# Osseointegration of implants surface treated with various diameters of $TiO_2$ nanotubes in rabbit

Cheul-Goo Kang

The Graduate School
Yonsei University
Department of Prosthodontics

# Osseointegration of implants surface treated with various diameters of $TiO_2$ nanotubes in rabbit

Directed by Professor June-Sung Shim

A Dissertation
Submitted to the Department of Prosthodontics
and the Graduate School of Yonsei University
in partial fulfillment of the
requirements for the degree of
Doctor of Dental Science

Cheul-Goo Kang

December 2012

This certifies that the Dissertation of
Cheul-Goo Kang is approved.

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Thesis advisor : June-Sung Shim

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[Thesis Committee : Keun-Woo Lee]

____________________________

[Thesis Committee : Yung-Bum Park]

____________________________

[Thesis Committee : Seong-Ho Choi]

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[Thesis Committee : Seung-Han Oh]

The Graduate School

Yonsei University

December 2012

# 감사의 글

대학에 입학한 지 22년 만에 배움의 끝이라 할 수 있는 박사과정을 마치게 되었습니다. 한편으로는 홀가분한 마음이지만 박사 학위가 다음 과정을 위한 연습이며 새로운 배움의 시작이라는 생각을 하게 됩니다. 한 사람의 새내기 대학생이 중년의 박사가 되기까지 가르침과 도움을 주셨던 많은 분들께 감사의 마음을 전하고 싶습니다.

무사히 학위를 마칠 수 있도록 박사 학위 과정 내내 이끌어 주신 심준성 지도 교수님께 먼저 깊은 감사를 드립니다. 그리고 부족한 논문에 조언과 격려를 아끼지 않으신 이근우 교수님, 박영범 교수님, 최성호 교수님과 오승한 교수님께도 감사의 말씀을 드립니다. 석사과정을 지도해 주신 정문규 교수님, 학문에 대한 열정으로 귀감이 되어주신 한동후 교수님, 박사과정 입학을 허락해 주신 문홍석 교수님 그리고 논문 작성에 도움이 되어 주신 이재훈 교수님, 김지환 교수님께도 감사 드립니다.

보철학에 입문 할 수 있도록 제자로 받아주신 전영식 선생님과 공중보건의 시절 보철학의 매력을 알려주신 양순봉 선배님께 뒤늦은 감사의 마음을 전합니다.

실험에 많은 도움을 주셨던 김무성 선생님과 논문 작성의 동반자가 되어주신 자미안 선생님에게 고맙다는 말을 전하며 실험과 논문 작성 내내 저와 함께 해 주신 박영범 교수님께 다시 한번 깊이 감사 드립니다.

학창시절을 같이 해준 승문, 정우, 호걸 매일 매일을 같이하며 늘 도움이 되어주는 승환, 광출, 동근, 윤수에게도 고맙다는 말을 전합니다.

마지막으로 가족들과 오늘의 기쁨을 함께하고 싶습니다. 삼형제를 한결 같은 믿음과 사랑으로 길러주신 부모님께 진심으로 감사 드립니다. 저도 자식을 신뢰하며 키울 수 있는 용기 있는 부모가 되었으면 좋겠습니다. 그리고 늘 제 편이 되어주시는 장인 장모님, 항상 저를 응원해주는 형제와 가족들에게 감사의 마음을 전합니다.

힘들고 어려운 일이 있어도 지우와 병준이를 바라보고 있으면 절로 미소가 지어지고 없던 힘도 솟아나는 느낌입니다. 이 아이들이 자라 자신의 연구 분야에 매진할 때에 뒤늦게 시작한 공부로 힘겨울 때면 아버지가 그들을 바라보며 힘을 얻었다고 말해주고 싶습니다. 공교롭게도 수업과 과제로 바쁜 학기에 첫째가 태어났고 실험과 논문 때문에 분주한 학기에 둘째가 태어났습니다. 남편의 도움이 절실할 시기에 남편 학업까지 뒷바라지 하느라 고생이 많았던 사랑하는 아내 미래와 함께 이 기쁨과 영광을 나누고자 합니다.

2012년 12월　강철구 드림

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<ABSTRACT>

# Osseointegration of implants surface treated with various diameters of $TiO_2$ nanotubes in rabbit

Cheul-Goo Kang

*Department of Prosthodontics*
*The Graduate School, Yonsei University*

(Directed by Professor June-Sung Shim)

The aim of this study was to evaluate the osseointegration of implants surface treated with various diameters of $TiO_2$ nanotubes (30 nm, 70 nm and 100 nm) in rabbit.

RBM surfaced implants (Osstem, Busan, Korea) with 3.5mm in diameter and 8.5mm in length were designated as control group and implants surface treated with various diameters of nanotubes (30nm, 70nm, 100nm) of the same shape were designated as experimental group. The implants were maintained unloaded for 4 and 12 weeks. After this period, the animals were sacrificed and Micro-CT evaluation, histomorphometric analysis (bone to implant contact; BIC, bone volume; BV) and removal torque test, were performed,

In micro CT analysis the bone volume results at 12weeks were significantly higher than at 4weeks ($p<0.05$). The results from micro CT examination showed that 30 nm and 70nm experimental group had the highest bone volume at 4 and 12weeks respectively, however, there were no statistical significant differences ($p>0.05$). In histomorphometric analysis the BIC and BV results at 12weeks showed significantly higher value than at 4weeks ($p<0.05$) and the BV results in three consecutive macro threads(Macro BV) at 12weeks showed higher value than at 4weeks, but there were no statistical significant differences ($p>0.05$). At

4weeks 70 nm experimental group had the highest BIC, BV and Macro BV result. At 12weeks 30nm nm experimental group had the highest BIC and BV result and control group had the highest Macro BV result, however there were no statistical significant differences($p>0.05$). The results from removal torque test showed that 70nm experimental group had the significantly higher RTV result compared to other groups at 4weeks ($p<0.05$). Micro CT, histomorphometric analyses, removal torque test results showed similar pattern that 70 nm experimental group had the highest value at 4weeks and 30nm experimental group had the highest value at 12weeks.

On the basis of results above, 30nm and 70nm $TiO_2$ nanotube may have positive effects on osteogenesis and osseointegration depending on the healing time. Further studies confirming the optimal nanotube diameter for earlier osseointegration, implantation in large defect region and drug delivery in the larger size animal model are necessary.

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Key words : $TiO_2$ nanotube, surface treatment, rabbit, osseointegration

# Osseointegration of implants surface treated with various diameters of $TiO_2$ nanotubes in rabbit

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## I. INTRODUCION

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 comes from inactivity of $TiO_2$ oxidation layer, the osseointegration of implant is also delayed because of them. Therefore, research groups have been trying to modify the $TiO_2$ surface to promote even earlier and better osseointegration[1, 2].

Schwartz et al. 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 condition play the important role in adhesion and proliferation of the cell and adsorption of protein during the early state of healing process[4, 5]. According to studies on the surface roughness of implants, rough−surfaced implants have sooner and stronger osseointegration clinically compared to smooth surfaced implants because 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 maximized survival rate is known to be 1−2 $\mu$m[2, 11].

In recent years, nanoscale surface modification has been attracting increasing attention. 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 MSCs [15−17]. $TiO_2$ nanotubes are hydrophilic, increase the surface area, and may provide increased channeling for the proper fluid exchange [15].

In previous study the cell behavior on the surface of $TiO_2$ nanotubes varied depending on the nanotube diameter. Oh et al. reported that on the 70nm $TiO_2$ nanotubes the adhesion of proteins, osteoblasts and MSCs showed the highest degree and the elongation and cellular activity of osteoblasts and MSCs were obtained on large(70, 100nm) $TiO_2$ nanotubes[16−19]. On the other hand, Park et al. 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 and differentiation[20, 21].

While there are many in vitro studies about $TiO_2$ nanotubes, there are not many animal studies on 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 treated with various diameters of $TiO_2$ nanotubes histomorphometically and biomechanically.

# II. MATERIALS AND METHODS

## 1. Implants and $TiO_2$ nanotube fabrication

### A. Implants

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

### B. $TiO_2$ nanotube fabrication

$TiO_2$ nanotube surfaces were processed on sixty machined surface implant in the department of dental biomaterials, college of dentistry, Wonkwang University, Iksan, Korea. Following is the brief description of the process.

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 2hours in order to crystallize the amorphous structure into an anatase structure.

Implants treated with various diameters (30㎛, 70㎛, 100㎛) of nanotubes were designated as experimental group.  Every group was divided into two categories according to healing time (4 weeks, 12 weeks).

Table I . Experimental groups classified by nanotube surface treatment

| Week | RBM surfaced | 30nm | 70nm | 100nm |
|---|---|---|---|---|
| 4 | 10 | 10 | 10 | 10 |
| 12 | 10 | 10 | 10 | 10 |

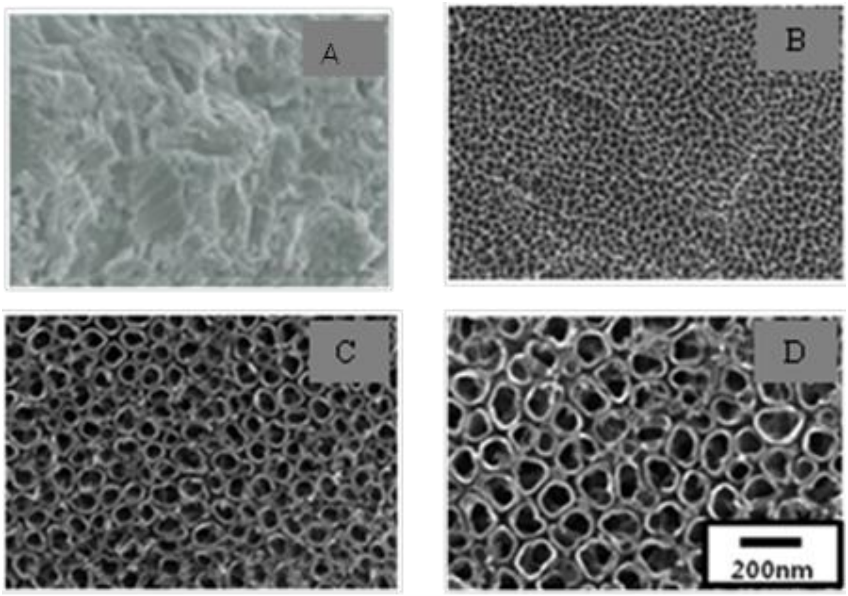


**Fig.1.** SEM images of an Resorbable Blasted Media surface used as control group(A) and $TiO_2$ nanotubes with various diameters, 30(B), 70(C), 100nm(D), processed by controlling anodizing potentials ranging from 5 to 20 V(Scale bar, 200㎛) courtesy from Prof. Seung-Han Oh in Wonkwang University

## 2. Experimental animals and surgical procedure

### A. experimental animals

Twenty rabbits (New Zealand white) of 6 weeks old, weighting approximately 3.5 kg each were used in this study. Animal selection and 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.

### B. surgical procedure

All surgical procedures were performed under general anesthesia. The animals were anesthetized with intravenously administered mixture of 30mg/kg of

Zolazepam (Zoletilⓡ virback Korea Co., Seoul, Korea) and 10mg/kg of Xylazine HCI (Rumpunⓡ, Bayer Korea, Seoul, Korea). After ten minutes later, the site of surgery was shaved and sterilized with povidon−iodine then further anesthetized with 2% lidocaine HCl with epinephrine 1:80000 by infiltration.

Implants were placed in the right femur of rabbit. After 8 weeks, implants of the same group were placed in the left femur of rabbit.  4 weeks after the second implantation animals were sacrificed by 2% para formaldehyde 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 and the last samples in each group were analyzed biomechanically

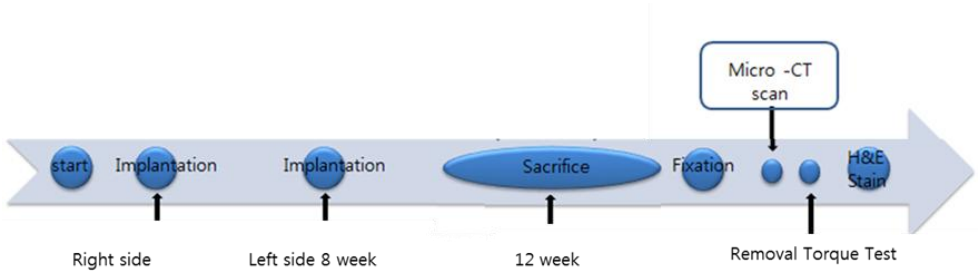


**Fig.2.** Diagram of experimental design protocol

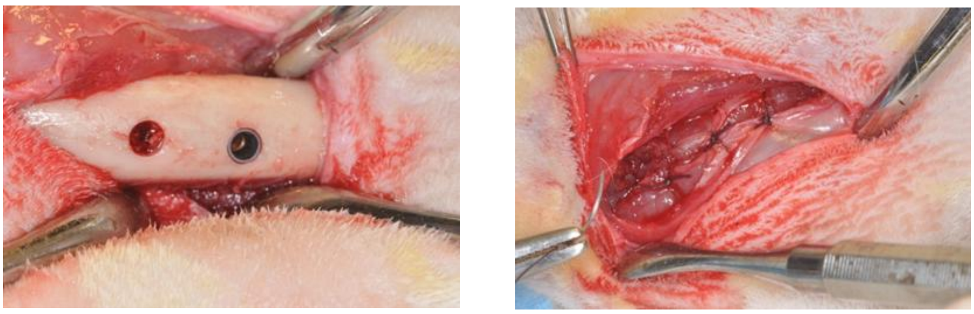

**Fig.3.** Pictures of surgical procedure and location of implants in the rabbit femur.

## 3. Evaluation method

### A. Micro-computed tomography (Micro CT) analysis

To evaluate the position of implants in the femur and new bone formation near the implant surface, mean bone volume within 400μm of implant surface was measured by micro CT (Skyscan 1076, Aartselaar, Belgium) at 18 μm pixel, 50 Kv and 30 μA.

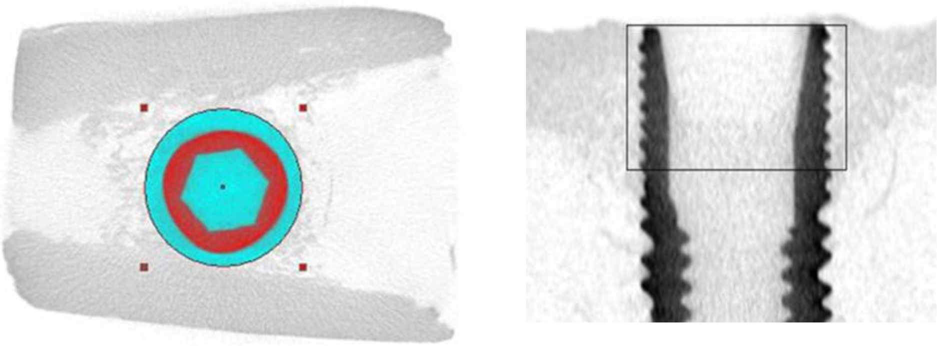

**Fig.4.** The defined area for measurement of new bone ( diameter 4.4mm x height 2 .5mm).

### B. Histologic and Histomorphometric analysis

The specimens were dehydrated through graded alcohols of 70%, 80%, 95%, 95%, 100% at 2 hour intervals for 1 week. The specimens were embedded in Technovit 7200(Heraeus KULZER, Dormagen, Germany) and alcohols (1:3,1:1,3:1 ratio) and sectioned in the bucco-lingual 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 ㎛ by microgrinding and polishing 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 microscopy Leica DM 2500, Leica Microsystems,Wetxlar, Germany) at x12.5, x50 magnification . The bone to implant contact ratio (BIC) was measured in the microthreads and bone volume was measured in the microthreads(BV) and three consecutive macro threads(Macro BV)using imaging analyses system (Image−Pro Plus 4.5 Media Cybernetics Inc., Silver Springs, MD, USA).


**Fig.5.** The defined area for measurement of new bone (H&E stain ; 12.5 magnification) A: Microthreads area, B: Three consecutive macro threads area.

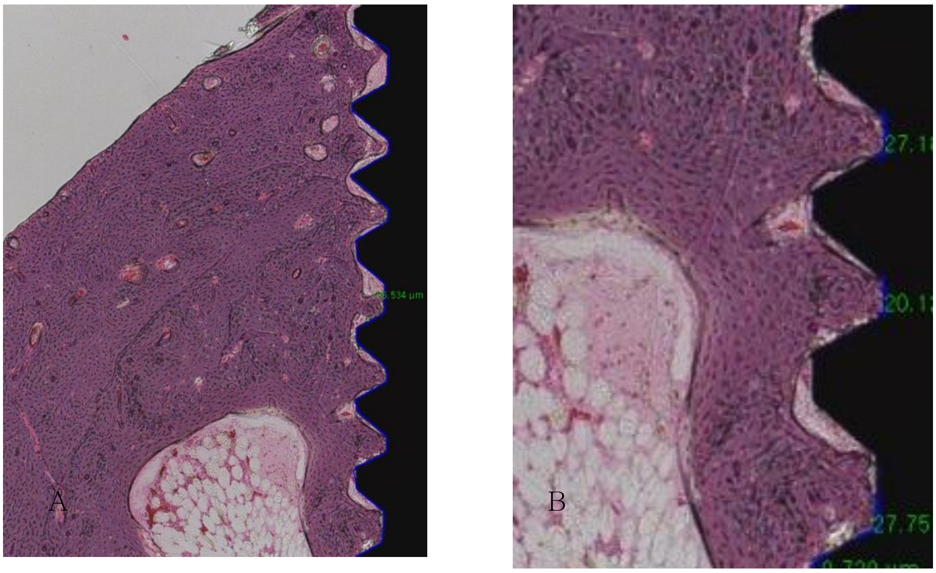


**Fig.6.** Calculation of Bone to Implant Contact. Total length of microthreads(A), Length of Bone to Implant Contact(B).

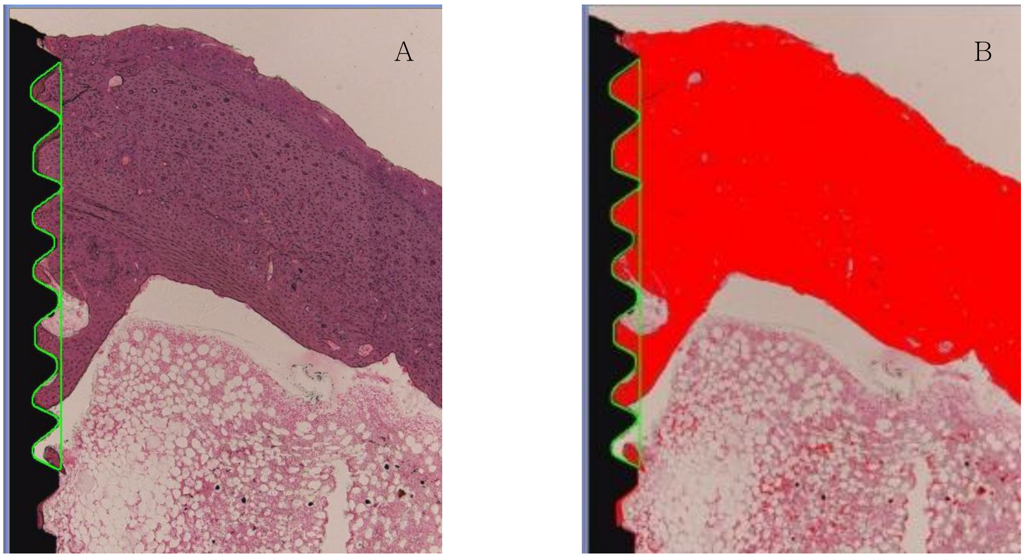


**Fig.7.** Calculation of Bone Volume. Total area of inter−thread space(A), Area of Bone Volume (B).

## C. Removal torque analysis

To evaluate the osseointegration of implants by biomechanically, removal torque analysis was performed at the same time of animal 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.


**Fig.8** Digital torque gauge (Mark-10, MGT12, New York, USA)

## D. 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/ BV/Macro BV and RTV analysis. The level of statistical significance was set at $p<0.05$.

# III. RESULTS

## 1. Clinical finding

Among twenty rabbits, four rabbits died from femur fracture or post-surgery stress. In addition we couldn't use 16 samples due to femur fracture at the site of implantation. Finally forty-eight samples were acquired then thirty samples were used for Micro CT and histomorphometric analysis and eighteen samples were used for removal torque analysis

## 2. Micro CT scan

The CT bone volume results at 12 weeks showed significantly higher value than at 4 weeks ($p<0.05$).

At 4 and 12 weeks, 30nm and 70nm experimental group had the highest bone volume, but there were no statistical significant differences ($p>0.05$) (Fig 9).

In micro CT images implants were positioned well in the middle of femur (Fig. 10)

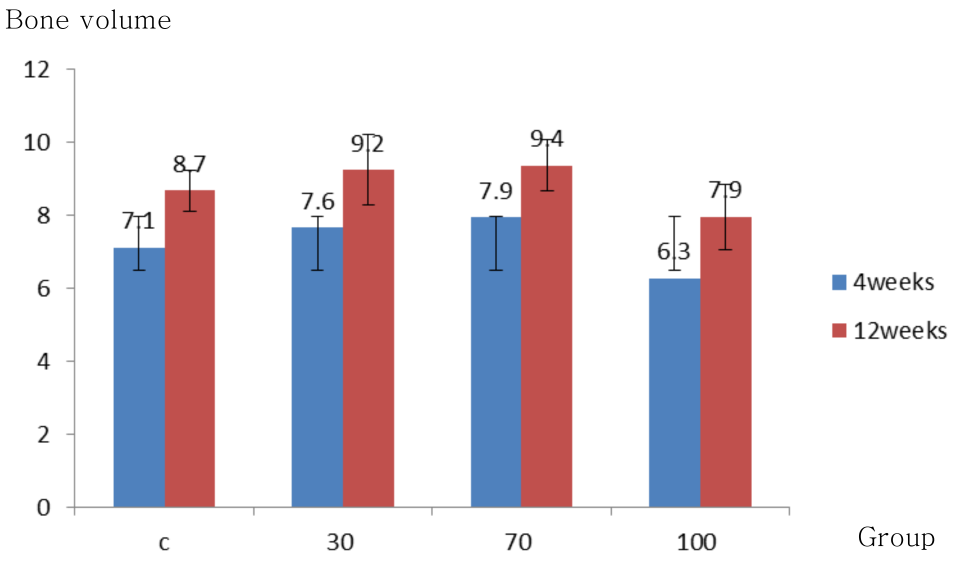


**Fig.9.** Measurement of Bone volume ($mm^3$) at 4 weeks and 12 weeks

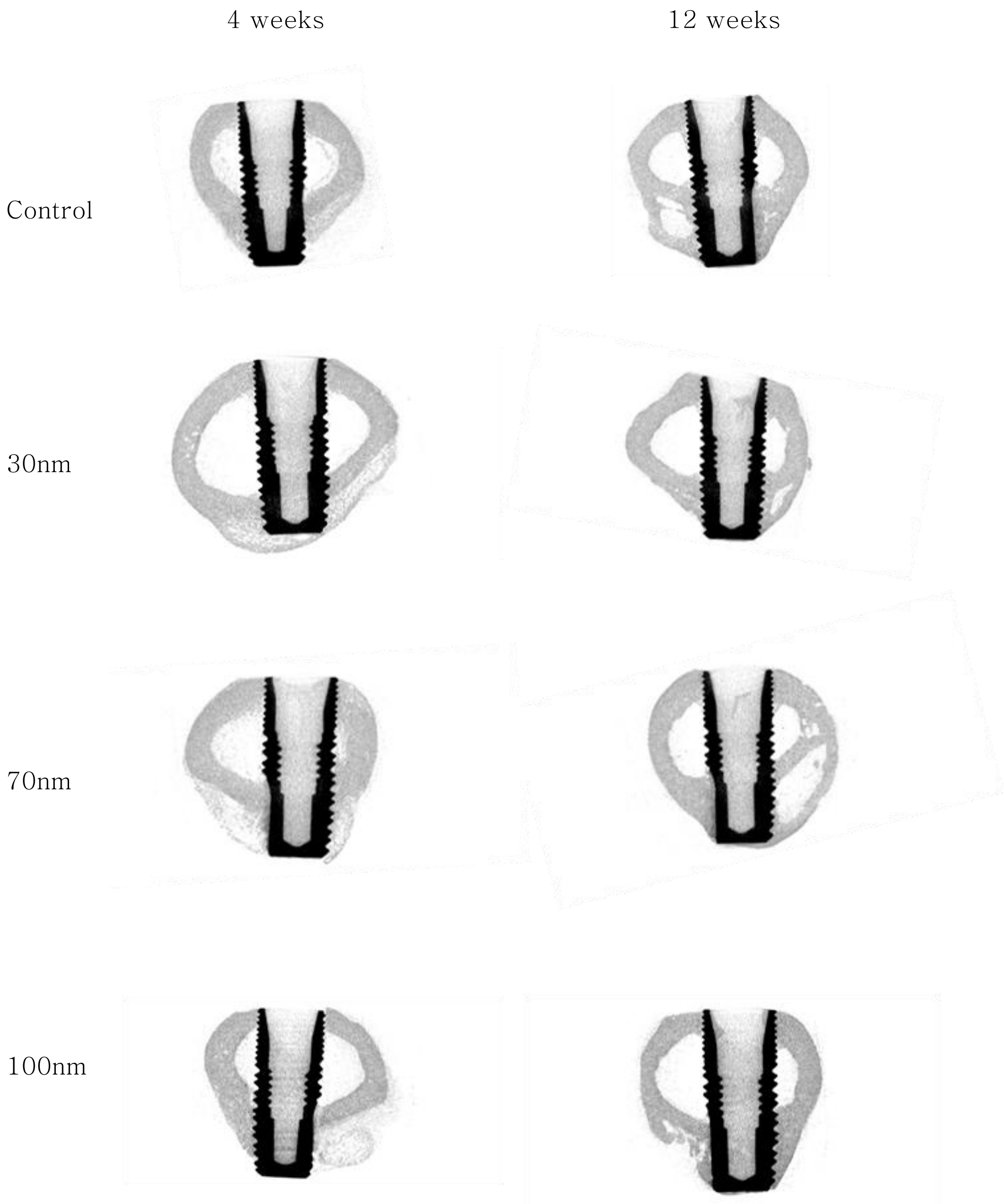


**Fig.10.** Micro CT images of representative sample of each group. All the implants were placed favorably in femur.

## 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 ($p>0.05$)

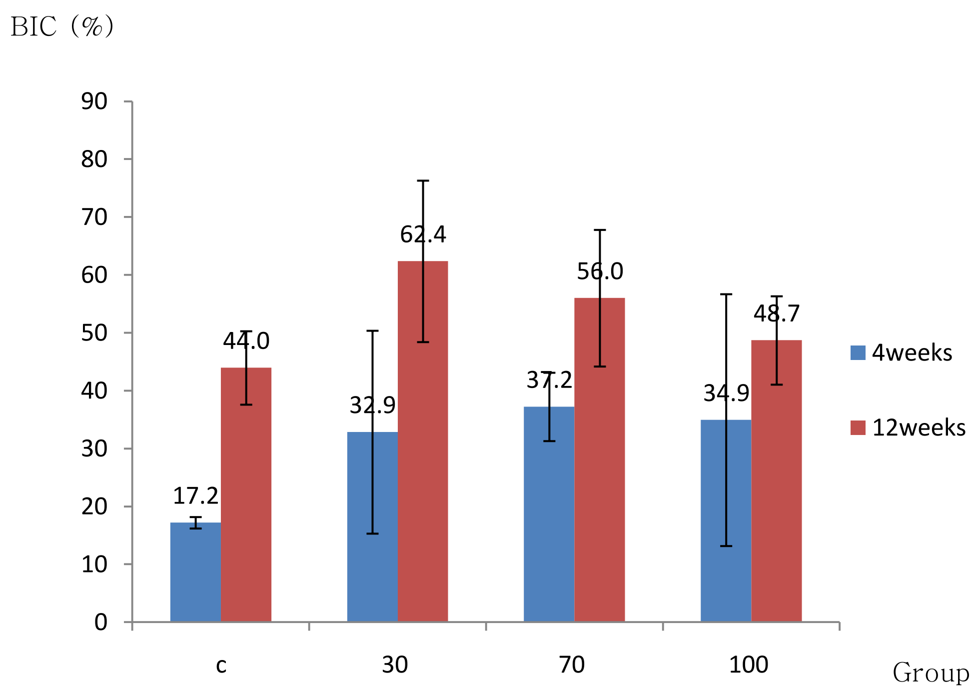


**Fig.11.** measurement of BIC (%) at 4 weeks and 12 weeks in defined area which is designated in micro threads

The BV results at 12 weeks showed significantly higher value than at 4 weeks ($p<0.05$).

At 4 weeks, 70nm experimental group had the highest BV value, but there were no statistical significant differences ($p>0.05$).

At 12 weeks, 30nm experimental group had the higher BV value than 100nm experimental group ($p<0.05$).

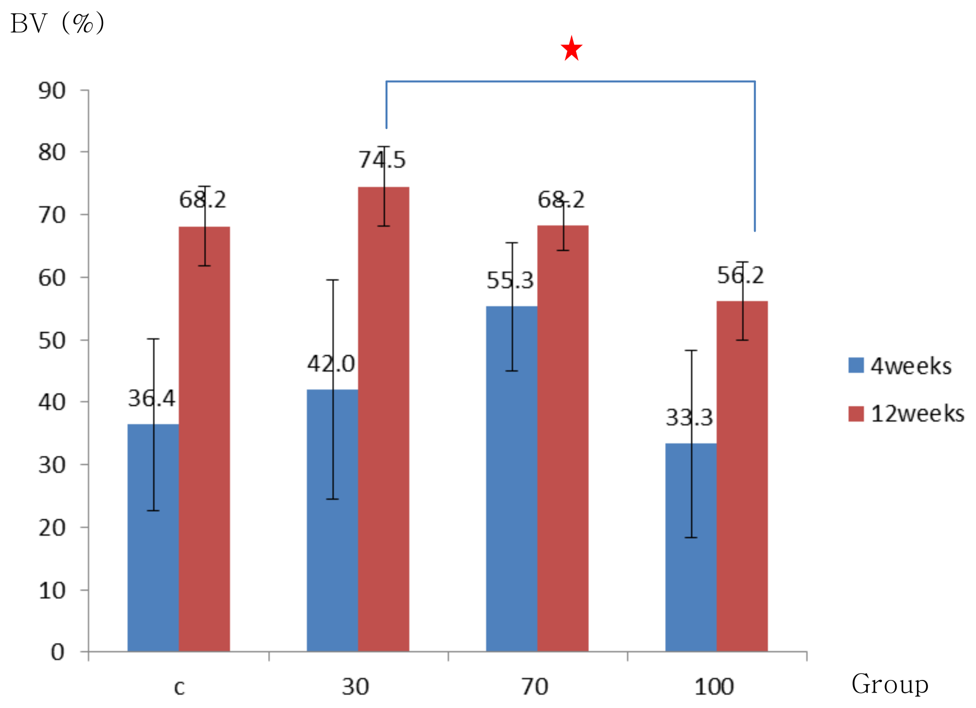


**Fig.12.** Measurement of Bone Volume ( % ) at 4 weeks and 12 weeks in defined area which is designated in micro threads

The bone volume results in three consecutive macro threads(Macro BV) at 12 weeks showed higher value than at 4 weeks, but there were no statistical significant differences ($p>0.05$).

At 4 weeks, 70nm experimental group had the higher Macro BV value than 100nm experimental group ($p<0.05$)

At 12 weeks, control group had the highest Macro BV value, but there were no statistical significant differences ($p>0.05$).

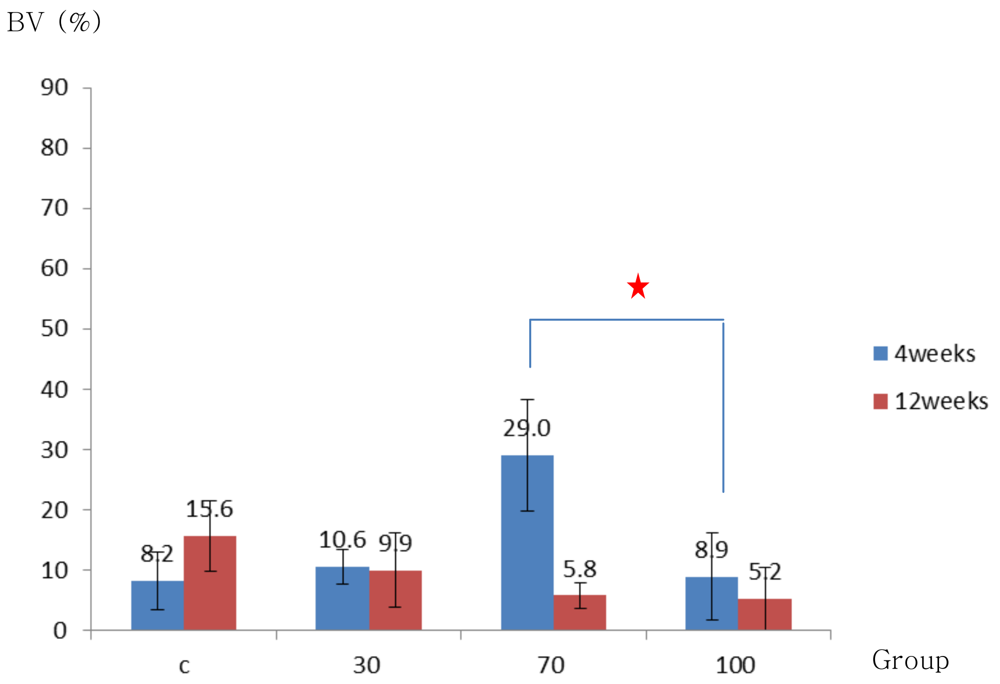


**Fig.13.** Measurement of Bone Volume ( % ) in defined area which is designated in three consecutive macro threads at 4 weeks and 12 weeks.

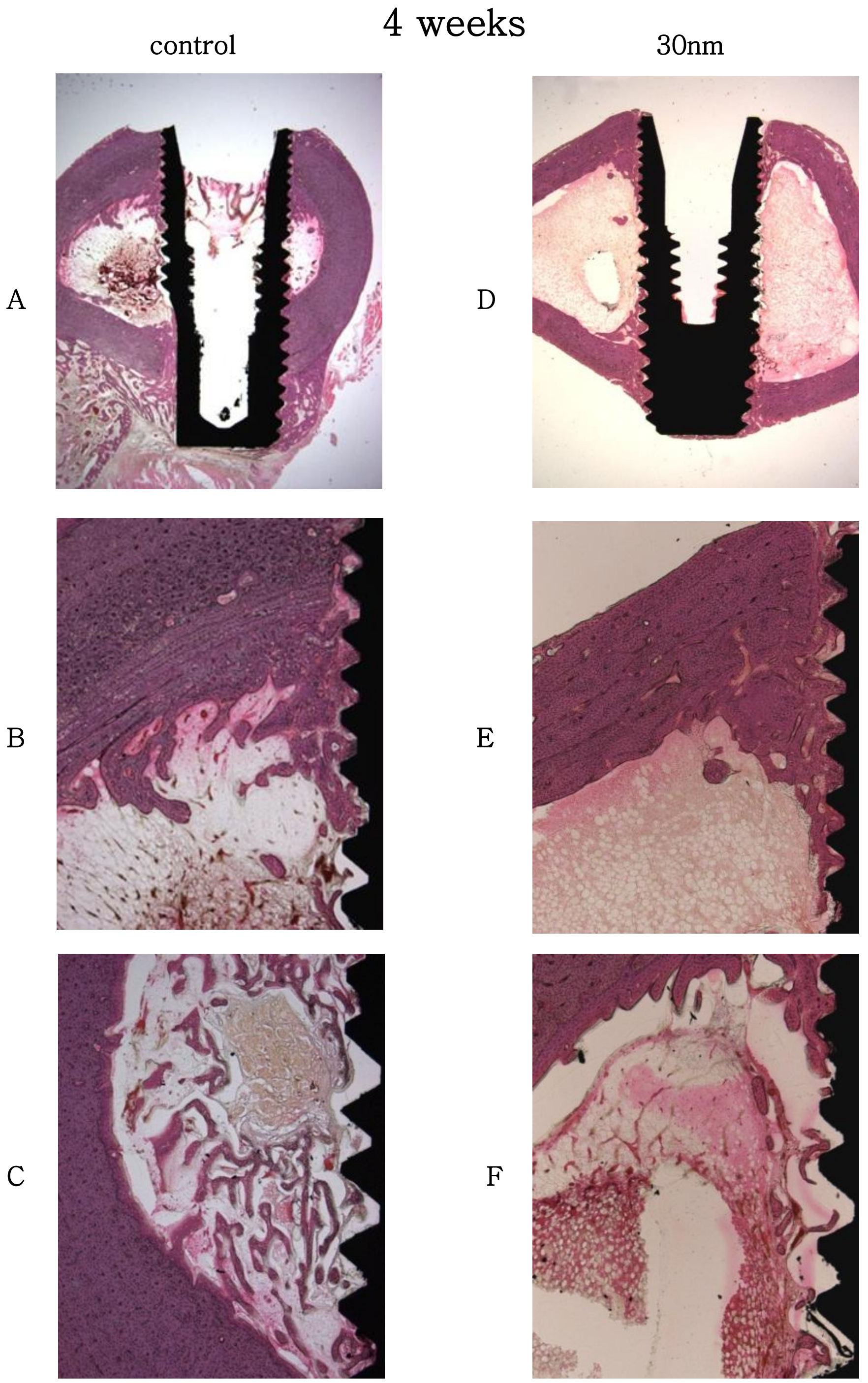


**Fig.14.** Histologic images of control (A,B,C) & 30 nm(D,E,F) group at 4 weeks after implantation.
A,D : H&E stained images at lower magnification (X12.5)
B,E : H&E stained images of microthreads in the A&D (X50)
C,F : H&E stained images of macrothreads in the A&D (X50)

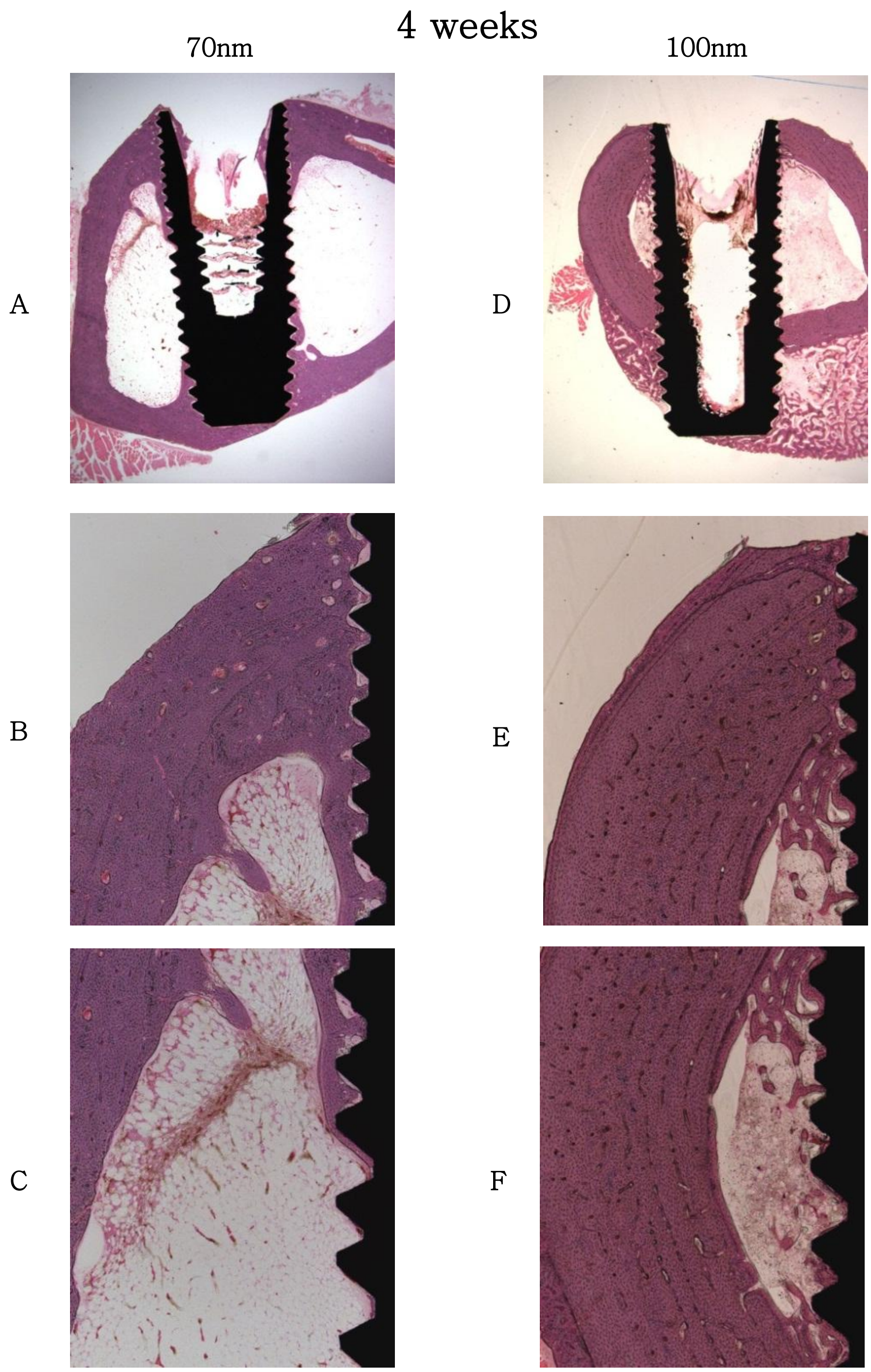


**Fig.15.** Histologic images of 70 (A,B,C) &100 (D,E,F) nm group at 4 weeks after implantation.
A,D : H&E stained images at lower magnification (X12.5)
B,E : H&E stained images of microthreads in the A&D (X50)
C,F : H&E stained images of macrothreads in the A&D (X50)

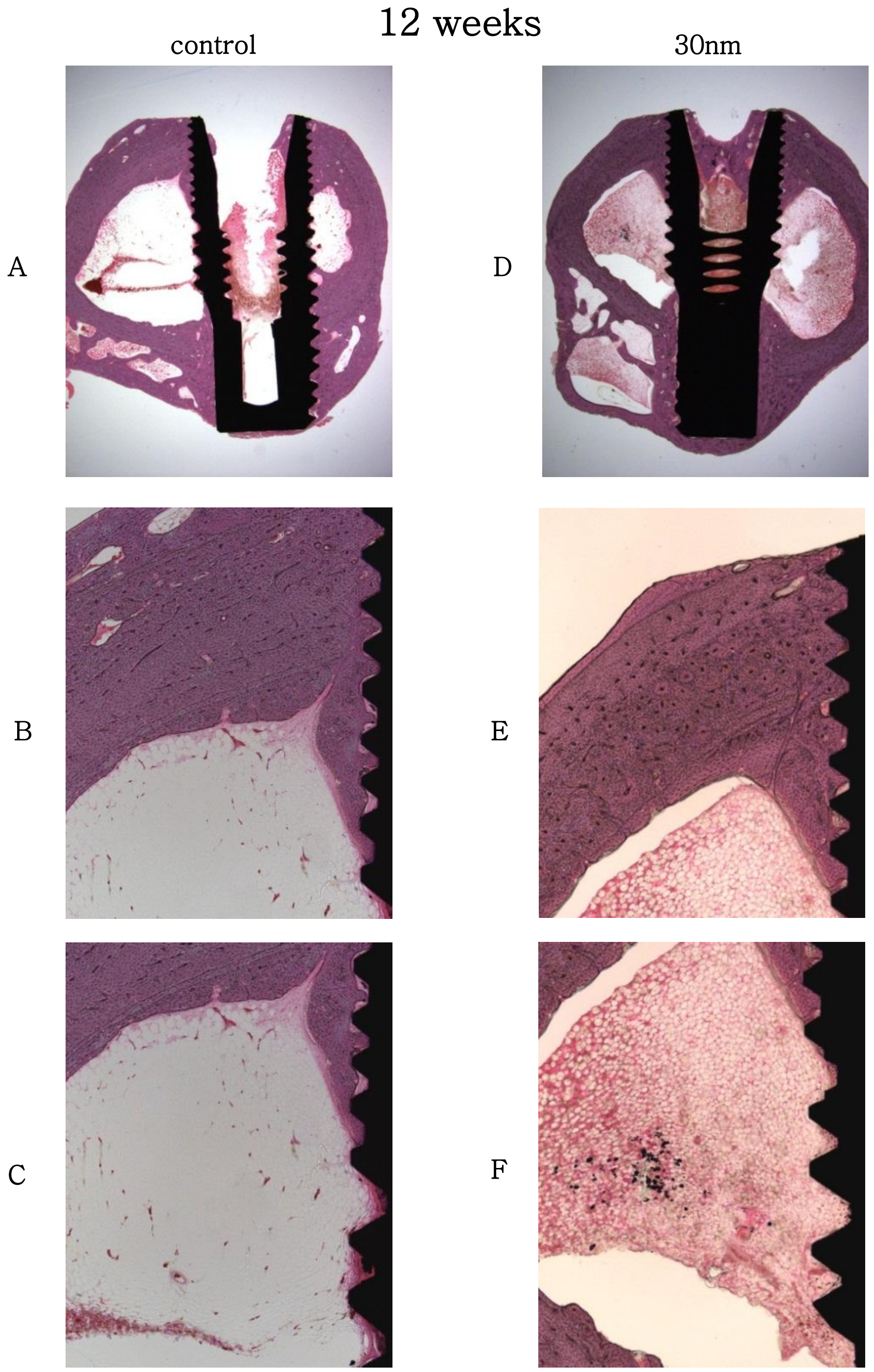


**Fig.16.** Histologic images of control(A,B,C) & 30 nm(D,E,F) group at 12 weeks after implantation.
A,D : H&E stained images at lower magnification (X12.5)
B,E : H&E stained images of microthreads in the A&D (X50)
C,F : H&E stained images of macrothreads in the A&D (X50)

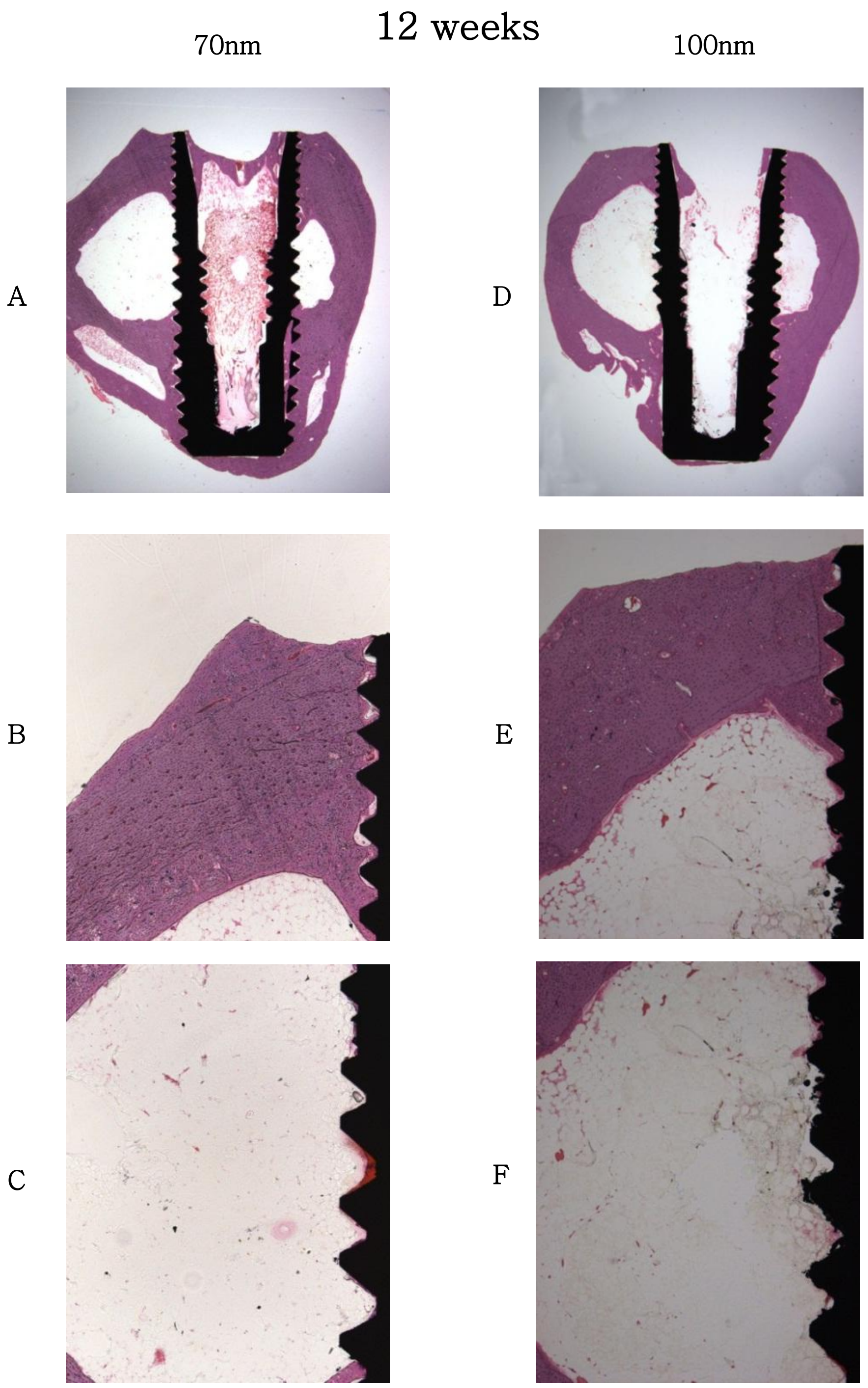


**Fig.17.** Histologic images of 70(A,B,C) &100(D,E,F) nm group at 12 weeks after implantation.
A,D : H&E stained images at lower magnification (X12.5)
B,E : H&E stained images of microthreads in the A&D (X50)
C,F : H&E stained images of macrothreads in the A&D (X50)

## 4. Removal torque measurement

At 4 weeks, 70 nm experimental group had the higher removal torque value than other groups ($p<0.05$)

We could not perform the statistical analysis because the number of specimens at 12weeks was not sufficient for the test.

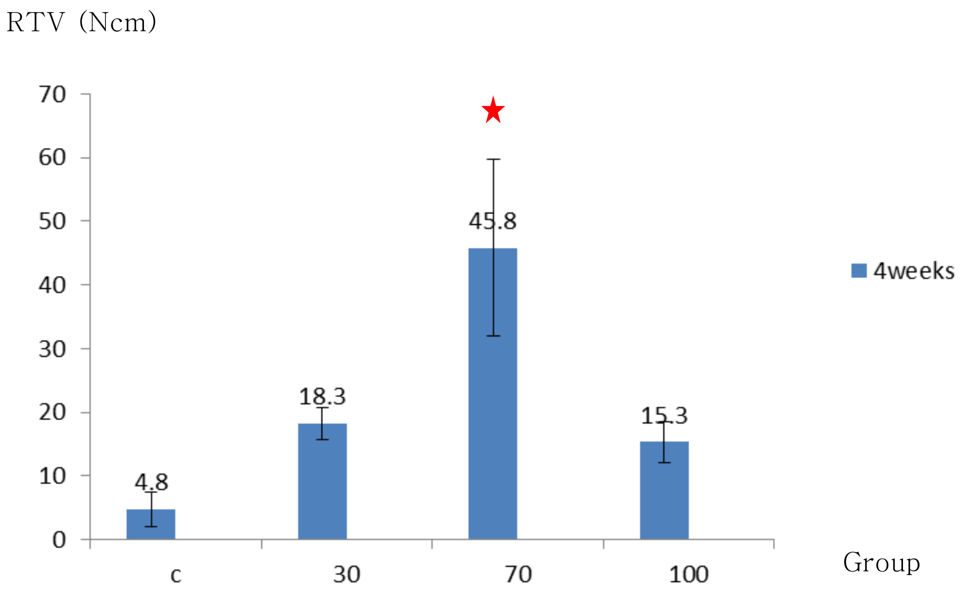


**Fig.18.** The mean of removal torque values at 4 weeks after implantation.

# IV. DISCUSSION

In the installation of implants, adhesion and differentiation of cells on the implant surface are critical factors for the successful osseointegration between the implant and the bone. With the effort to enhance the cell adhesion and osteogenesis of cells on the implant surface, there have been studies on modifying the $TiO_2$ surface by processing nanostructures on the oxide surface[12]. Vertically aligned and laterally spaced $TiO_2$ nanotubes created by electrochemical anodization are hydrophilic, increase the surface area and may provide increased channeling for the proper fluid exchange. In addition , the advantages of $TiO_2$ nanotube includes simple, low cost, flexible manufacturing and the possibility for their usage as a drug or growth factor delivery system [15].

In vitro studies show that the $TiO_2$ nanotube improved osteoblast adhesion, proliferation and mesenchymal cell differentiation [16-21]. In animal studies, implants surface treated with $TiO_2$ nanotubes demonstrated a significant increase in new bone formation and gene expression associated with bone formation and remodeling during the osseointegration period [22, 23]. To our knowledge, there are not many animal studies on the effect of various nanotube diameters on osseointegration of titanium implants, and the optimum $TiO_2$ nanotube diameter is still controversial [16, 19-21].

Therefore, it is important to carry out an in vitro study using small, medium and large animals to compare the osseointegration of the implants surface treated with various diameters of $TiO_2$ nanotubes. The purpose of this study was to evaluate the osseointegration of implants surface treated with various diameters of $TiO_2$ nanotubes depending on the healing time in rabbit based on the previous study which used the rat model system.

In the previous study, rat model have been used. In order to investigate the ossteointegraion in the larger animal, rabbits(New Zealand white) were used in this study. The rabbit is one of the most commonly used animals for medical research, being used in approximately 35% of musculoskeletal research studies, due to ease of handling and size[24]. We installed 4 implants in a rabbit in order to compare the osseointegration processes between modelling stage(4weeks) and remodelling stage(12weeks) in the same animal[25]. And we installed the implants in the middle of rabbit femur where the quality of bone is poor to observe the surface characteristic of the implant. In this study, femur fractures occurred in more than expected times. For the rabbit model, implants are not recommended to be larger than 2mm in diameter and 6mm in length because the bone is brittle[26]. In this study we use real size (3.5mm*8.5) implant which was the relatively large for the rabbit. Specially designed implant for the rabbit can reduce the trauma in further study.

Implants treated with various diameters of nanotubes (30nm, 70nm, 100nm) were designated as experimental group which was recommended for optimal in previous studies [16, 20, 23]. Half of the samples in each group were measured and compared the bone area near the implants to evaluate the osseointegration of implants radiologically , histomorphometrically and the last samples in each group were measured the implant removal torques to evaluate the osseointegration of implants biomechanically

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, non-destructive 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[27]. Futami et al stated that the affected areas in the installation of implant are within 100μm drilling sites [28] and Kenzora et al stated that the affected areas are within 500μm drilling sites[29]. In this study we set the affected area within 400μm from the implant surface.

In micro CT analysis the bone volume results at 12 weeks were significantly higher than at 4weeks ($p<0.05$). It can be stated that osseointegration of the implant was enhanced by the bone remodeling and maturation over 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 stage. Although there was no significant differeces, 30nm and 70nm experimental group had the highest bone volume at 4 and 12weeks (Fig 9). From the result of the micro CT analysis it can be stated that the implants surfaced treated with 30nm and 70nm $TiO_2$ nanotubes show higher bone formation than control group.

In histomorphometric analyses, the bone to implant contact ratio (BIC) and bone volume were measured in the microthreads(BV) and three consecutive macro threads(Macro BV). Although histomorphometry is a destructive method and there is uncertainty whether the analysis of histological sections represent the entire osseous situation, the histomorphometric evaluation of the bone−implant contact (BIC) and bone area within the threads(BV) was established as the most common method and was applied in the majority of subsequent studies[30−32].

In histomorphometric analyses, BIC and BV results at 12weeks were significantly higher than at 4weeks ($p<0.05$). It can be stated that osseointegration of the implant was enhanced by the bone remodeling and maturation over time. Interestingly, though there was no statistical significance, the Macro BV results at 4 weeks were higher than at 12 weeks. Perhaps the modelled bone caused by favorable surface characteristics during the healing period was resorbed during remodeling period because there was little of functional stress and cellular component in the cancellous bone. 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 surface treated with $TiO_2$ nanotube in modelling stage. Although there was no significant differeces, in histomorphometric analyses 70 nm experimental group had the highest BIC and BV result at 4weeks and 30nm nm experimental group had the highest BIC and BV result at 12weeks (Fig 11,12,13).

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[33]. In removal torque test, 70 nm experimental group had the significantly higher value than other groups at 4weeks ($p<0.05$) (Fig 18). These findings might explain the higher BIC and BV results of the 70 nm experimental group than other groups at 4weeks. We could not perform the statistical analysis because the number of specimens at 12 weeks was not sufficient for test. however the mean removal torque at 12 weeks was higher than at 4 weeks and 30nm experimental group had the higher result than other experimental groups.

In this study, 30nm and 70nm experimental groups showed more new bone formation and bone implant fixation than control group. This result is in agreement with previously published data where pull-out testing indicated that $TiO_2$ nanotubes significantly improved bone bonding strength compared with $TiO_2$ grit-blasted surfaces in rabbit tibias[34]. 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 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 (80,100nm) [16, 19]. They are also in accordance with von Wilmovosky et al, who reported that the highest level of osteocalcin was observed in the 70 nm nanotube implant[23].

The spaces and gaps between the nanotubes increase with increasing diameter, therefore larger diameter nanotubes may be the more advantageous for allowing fluid and nutrient flow to occur[16]. And protein aggregates adhere to the top wall surface of $TiO_2$ nanotubes, owing to the presence of empty nanotube pore spaces and the gap between adjacent nanotubes. Therefore, MSCs are forced to elongate and stretch to search for protein aggregates to establish initial contact and this elongated morphology probably causes cellular cytoskeletal tension and stress[19, 22]. Because various kinds of physical stresses from the substrate morphology and topography can accelerate stem cell differentiation into a specific cell lineage[35], it can be stated that the larger the size of nanotube, the more the cellular cytoskeletal tension and stem cell differentiation . In this study our results support this hypothesis.

Our results also show that when healing is completed and remodeling is progressed at 12 weeks, 30nm experimental groups showed a good radiological, histological biomechanical results. This results are in accordance with Park et al. reporting 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. So almost identical response of MSCs, HSCs, and osteoclasts to the 15nm 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 $TiO_2$ nanotubes is a complex biological process and not fully understood yet. And the vitro and vivo study searching for the optimal $TiO_2$ nanotube diameter have shown conflicting results depending on surface chemistry, crystalline structure, roughness, cell−type, spices 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 and are not limited to surface chemistry, surface energy/tension/wettability, 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 systems 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, which is a long bone, 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 rates of growth and rehabilitation as well as other variations that may happen during surgery. However, implants surface treated with $TiO_2$ nanotubes showed good osseointegration of the implant compared to the control implant group. In 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 exhibited a stronger osseointegration than other groups. Therefore, depending on healing time 30nm and 70nm $TiO_2$ nanotube could be beneficial to osseointegration of implants compared to control and 100nm nanotubes. But, further investigation with the increasing number of implants would be necessary to have a meaningful study result because the statistical significance is not enough in this study.

Future trends of implant concern the modifications of surface roughness at the nanoscale level, the incorporation of biological drugs for earlier implantation and earlier loading and implantation in large defect region [1]. Because of 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. Therefore, based on this experiment, preclinical studies confirming the optimal nanotube diameter for earlier osseointegration, implantation in large defect region and drug delivery in the larger size animal model are necessary in the future.

# V. CONCLUSION

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 treated with various diameters of $TiO_2$ nanotubes histomorphometically and biomechanically. Within the limitations of this study, the following conclusions were made.

1. The results from micro−CT, histomorphometric analysis showed that the bone volume, BIC and BV results at 12weeks were higher than at 4weeks ($p<0.05$). It can be stated that osseointegration of the implant was enhanced by the bone remodeling and maturation over time.
2. Although there were no statistical significant differences, the results from histomorphometric analysis showed that the BV results in three consecutive macro threads at 4weeks were higher than at 12weeks ($p>0.05$). 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 surface treated with $TiO_2$ nanotubes in modelling stage.
3. The results from Removal torque test showed that 70 nm experimental group had the higher removal torque value than other groups at 4weeks ($p<0.05$).
4. Micro CT, histomorphometric analysis, removal torque test results showed similar pattern that 70 nm experimental group had highest value at 4weeks and 30nm nm experimental group had highest value at 12weeks.

On the basis of above results, 30nm and 70nm $TiO_2$ nanotube may have positive effects on osteogenesis and osseointegration depending on the healing time.

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< ABSTRACT(IN KOREAN)>

# 토끼에서 다양한 직경의 $TiO_2$ 나노튜브로 표면 처리된 임플란트의 골유착


<지도교수 심 준 성 >

연세대학교 대학원 치의학과

강 철 구



본 연구에서는 서로 다른 직경의 티타니아 나노튜브 표면이 생체 내에서 골형성 및 골유착에 미치는 영향을 토끼를 이용한 동물실험을 통해 조직학적, 생역학적으로 평가하였다.

토끼 대퇴골에 식립할 나사형의 티타늄 임플란트를 80개 제작한 후, 이를 30 nm, 70 nm, 100 nm 직경의 나노튜브로 표면처리한 실험군과 RBM처리한 타이타늄 표면의 대조군으로 나누고 다시 관찰 기간별로 4주 관찰군과 12주 관찰 군으로 나누었다. 토끼의 우측 대퇴골에 12주관찰 군을 식립 한 후 8주 후에 좌측 대퇴골에 4주 관찰 군을 식립 하였다. 다시 4주 후에 토끼를 희생하여 시편의 절반은 마이크로 CT촬영으로 신생 골량을 측정한 후 탈회 조직시편을 제작하고 광학현미경 하에서 임플란트 주위 골면적을 계측하였다. 나머지 절반의 시편으로 뒤틀림 제거력을 측정하여 비교, 분석하였다.

micro CT, 조직계측학적 분석에서 12주 희생군의 bone volume, BIC수치, BV수치가 4주 희생군의 수치보다 유의하게 높았다 ($p<0.05$). 마이크로 CT 분석에서 4주와 12주 희생군에서 30nm, 70nm군이 높은 신생골 량을 보였으나 통계학적인 유의차는 없었다 ($p>0.05$). 조직 계측학적 분석에서 BIC수치는 4주군에서 70 nm 실험군이 12주군에서는 30nm 실험군이 가장 높은 수치를 보였으나 통계학적 유의차는 없었다($p>0.05$). 조직 계측학적 분석에서 BV수치는 4주군에서 70 nm 실험군이 가장 높은 수치를 보였으나 통계학적 유의차는 없었다($p>0.05$). 12주군에서 30 nm 실험군이 100nm실험군 보다 유의하게 높은

수치를 보였다($p<0.05$). Macro thread 상위3개에서 측정한 BV수치는 4주군에서 70 nm 실험군이 100nm실험군보다 유의하게 높은 수치를 보였다 ($p<0.05$). 12주군에서 대조군이 가장 높은 수치를 보였으나 통계학적으로 유의한 차이는 없었다 ($p>0.05$). 뒤틀림 제거력 평가에서는 4주군에서 70nm 실험군이 다른 군에 비해 통계적으로 유의하게 높은 수치를 나타내었다($p>0.05$).

Micro CT, 조직계측학적 분석, removal torque test 에서 4주 희생군에서는 70 nm 실험군이 가장 높은 수치를 보이고 12주 희생군에서는 30nm nm 실험군이 가장 높은 수치를 보이는 경향을 보였다.

본 연구에서의 제한된 실험 결과를 토대로 30nm 와 70nm 티타니아 나노튜브가 토끼에서 임플란트의 골유착 및 골형성에 영향을 긍정적 영향을 미칠 수 있으며, 이러한 효과가 상위동물의 악골에서는 어떻게 나타나는지에 대한 추가적인 연구가 필요하리라 사료된다.

핵심되는 말 : 티타니아나노튜브, 표면처리, 토끼 , 골유착, 임플란트, 뒤틀림제거력