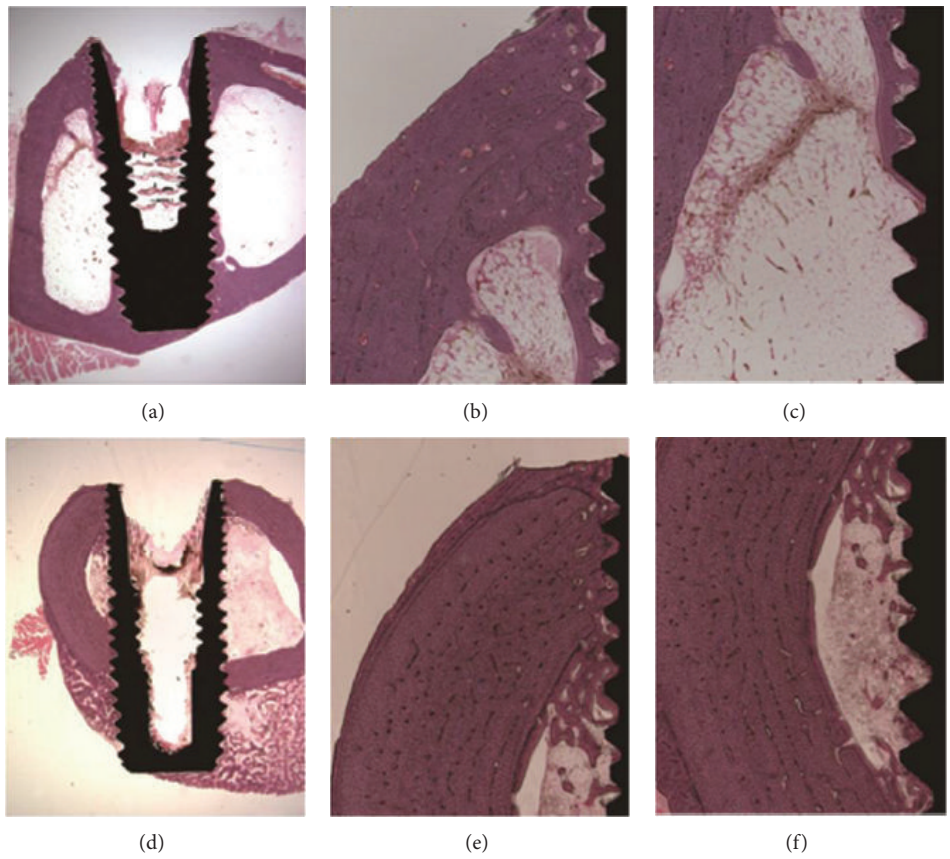


Figure 12: Histologic images of 70 ((a), (b), and (c)) and 100 ((d), (e), and (f)) nm groups at 4 weeks after implantation. ((a), (d)) H&E stained images at lower magnification (×2.5), ((b), (e)) H&E stained images of microthreads in the A&D (×50), and ((c), (f)) H&E stained images of macrothreads in the A&D (×50).

rabbit model, implants are not recommended to be larger than 2 mm in diameter and 6 mm in length because the bone is very brittle [24]. In this study, we used commercial size (3.5 mm ∗ 8.5 mm) implant, which might have been too large for the rabbit and therefore had resulted in femur fracture; specially designed implant for the rabbit would be required in the future study to reduce the fracture.

Implants treated with various diameters of nanotubes (30 nm, 70 nm, and 100 nm) were designated as experimental groups since those showed the best results from the previous studies [16, 20, 25]. Half of the samples in each group used to measure bone area near the implants in order to evaluate the osseointegration of implants radiologically and histomorphometrically. The remaining samples in each group were used to measure the removal torque values and evaluate the osseointegration of implants.

In micro-CT analysis, we measured the bone area near the implants to evaluate the osseointegration of implants radiologically. Micro-computed tomography is an efficient, nondestructive, and reproducible three-dimensional imaging technique that analyses bone architecture and density under various conditions without sophisticated specimen preparation. Although the micro-CT evaluation has limited ability to measure bone adjacent to the implant surface, it can possibly be used for studies designed to compare different groups of experiments [26]. Futami et al. [27] stated that the affected areas in the installation of implant are within 100 μm drilling sites and Kenzora et al. [28] stated that the affected areas are within 500 μm drilling sites. In this study, we set the affected area to be within 400 μm from the implant surface.

In micro-CT analysis the bone volume results at 12 weeks were significantly higher than at 4 weeks ($P < 0.05$). It can be stated that osseointegration of the implant was enhanced by the bone remodeling and maturation over the time. This finding meets the purpose of our study to compare the osseointegration of implants surface-treated with TiO_2 nanotube between late modeling and late remodeling stages. Although there were no significant differences, 30 nm and 70 nm experimental groups showed the higher bone volume than other groups at 4 and 12 weeks (Figure 5). From the result of the micro-CT analysis, it can be stated that the implants surfaced-treated with 30 nm and 70 nm TiO_2 nanotubes show higher bone formation than control group.

In histomorphometric analysis, the BIC and bone volume were measured in the microthreads (BV) and three consecutive macrothreads (macro-BV). Although histomorphometry is a destructive method and there is uncertainty whether the analysis of histological sections represents the entire osseous status, the histomorphometric evaluation of the bone implant contact (BIC) and bone area within the threads

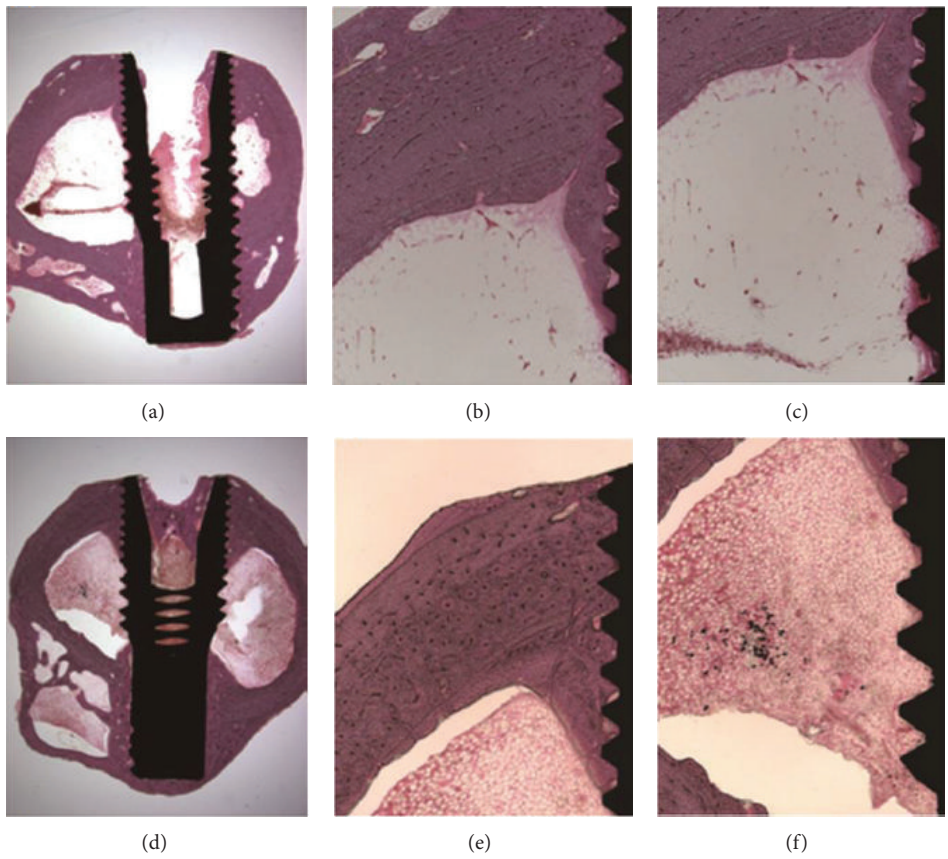


Figure 13: Histologic images of control ((a), (b), and (c)) and 30 nm ((d), (e), and (f)) groups at 12 weeks after implantation. ((a), (d)) H&E stained images at lower magnification (×12.5), ((b), (e)) H&E stained images of microthreads in the A&D (×50), and ((c), (f)) H&E stained images of macrothreads in the A&D (×50).

(BV) was established as the most common method and was applied in the majority of subsequent studies [29–31]. In histomorphometric analyses, BIC and BV results at 12 weeks were significantly higher than at 4 weeks ($P < 0.05$). Interestingly, although there was no statistical significance, the macro-BV results at 4 weeks were higher than at 12 weeks. This may be because modelled bone resulted from favorable surface characteristics during the healing period which was then resorbed during remodeling period because there were little functional stress and cellular component in the cancellous bone. Also, it can be stated that even in the situation where the bone is difficult to be generated there was new bone formation near the implants which were surface-treated with TiO_2 nanotube in modelling stage. Although there was no significant difference in histomorphometric analyses, 70 nm experimental group had the highest BIC and BV result at 4 weeks and 30 nm experimental group had the highest BIC and BV result at 12 weeks (Figures 11, 12, 13, and 14).

Removal torque test has been used for one of the ways to evaluate new bone formation since Johansson et al. said that a directly proportional relationship exists between removal torque and BIC [32]. In removal torque test, 70 nm experimental group had significantly higher value than any other groups at 4 weeks ($P < 0.05$) (Figure 10). These findings might explain the higher BIC and BV results of the 70 nm experimental group than any other groups at 4 weeks. We could not perform the statistical analysis because the number of specimens at 12 weeks was not sufficient for testing. However, the mean removal torque at 12 weeks was higher than at 4 weeks and 30 nm experimental group had the higher result than other experimental groups.

In this study, 30 nm and 70 nm experimental groups showed more new bone formation and bone implant fixation than control group. This result is in agreement with previously published study where pull-out testing indicated that TiO_2 nanotubes significantly improved bone bonding strength compared with TiO_2 grit-blasted surfaces in rabbit tibias [33]. We hypothesize that the topography of the TiO_2 nanotubes more closely resembles the porous structure of native bone tissue, allowing more optimal interactions for contact osteogenesis.

In this study, micro-CT investigation, histomorphometric analysis, and removal torque test showed similar patterns. The 70 nm experimental group at 4 weeks and the 30 nm experimental group at 12 weeks showed more new bone formation and exhibited a stronger osseointegration than other groups.

Our results showing good radiological, histological, biomechanical results in the 70 nm experimental group at 4

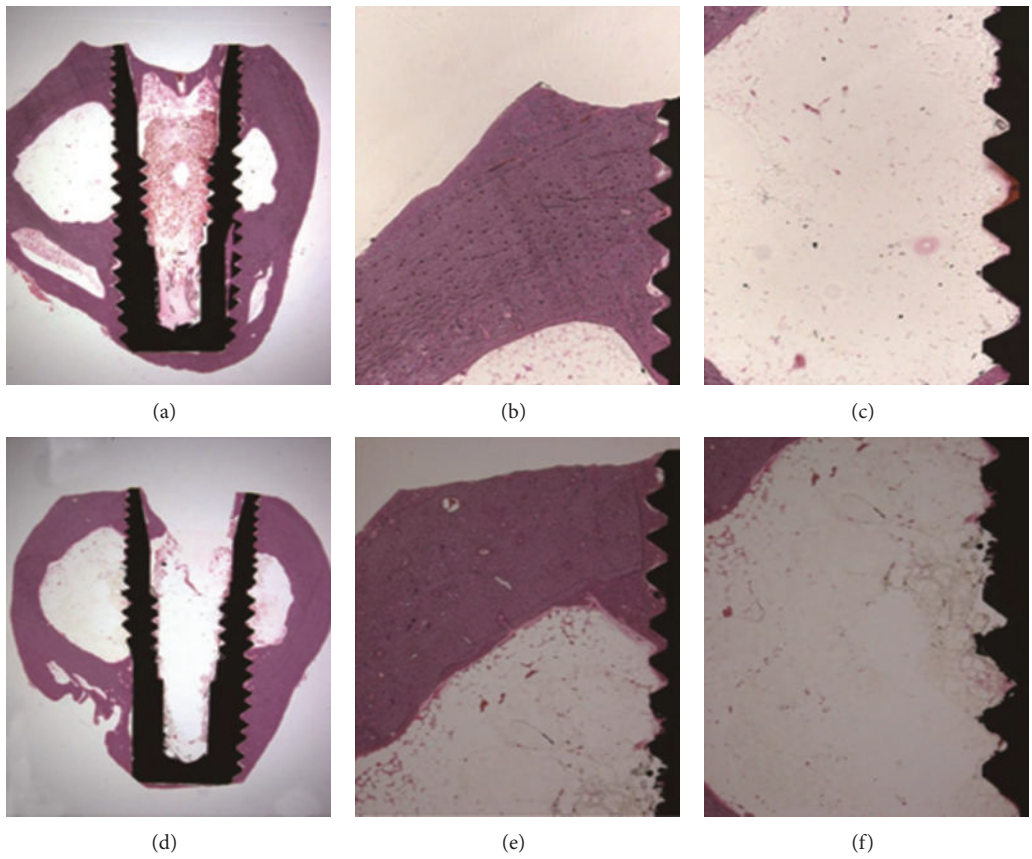


Figure 14: Histologic images of 70 ((a), (b), and (c)) and 100 ((d), (e), and (f)) nm groups at 12 weeks after implantation. ((a), (d)) H&E stained images at lower magnification (×12.5), ((b), (e)) H&E stained images of microthreads in the A&D (×50), and ((c), (f)) H&E stained images of macrothreads in the A&D (×50).

weeks are in accordance with Oh et al. reporting that the optimal elongation and cellular activity of osteoblasts and stem cells were obtained in large diameters (80, 100 nm) [16, 19]. They are also in accordance with von Wilmowsky et al. [25], who reported that the highest level of osteocalcin was observed in the 70 nm nanotube implant.

Once healing is completed and remodeling has been progressed at 12 weeks, 30 nm experimental groups showed good radiological, histological, and biomechanical results. These results are in accordance with Park et al. who reported that a spacing of 15 nm provides the optimum length scale for integrin clustering and focal contact formation, inducing osteoblasts, MSCs, and osteoclasts proliferation, migration, and differentiation [20, 21]. In addition, the maintenance of an appropriate balance of bone resorption and bone remodeling during and after wound healing is important for stable integration of the implants. In most aspects, osteoblasts and osteoclasts behave different *in vitro* and *in vivo*. Thus, the similar response of MSCs, HSCs, and osteoclasts to the 15 nm spacing suggests that this nanoscale spacing may be a universal scaffold at least for bone remodeling-associated cells [20, 21].

However, the cellular and molecular mechanism responsible for the favorable osteogenesis responses to TiO_2 nanotube is a complex biological process and is not fully understood yet. And the *in vitro* and *in vivo* studies searching for the optimal TiO_2 nanotube diameter have shown conflicting results depending on surface chemistry, crystalline structure, roughness, cell type, species of animal, and other experimental conditions. There are several material factors that affect how the proteins adhere, unfold and how the surface is perceived by the cell. These include surface chemistry, surface energy/tension/wet ability, surface roughness, crystal structure, surface charge, feature size, feature geometry, and other mechanical properties such as elasticity [15]. Therefore, in order to find the optimal diameter of the TiO_2 nanotube, the studies in various surface conditions of nanotube and in various kinds of animal models should be comparatively analyzed.

The limits of this study include that the number of samples of the experimental groups was too small and the femur was used as the model instead of the jaw bone. In addition, it was not possible to avoid the differences in the thickness of the cortical bone or the rate of growth and rehabilitation as well as other variations that might have happened during surgery. However, the implants that were surface-treated with TiO_2 nanotube showed good osseointegration compared to the control group. From the time point of view, the 70 nm experimental group at 4 weeks and the 30 nm experimental group at 12 weeks showed more new bone formation

and a stronger osseointegration than other groups. Therefore, depending on healing time, both 30 nm and 70 nm TiO_2 nanotubes could be beneficial to osseointegration of implants compared to control and 100 nm nanotubes. However, further investigation with the increasing number of implants would be necessary to yield a meaningful result because statistically significant difference was not found.

Future trends of implant will be concerned about the modifications of surface roughness at the nanolevel and the incorporation of biological drugs for earlier osseointegration and loading in large defect region. Due to their simple manufacturing and the possibility for the usage as a drug delivery system, TiO_2 nanotubes can be a useful method for future implant surface treatment [3]. Therefore, based on this experiment, preclinical studies confirming the optimal nanotube diameter for earlier osseointegration, implantation in large defect area, and drug delivery in large-sized animal model are necessary.

5. Conclusion

Within the limitations of this study, the results from micro-CT and histomorphometric analysis showed that the bone volume, BIC, and micro-BV results at 12 weeks were higher than at 4 weeks ($P < 0.05$). However, BV results in three consecutive macrothreads at 4 weeks were higher than 12 weeks ($P > 0.05$). Also, removal torque test showed that 70 nm experimental group had higher removal torque value than any other groups at 4 weeks ($P < 0.05$). Overall, micro-CT, histomorphometric analysis, and removal torque test results all showed similar pattern; 70 nm experimental group had highest value at 4 weeks and 30 nm experimental group had highest value at 12 weeks. Therefore, on the basis of above results, it can be concluded that both 30 nm and 70 nm TiO_2 nanotubes may have positive effects on osteogenesis and osseointegration depending on the healing period.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Cheul-Goo Kang and Young-Bum Park equally contributed to this study. Seunghan Oh is cocorresponding author for production of TiO_2 nanotube.

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