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## I. Introduction

Titanium-based dental implants are widely used to treat edentulous patients because of their excellent biocompatibility and osseointegration. Osseointegration has been predictable with adequate bone, while the results were not unreliable when local bone was inadequate due to conditions such as poor bone quality, old age, osteosclerosis and osteoporosis.<sup>1</sup> The suitability of several biomedical materials for improving local bone condition has been studied, particularly bone graft materials and dental implant surfaces. Many studies have been directed towards improving the osseointegration of dental implants by modifying the implant surface thereby increasing the implant success rate and shortening the healing period, especially in areas of poor-quality bone.<sup>2-3</sup> Surface topography and chemical modification are two important elements that influence the bone/implant interface.<sup>4</sup> Biochemical modification refers to the immobilization of bioactive peptides or growth factors such as bone morphogenetic protein (BMP) on the implants' surfaces.<sup>5</sup>

Treating the implant surface with rhBMP-2 to promote osseointegration has been studied recently. Shi et al.<sup>6</sup> developed a sustained BMP-2 release system based on rhBMP-2 encapsulated in poly-D,L-lactide-co-glycolide microspheres, which were then loaded into chitosan/collagen scaffolds, and demonstrated improved osseointegration ability. Chatzinikolaidou et al.<sup>7</sup> demonstrated the feasibility of enhancing peri-implant osseointegration and gap bridging by immobilizing rhBMP-2 on implant surfaces. Implants soaked in 2 mL of sterile water containing 1.0 mg rhBMP-2 with polylactic acid as the carrier exhibited both qualitative and quantitative improvements in osseointegration at the implant-bone interface.<sup>8</sup> However, procedures for attaching rhBMP-2 to a titanium surface are still under study and thus far, the materials used in attaching rhBMP-2 to titanium were the simple delivery agents, whose functions were not related to the osseointegration of the dental implants.

To overcome this, materials that promote osteogenesis are necessary as a delivery system for BMP.<sup>9, 10</sup> Hydroxyapatite (HAp) has been applied to clinically approved bone substitutes due to its high osteoconductivity and maintenance of a constant shape. It also exhibits high affinity for rhBMP-2, evenly

distributes rhBMP-2 under pressure, and causes minimal foreign body reactions, and is therefore considered useful as a BMP carrier.<sup>11</sup> Lee et al.<sup>9</sup> reported excellent results when rhBMP-2 was combined with HAp as bone-grafting material in a rabbit spinal fusion model. However, the bonding strength between HAp coatings and titanium implant surfaces is reported to be relatively low, ranging from 20–30 MPa.<sup>12</sup> It is essential to improve the mechanical properties of HAp coatings in the implant system. Investigators have focused on nanotechnology, concentrating particularly on nanohydroxyapatite (nHAp), which, in combination with collagen fibers, constitutes bone, and is structurally similar to bone crystals.<sup>12,</sup>

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Collagen hydrogel, especially those made with type I collagen, is the major organic component of the extracellular bone matrix; it positively affects the osteogenic differentiation and is required for the formation of a mineralized matrix. It has also been the promising matrices for drug delivery and tissue engineering applications.<sup>14, 15</sup>

As it has been suggested that nHAp improves biocompatibility and mechanical properties more effectively than conventional HAp<sup>16</sup>, our previous study (unpublished data) investigated the biological properties of nHAp using the calvarial defect, ectopic bone formation, and rabbit tibial implant installation models. nHAp mixed with collagen hydrogel showed more calcification in the calvarial defect and rat thigh muscle. Titanium implants coated with collagen hydrogel and nHAp showed more new bone formation than uncoated implant when inserted in rabbit tibia.

Therefore, a surface coating with a collagen hydrogel containing rhBMP-2 and nHAp was hypothesized to improve osseointegration further. The purpose of this study was to evaluate the osteoinductivity of collagen hydrogel, nHAp and recombinant human bone morphogenetic protein 2 (Col/nHAp/BMP-2) complex and to investigate whether the titanium surface coated with Col/nHAp/BMP-2 complex can enhance osseointegration effectively.

## **II. Materials and Methods**

### **1. Col/nHAp/BMP-2 complex**

#### **1.1. Fabrication of Col/nHAp/BMP-2 complex**

The complex consisted of collagen hydrogel, nHAp, and rhBMP-2. A type I atelocollagen powder (Koken Corp., Osaka, Japan) was used for the collagen hydrogel. nHAp solution with 50-nm-sized particles, was provided by Bio-Alpha Corporation (Seoul, Korea). *E-coli* derived rhBMP-2 was provided by Daewoong Pharmaceutical Corporation (Seoul, Korea).

Type I atelocollagen was dissolved at a concentration of 5 mg/mL in 0.001 N HCl with stirring at 4°C and chondroitin-6-sulfate, nHAp, and rhBMP-2 were added at concentrations of 0.25 mg/mL, 0.03 mg/mL, and 15 µg/mL, respectively.

#### **1.2. Expression of osteogenic markers in vivo**

The animal experiments were approved by the Animal Care and Use Committee of Seoul National University (SNU-110223-1). For all experiments, the animals were housed and handled in strict accordance with the animal care guidelines of the Institute of Laboratory Animal Resources at Seoul National University. 20 µL of collagen hydrogel (Col), collagen hydrogel with nHAp (Col/nHAp), or Col/nHAp/BMP-2 complex were injected into subcutaneous pockets of nude mice.

The mice were sacrificed and examined 4 weeks after injection. Hematoxylin and eosin (H & E) staining, Masson's trichrome (MT) staining, and immunochemistry were performed to assess bone formation and angiogenesis. Anti-osteonectin (1:500 dilution, AB 1858, Chemicon, Carlsbad, CA, USA) was used for osteonectin staining and anti-CD31 monoclonal antibody (Dako, Carpinteria, CA, USA) for CD31 staining, followed by standard immunohistochemical procedures. The images were examined under a light microscope (System Microscope CX 40-32PH, Olympus, Center Valley, PA, USA).

## **2. Titanium coated with Col/nHAp/BMP-2 complex**

### **2.1. Titanium disc coated with Col/nHAp/BMP-2 complex**

#### **2.1.1. Preparation of Col/nHAp/BMP-2 complex coated disc**

Grade-4 titanium discs with an 8mm diameter were used in this experiment. The surface of each titanium disc was etched by sonification of the discs in Kroll's reagent (4.0% HF, 7.2% HNO<sub>3</sub>, and 88.8% water) for 10 minutes. The discs were then washed ultrasonically for 10 minutes each in acetone and triple-distilled water and placed in 40% HNO<sub>3</sub> for 40 minutes to passivate the surface.

The prefabricated Col/nHAp/BMP-2 complex described previously was used. 500  $\mu$ L of the sol state complex was spread onto the titanium disc with a micropipette, and the disc was dried for 24h at 4°C.

#### **2.1.2. Surface characteristics of titanium disc**

##### **Surface roughness**

The surface was scanned using Nanomen II Atomic Force Microscopy (AFM; Veeco, NY, USA). Scans and readings were taken by the tapping mode AFM at random sites (1 mm<sup>2</sup> each) for 3 discs using a 5-mm scan head. A cantilever with an attached tip was oscillated at its resonant frequency to scan across the sample surface. Typical amplitudes were 20–100 nm, which could reflect the variation when the tip scanned over bumps or depressions on a surface. The Van der Waals forces were 200 pN or less. The surface roughness (root mean square) was calculated using the NanomenII software.

##### **Surface wettability**

To measure the contact angles, a contact angle analyzer (DSA100, Kross, Germany) was used with 2 $\mu$ m deionized water. The contact angle of the water droplet was measured after 10 seconds on both sides of the water droplet, and 5 samples per group were evaluated.

### **2.1.3. Release profile of rhBMP-2**

A complex containing 200ng of rhBMP-2 was coated onto the titanium surfaces. Release of rhBMP-2 from titanium discs coated with Col, Col/BMP-2 or Col/nHAp/BMP-2 were determined by first incubating the discs with PBS at 37°C in sealed tubes blocked with bovine serum albumin, and then recovering the released media at 1, 2, 3, 5, 7 and 9 days, followed by replacement with new PBS. The rhBMP-2 was measured indirectly in the release media using the human BMP-2 super X-ELISA kit obtained from Antigenix America (Huntington Station, NY, USA). Three samples from each group were evaluated for loading and release.

### **2.1.4. Osteoblastic differentiation of bone marrow mesenchymal stem cells**

A primary culture of mesenchymal stem cells was isolated from the bone marrow of human donors with the approval of the patients and the Institutional Review Board of St. Mary's Hospital, Catholic University. The mononuclear cell fraction was isolated by Ficoll (0.77 g/mL) density gradient centrifugation. The bone marrow mesenchymal stem cells (BM-MSCs;  $2 \times 10^5$  cells) were seeded onto the 5×5 cm titanium discs. The medium consisted of DMEM containing 10% fetal bovine serum, 10mM  $\beta$ -glycerophosphate, 50  $\mu$ M Asc-2-P, and  $10^{-7}$  M dexamethasone (all from Sigma Aldrich, St. Louis, MO, USA), and the medium was changed every 3 to 4 day.

#### **Flow cytometry to evaluate loss of stemness**

Antibodies against the human antigens CD90 and CD73 were purchased from BD Sciences (San Jose, CA, USA), and the antibody against CD105 was purchased from Ansell (Bayport, MN, USA). The fluorescence intensity of the cells was evaluated by a flow cytometer (FACScan; BD Sciences), and the data were analyzed using the CellQuest software (BD Sciences).

#### **Biochemical assays for alkaline phosphatase, glycosaminoglycan, and osteopontin production**

Alkaline phosphatase (ALP) activity was measured from the cell supernatant using a colorimetric

SensoLyte pNPP alkaline phosphatase assay kit (AnaSpec, Fremont, CA, USA). The total intracellular sulfated glycosaminoglycan (GAG) content was measured using a Blyscan sulfated glycosaminoglycans assay kit (Bioassay, Hayward, CA, USA). The concentration of osteopontin (OPN) protein in the culture medium was measured using a Quantikine human osteopontin immunoassay kit (R&D systems, Minneapolis, Minnesota, USA). The samples were dissolved in dissociation reagent, and the absorbance was measured at 656 nm by using a microplate reader (VERSAmix tunable microplate reader, Molecular Devices Corp., Sunnyvale, CA, USA).

### **Polymerase chain reaction for osteoblastic markers**

Polymerase chain reaction (PCR) analysis was performed to compare the expression of osteogenic markers such as type I collagen, type III collagen, osteonectin, OPN, BMP-2, osteoprotegerin, and GAPDH (as an internal control). Total cellular RNA was isolated by Trizol reagent (Invitrogen, CA, USA) and cDNA was synthesized by reverse transcription. The primers used for PCR were purchased from Bioneer (Daejeon, Korea), and their sequences, reaction conditions, and product sizes are summarized in Table 1. The PCR products were then analyzed on 2% agarose gels and visualized by SYBR Safe DNA gel staining (Invitrogen). The Image J software (Wayne Rasband, National Institutes of Health, USA) was used for the quantitative analysis of PCR amplicons on digitized gel images.

## **2.2. Preparation of implants coated with Col/nHAp/BMP-2 complex and in vivo study**

### **2.2.1. Preparation of implant**

The same complex described previously was used. Titanium implants treated with resorbable blasted media (RBMs) were used as the negative control while implants coated with Col/nHAp/BMP-2 complex were used as the experimental group. The diameter and length of the implant were 3.3 and 7 mm, respectively. 20  $\mu$ L of the sol state complex were spread onto each implant by micropipette and the implants were dried for 24 hours at 4°C.

### **2.2.2. Adherence of Col/nHAp/BMP-2 on implant surface**

Three Col/nHAp/BMP-2 coated dental implant were placed into the commercially fabricated artificial bone (polyurethane form), which were anatomically similar to D4 bone. Then, the implants were retrieved to measure the remaining amount of BMP-2 using the human BMP-2 super X-ELISA kit obtained from Antigenix America (Huntington Station, NY, USA).

### **2.2.3. Implantation on the rabbit tibia**

Six male New Zealand white rabbits with an average weight of 3.3 kg were used. Two uncoated implants were randomly placed in the right or left tibia of each animal and 2 implants coated with Col/nHAp/BMP-2 complex were placed in the contralateral side tibia. The animals were sacrificed after 4 weeks. The implants on the proximal side were used for histomorphometric analysis and those on the distal side were used for the removal torque test. Static torque was applied at a linearly increasing rate of 9.5 Ncm/second. The strength and rate of osseointegration of the bone implant were evaluated by the peak removal torque values.

### **2.2.4. Histomorphometric analysis**

The fixated specimens were dehydrated in a graded ethanol series using a dehydration system with agitation and a vacuum, and were embedded in light-curing methacrylate (Technovit 7200 VCL, Kulzer, Wehrheim, Germany). The implants were cut along a mid-axial coronal-apical plane by the sawing-and-grinding technique (EXAKT Apparatebau, Norderstedt, Germany). The bone implant contact (BIC) ratio and new bone formation area inside the thread area of the upper 3 threads were measured.

## **3. Statistics**

Statistical analysis was performed using a commercially available software program (SPSS 18.0; SPSS Inc., Chicago, IL, USA). Continuous variable data were presented as the mean  $\pm$  standard deviation. Multiple comparisons for biochemical assay were made using analysis of variance and post-hoc Tukey

tests after verification of normal distribution with the Kolmogorov-Smirnov test. Histomorphometric data and removal torque in vivo experiments were evaluated statistically by the Wilcoxon signed ranks test due to the sample size and data structure. The difference between means was considered significant when p-values were less than 0.05.

### **III. Results**

#### **1. Expression of osteogenic markers in vivo with Col/nHAp/BMP-2 complex**

By the 4th week, no obvious bone formations were observed for any of the injected materials, either macroscopically or microscopically (Figure 1A–I), although many cells had grown into both Col/nHAp and Col/nHAp/BMP-2. Additionally, the injected materials were partially degraded. The application of von Kossa staining revealed the absence of mineralization in the Col group (Figure 1J), with a higher intensity observed for Col/nHAp group than for Col/nHAp/BMP-2 group (Figure 1K, L). The differentiation of osteoblasts was confirmed by staining for osteonectin. The staining for osteonectin was limited in the case of Col enrichment, whereas the strongest staining was observed in Col/nHAp/BMP-2 group (Figure 1M–O). To evaluate angiogenesis, the tissues surrounding the injected composite solution were stained for CD 31. A higher degree of CD31 staining and angiogenesis was observed in Col/nHAp/BMP-2 group than in Col/nHAp group (Figure 1P–R).

#### **2. Titanium coated with Col/nHAp/BMP-2 complex**

##### **2.1. Titanium discs coated with Col/nHAp/BMP-2 complex**

###### **2.1.1. Surface characteristics of titanium discs**

Col/nHAp/BMP-2 increased the surface roughness more than Col, Col/BMP-2, or the bare titanium surface (Figure 2). The root-mean-square were higher in Col/nHAp/BMP-2 than in the negative control at 872nm and 357nm, respectively.

The water contact angles on Col/nHAp/BMP-2 were lower than those on the bare titanium surface at  $58.4\pm 3.0^\circ$  and  $70.5\pm 8.2^\circ$ , respectively (Figure 3). These results indicate the increased hydrophilicity of Col/nHAp/BMP-2 coating.

### **2.1.2. Release profile of BMP-2 from the disc**

The surfaces treated with Col/nHAp/BMP-2 exhibited a BMP-2 release of approximately 50% after 24 hour of incubation and approximately 75% after a 48-hour incubation, while the surfaces coated with Col/BMP-2 exhibited 70% initial release within 24 hour followed by an 88% release at 48 hour (Figure 4). Therefore, most of the coated BMP-2 was released within 5 days from Col/nHAp/BMP-2 and within 3 days from Col/BMP-2.

### **2.1.3. Osteoblastic differentiation of BM-MSCs**

#### **Flow cytometric analysis to evaluate loss of stemness**

BM-MSCs were tested for the presence of mesenchymal stem cell markers, including the antigens CD73, CD90, and CD105, by flow cytometry (Figure 5A). The BM-MSCs exhibited a high expression of these markers prior to differentiation. That expression was down-regulated following culturing on the titanium surface coated with Col/nHAp/BMP-2 after 2 weeks, indicating loss of stemness.

#### **Biochemical assays for ALP, GAG, and OPN production**

The BM-MSCs were cultured for 14 days, and assays for ALP, GAG, and OPN production were performed to evaluate bone induction (Figure 5B). In the cells grown on the Col/nHAp/BMP-2 surface, the ALP activity and the GAG and OPN levels were significantly higher than those in the cells grown with Col enrichment alone. Cells on the Col/nHAp/BMP-2 surface also exhibited higher ALP activity and GAG and OPN levels than those on the Col/nHAp surface.

ALP activity is a phenotypic marker of osteoblasts and is characteristic of differentiation.<sup>17</sup> GAG is the major organic extracellular matrix component, and changes in its composition are frequently seen in physiological and pathological remodeling processes such as bone formation or scarring.<sup>18</sup> The expression of OPN by osteoblasts early in bone development is consistent with the formation of the bone matrix.<sup>19</sup> Thus, higher ALP activity and greater expression of GAG and OPN in BM-MSCs on Col/nHAp/BMP-2

indicated that nHAp with BMP-2 can induce BM-MSCs to osteoblastic differentiation after 14 days of culturing. These activities also increased on Col/nHAp although to a lesser extent than for Col/nHAp/BMP-2. These results demonstrated the osteoinductive capacity of nHAp itself and the synergistic effect of coupling BMP-2 with nHAp.

### **PCR for osteoblastic markers**

As seen in Figure 5C, the levels of type III collagen, osteocalcin and osteoprotegerin were highly increased in BM-MSCs grown on Col/nHAp/BMP-2 as compared with the other groups. A similar expression pattern was observed for type I collagen in cells grown on Col/nHAp. Both Col/nHAp and Col/nHAp/BMP-2 groups had increased expression of OPN, osteonectin and BMP-2 as compared with the Col group.

Osteocalcin is secreted solely by osteoblasts and is believed to play a role in bone mineralization and calcium ion homeostasis.<sup>20</sup> Osteoprotegerin can reduce the production of osteoclasts by inhibiting the differentiation of osteoclast precursors into osteoclasts, and it also regulates the resorption of osteoclasts in vitro and in vivo.<sup>21</sup> Type I collagen is the main component of the organic part of bone, whereas type III collagen, a major component of the extracellular matrix, is commonly found alongside type I collagen and is produced before the tougher type I collagen is synthesized.<sup>22, 23</sup> OPN and osteonectin levels are the early marker of bone development. Considering these results and those from previous studies, it is possible that BMP-2 combined with nHAp stimulates the formation and mineralization of the bone due to their chemical or structural characteristics.

## **2.2. Preparation of implant coated with Col/nHAp/BMP-2 and in vivo study**

### **2.2.1. Adherence of Col/nHAp/BMP-2 on implant surface**

After the retrieval of the implants coated with Col/nHAp/BMP-2, 55.7±13.9% of BMP-2 remained on

the implant surface.

### **2.2.2. Implantation on the rabbit tibia**

Following 4 weeks of healing, the implants coated with Col/nHAp/BMP-2 demonstrated greater new bone area than the uncoated implants ( $73.69 \pm 5.88\%$  and  $62.24 \pm 12.52\%$ , respectively;  $p=0.046$ ,  $n=6$ ). There were no statistical differences in BIC or removal torque between the coated and uncoated implants, although the mean values of BIC and removal torque were higher in the implants coated with Col/nHAp/BMP-2 ( $33.46 \pm 6.91\%$  vs.  $31.36 \pm 3.22\%$  with  $p=0.345$  for BIC and  $26.63 \pm 4.41\text{N}\cdot\text{cm}$  vs.  $22.90 \pm 2.33\text{N}\cdot\text{cm}$  with  $p=0.225$  for removal torque; Figure 6).

#### IV. Discussion

The goal of this study was to develop a surface-modified implant that allows the active growth factor rhBMP-2 to promote osseointegration. First, the biological properties of Col/nHAp/BMP-2 complex were evaluated by injecting materials into mouse subcutaneous pockets. Then, the biological response to titanium coated with Col/nHAp/BMP-2 complex was evaluated *in vitro* and *in vivo*.

The amount of rhBMP-2 injected into the mouse pockets and coated per implant in the rabbit tibias was 300ng. Watering et al.<sup>24</sup> used from 2 to 20  $\mu\text{g}$  of rhBMP-2 in a mouse ectopic bone formation model. In addition, Bae et al.<sup>25</sup> used a minimum 10 $\mu\text{g}$  of rhBMP-2 to induce the ectopic bone formation in rabbit muscles. Unlike these ectopic bone formation models, the interfaces between the bone tissues and implants were narrow and coating materials were released from these narrow contact areas. This necessitates reducing the concentration of released materials and we decided to use a small amount of rhBMP-2. Our research based on the study of Zhang et al.<sup>26</sup> who cultivated the periosteal cells with 500 ng/ml rhBMP-2 and then seeded the cells into pre-wetted nHAp/collagen/ poly(L-lactic acid) scaffolds. After the implantation of these scaffolds into rat muscle, bone formation was found. Another study by Zhang et al.<sup>11</sup> also used a relatively low dose of BMP-2. They reported that a BMP-2 gene-fibronectin-apatite composite layer expressed 100~600 pg/ml of BMP-2 and enhanced bone formation in a rat calvarial defect model. In our study, the injection of Col/nHAp/BMP-2 complex to the mouse subcutaneous pocket did not show obvious bone formation after 4 weeks in any of the groups either macroscopically or microscopically. However, the complex caused the strong expression of osteogenic markers at the molecular level, such as osteonectin, an early marker of osteoblast differentiation, and CD31, a marker of angiogenesis, indicating the potential for bone formation after long periods.<sup>27</sup>

Implants coated with Col/nHAp/BMP-2 complex showed increased osseointegration values in general with significant improvement in new bone area. The present coating dose of rhBMP-2 is very low (300ng per implant) compared to current clinical standards which ranging from 2  $\mu\text{g}$  to 30  $\mu\text{g}$  in rat and rabbit.<sup>25</sup>

<sup>28</sup> Specifically, Lee et al.<sup>29</sup>, whose experimental design was similar to ours, coated the RBM implant with

14.5 µg of rhBMP-2 and found a significant increase in BIC as compared to the uncoated RBM implant in rabbit. Our results demonstrated that even a small amount of rhBMP-2 has efficacy in promoting the osseointegration of dental implants. This could be explained by the role of rhBMP-2 in bone formation and the narrow interface between the bone tissues and implants described previously. Recently, it has been reported that rhBMP-2 promotes the adipogenic differentiation of periodontal ligament stem cells both in vitro and in vivo.<sup>30</sup> Other studies have also reported that rhBMP-2 significantly enhances the adipogenic as well as osteogenic potential of alveolar bone-derived stromal cells in a dose- and time-dependent manner.<sup>31</sup> Considering the dose dependent adverse role of rhBMP-2, the amount of rhBMP-2 in our study would be sufficient for osteogenesis in dental implant. Still, additional considerations remain, because the BIC and removal torque were not significantly elevated and further studies with increased the rhBMP-2 doses are necessary.

Several techniques are available for coating the titanium implants with nHAp and rhBMP-2, including electrophoresis, hydrothermal processes, and electrochemical processes.<sup>1</sup> We coated the implant surface by spreading the sol state Col/nHAp/BMP-2 complex to the titanium surface using a micropipette. After being coated with Col/nHAp/BMP-2 solution, the surface roughness and wettability was increased than uncoated titanium surface. Titanium surfaces with both excellent wettability and micron-/submicron-scale complex roughness can synergistically enhance osseointegration through fast blood clot stabilization and shortened wound healing time.<sup>32</sup> It has been reported that nHAp significantly increases surface wettability due to its high specific areas and the special microstructures of nHAp such as voids within the grain; these support our results of increased hydrophilicity in Col/nHAp/BMP-2 coated titanium.<sup>33</sup>

In the release kinetics of rhBMP-2, most of the coated rhBMP-2 was released within 5 days for the Col/nHAp/BMP-2 group. Although rhBMP-2 incorporated with nHAp exhibited a slower release pattern than rhBMP-2 alone, recent studies have reported more sustained release patterns.<sup>34</sup> Despite the relatively rapid release of rhBMP-2 as compared with other studies, our implant showed increased new bone formation. Wen et al.<sup>10</sup> reported that despite the relatively rapid release of rhBMP-2 in polyethylene

glycol (PEG) hydrogel, the PEG hydrogel group had the peak values for new bone growth. Most of the coated BMP-2 from the PEG hydrogel was released within 5 days similar to our Col/nHAp/BMP-2 complex. Hence, the release of rhBMP-2 release in more than 5 days appears to be enough to enhance osseointegration.

During the implant placement, coated materials were thought to be scraped off from the titanium surface. Our results showed that  $55.7\pm 13.9\%$  of the rhBMP-2 remained after insertion and retrieval in artificial bone. Although there are no reports that evaluated the percentage of rhBMP-2 remaining after implantation, a carrier that maintained more than 50% of rhBMP-2 was satisfactory and collagen hydrogel was suggested to be an appropriate carrier of rhBMP-2 for coating dental implants. To improve this further, methods for bonding rhBMP-2 tightly to titanium surface, such as the fabrication of nanotubular structures on a Ti surface by anodizing or chemical immobilization can be applied.<sup>7, 35</sup>

The culturing of BM-MSCs on Col/nHAp/BMP-2 coated titanium showed the decreased expression of stem cell marker and increased expression of osteogenic markers such as ALP activity, GAG, OPN, osteocalcin, osteonectin and osteoprotegrin. These indicate that Col/nHAp/BMP-2 complex induced the osteoblastic differentiation of the MSCs. Osteoblastic differentiation is a well known activity of rhBMP-2 while there has been ambiguity about the osteoinductivity of nHAp. Conventionally, HAp is not considered osteoinductive.<sup>36</sup> However, Yuan *et al.*<sup>37</sup> suggested that osteoinduction by HAp is dependent not only on its chemical composition, but also on the method by which the material is prepared, affecting its microstructure. Following the development of nanoscale HAp, Götz *et al.*<sup>38</sup> detected signs of early osteogenesis after nHAp injection. To explain the mechanism by which nHAp stimulates osteoinduction, Suto *et al.*<sup>39</sup> demonstrated that nHAp increases BMP-2 expression at both the gene and protein level through activation of the p38 mitogen-activated protein kinase pathway. Our in vitro analyses of BM-MSC culturing were consistent with the results of Suto *et al.* as nHAp itself affected the osteoblastic differentiation.

While implants coated with Col/nHAp/BMP-2 complex showed increased osseointegration values in

general with significant improvement in new bone area, the BIC and removal torque were not significantly increased . We evaluated the results of dental implants coated with 300ng of rhBMP-2 after 4 weeks of implant placement. It is necessary to evaluate the osteogenic capacity with various concentration of rhBMP-2 and with diverse healing periods to develop surface-modified implants with Col/nHAp/BMP-2 for clinical use.

## **V. Conclusions**

A collagen hydrogel/nHAp/rhBMP-2 complex injected into mouse subcutaneous pocket induced the expression of osteogenic markers. Titanium surfaces coated with this complex displayed increased surface wettability and roughness, maintained the release of rhBMP-2 and increased the expression of osteogenic markers. The implants coated with this complex also exhibited significantly increased new bone in rabbit tibias. These results suggest that surface coating with collagen hydrogel/nHAp/rhBMP-2 has the potential to improve the osseointegration of dental implants.

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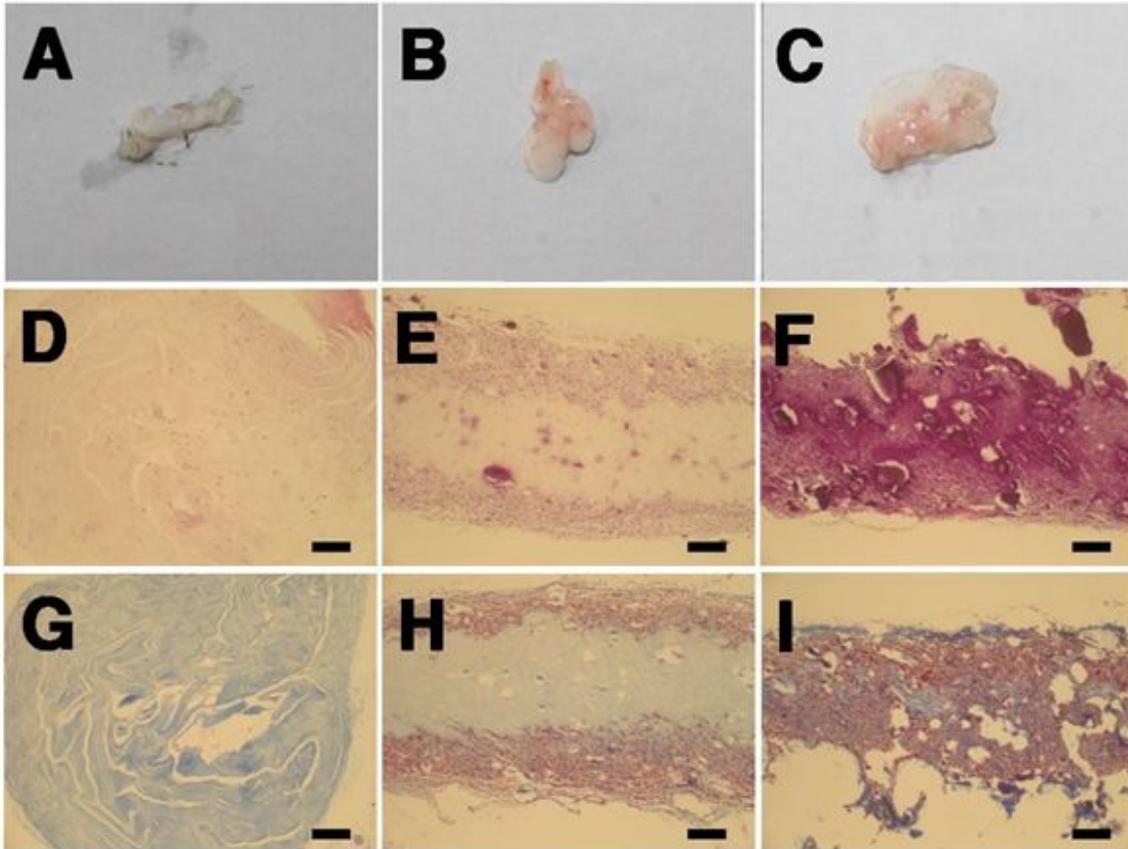
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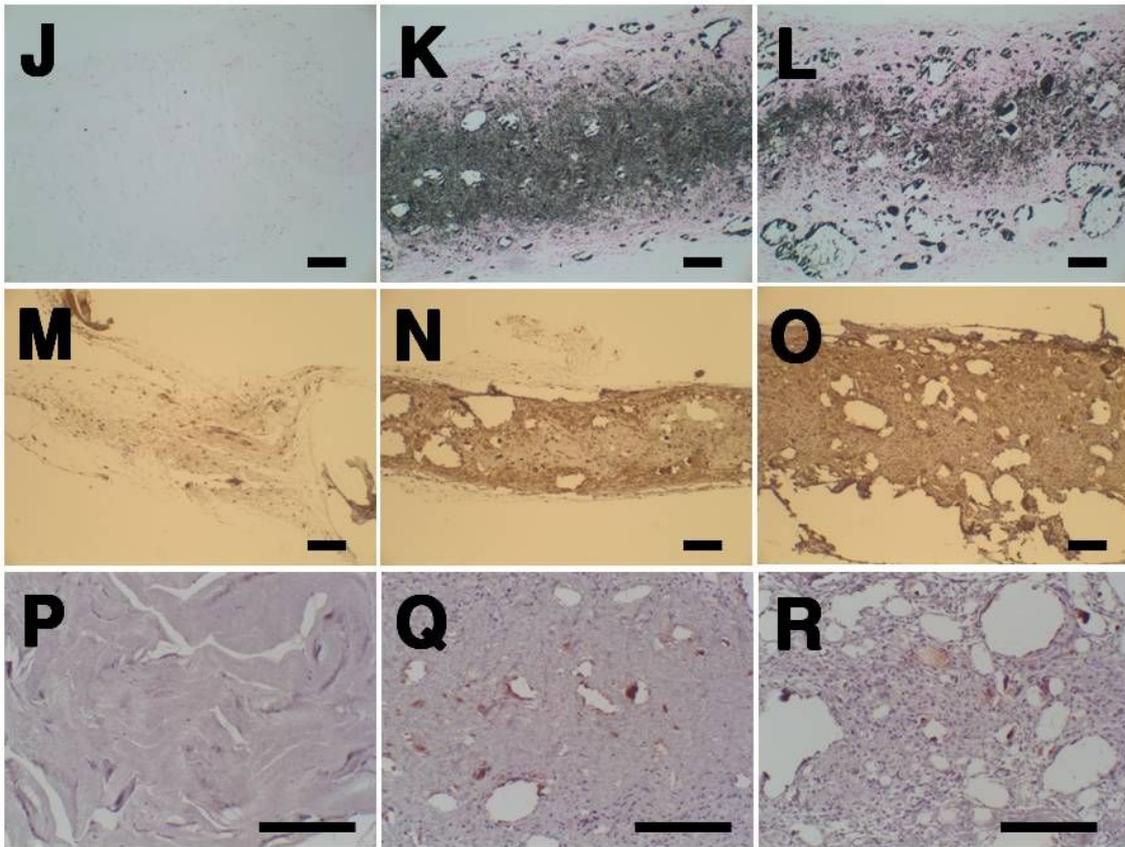
**Table**

Table 1. Primer sequences, reaction conditions, and product size used for PCR to evaluate the expression of osteoblastic biomarkers in bone marrow mesenchymal stem cell

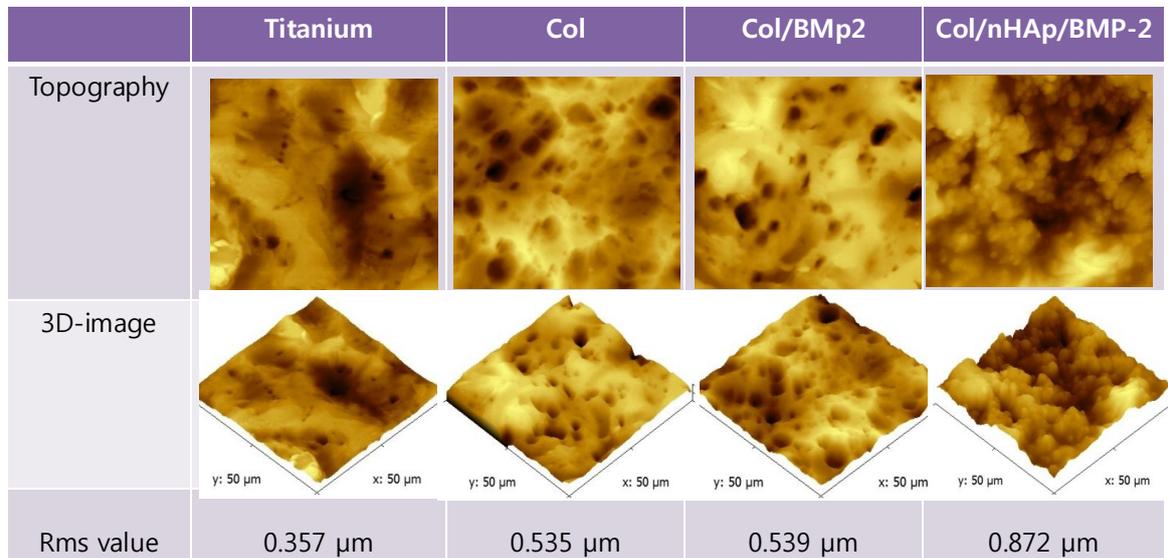
Gene	Sequence	Product Size (bp)	Annealing (°C)	Cycles
BMP-2	F : GTC CAG CTG TAA GAG ACA CC R : GTA CTA GCG ACA CCC ACA AC	316	54	31
Collagen1	F : GAA AAC ATC CCA GCC AAG AA R : CAG GTT GCC AGT CTC CTC AT	270	57	23
Collagen3	F : CAG GTG AAC GTG GAG CTG C R : TGC CAC ACG TGT TTC CGT GG	661 849	57	23
Osteonectin	F : CCA GAA CCA CCA CTG CAA AC R : GGC AGG AAG AGT CGA AGG TC	161	57	23
Osteopontin	F : TCG CAG ACC TGA CAT CCA GT R : TCG GAA TGC TCA TTG CTC TC	267	57	32
GAPDH	F : ACC ACA GTC CAT GCC ATC AC R : TTC ACC ACC CTG TTG CTG TA	450	55	25

Figures and legends

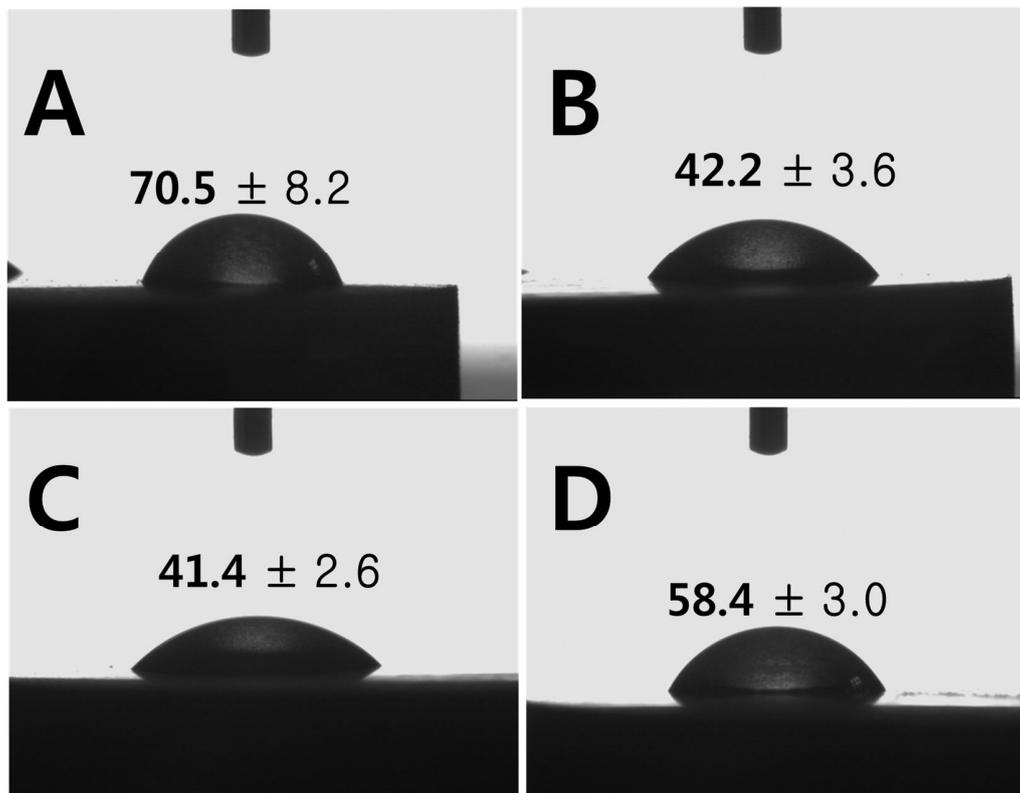




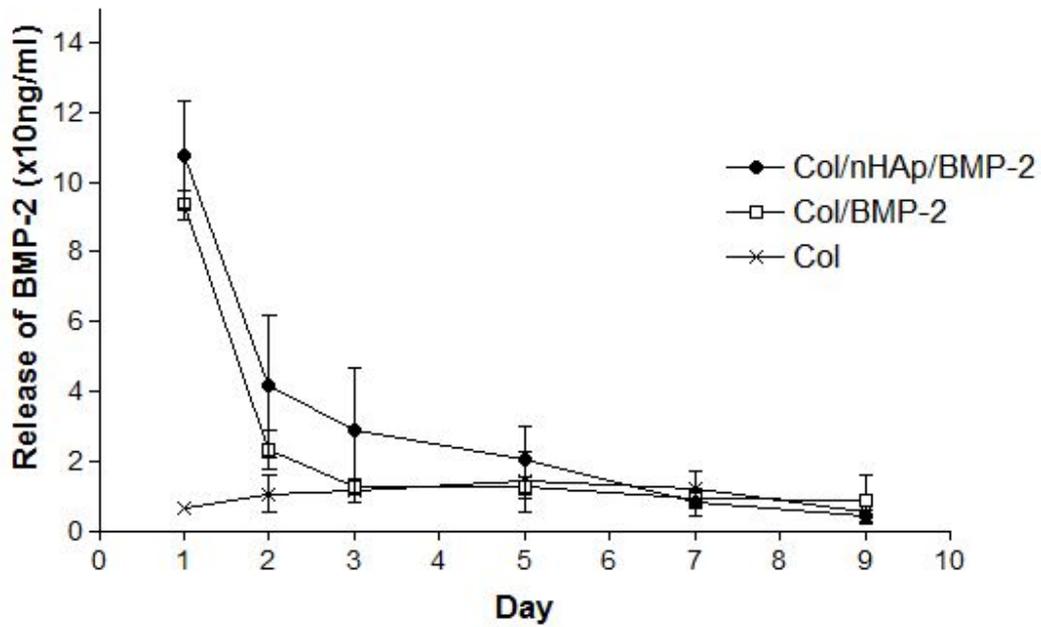
**Fig 1. Histological images of the mouse subcutaneous pocket injection model.** Collagen(A, D, G, J, M, P), collagen/nHAp (B, E, H, K, N, Q), and collagen/nHAp/BMP-2 (C, F, I, L, O, R). Macroscopic images (A–C), H&E staining(D–F), MT staining (G–I), Von-Kossa staining(J–L), osteonectin staining(M–O), and CD31 staining(P–R) (magnification: D–O×100, P–R x 200, scale bar=200  $\mu$ m). At the 4th week, no obvious bone formations were observed for any of the injected materials either macroscopically or microscopically (Figure 1A–I). Von Kossa staining revealed the higher intensity in Col/nHAp and Col/nHAp/BMP-2 than that in Col. (Figure 1J–L). Staining for osteonectin was limited in the case of Col enrichment, whereas the strongest staining was observed in Col/nHAp/BMP-2 (Figure 1M–O). A higher degree of CD31 staining and angiogenesis was observed in Col/nHAp/BMP-2 than in Col/nHAp (Figure 1P–R).



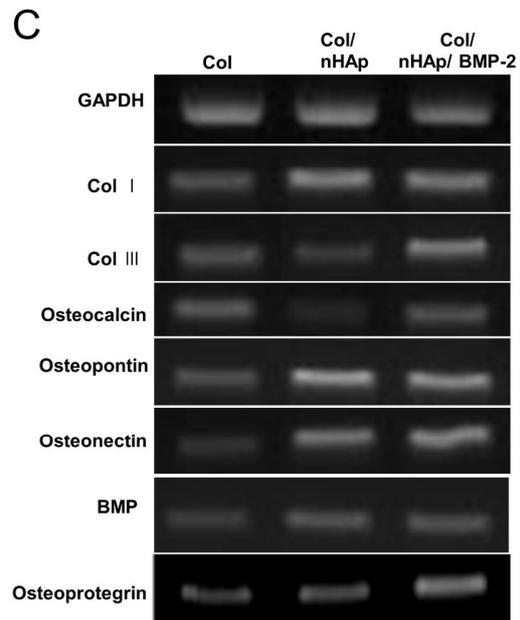
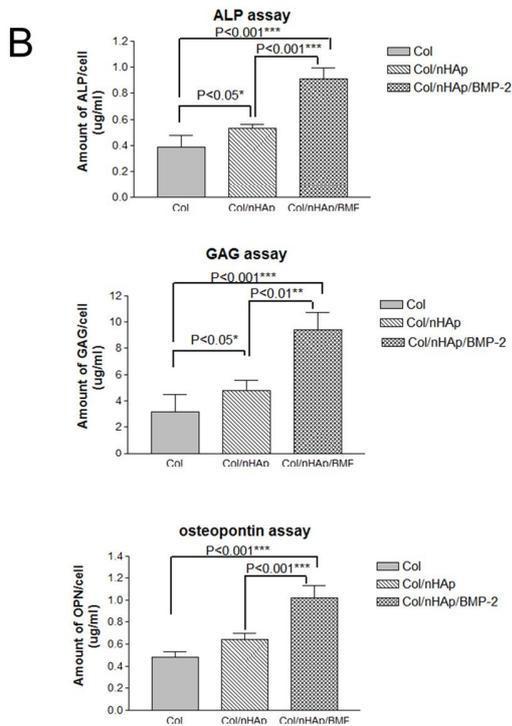
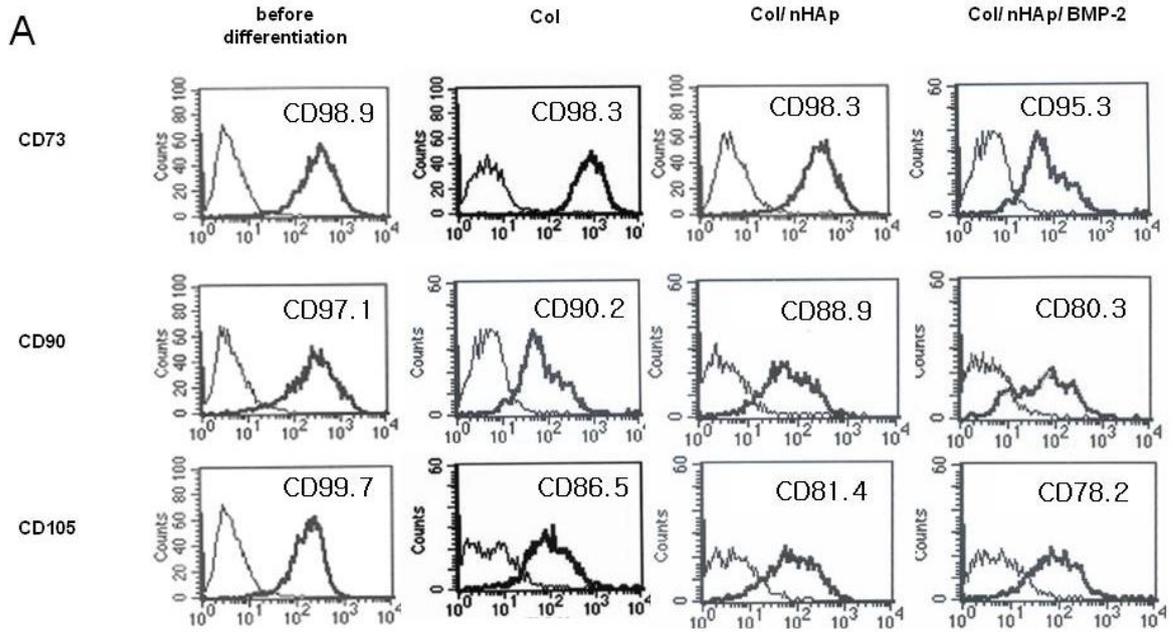
**Fig 2. Topographic and 3-dimensional atomic force microscopic images of the coatings.** Titanium, untreated titanium disk; Col, collagen-coated titanium surface; Col/BMP-2, collagen/BMP-2 coated titanium surface; Col/nHAp/BMP-2, collagen/nHAp/BMP-2 coated titanium surface. Coating with Col/nHAp/BMP-2 increased the surface roughness than the other titanium surfaces.



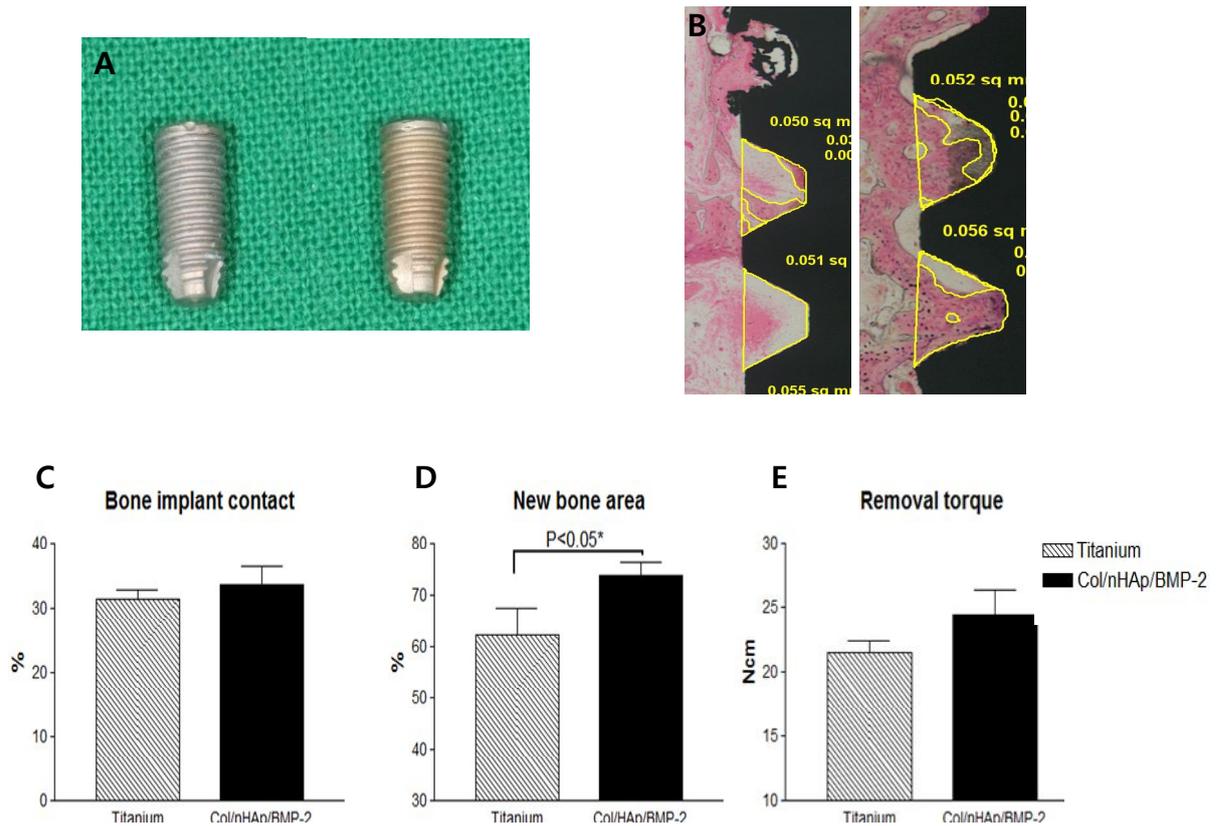
**Fig 3. Static water contact angle on titanium surface.** (A) Negative control (RBM surface titanium), (B) collagen-coated surface, (C) collagen/BMP-2 coated surface, and (D) collagen/nHAp/BMP-2 coated surface. The water contact angles on Col/nHAp/BMP-2 were lower than bare titanium surface indicating the increased surface wettability of Col/nHAp/BMP-2 coating.



**Fig 4. In vitro release curve of BMP-2 from various surface-treated titanium disks.** The results are shown as an average  $\pm$  standard deviation (n=3) values. An increase in BMP-2 release was observed within 24 h. A sustained release in the collagen/nHAp/BMP-2 group was observed between 1–5 day. The values presented are the mean  $\pm$  standard deviation



**Fig 5. Bone marrow-derived mesenchymal stem cell (BM-MSC) differentiation on differently treated titanium disks.** (A) Flow cytometry of typical CD markers present on BM-MSCs cultured on the Col, Col/nHAp, or Col/nHAp/BMP-2 coated titanium disks. CD73<sup>+</sup>, CD90<sup>+</sup> and CD105<sup>+</sup> cells were observed prior to differentiation. Reduction in mesenchymal CD73<sup>+</sup>, CD90<sup>+</sup> and CD105<sup>+</sup> cells was observed in the Col/nHAp/BMP-2 group. (B) Biochemical assays for alkaline phosphatase (ALP) activity and intracellular glycosaminoglycan (GAG) and osteopontin expression in BM-MSCs. Cells on Col/nHAp/BMP-2 exhibited the highest expression. The values presented are the mean  $\pm$  standard deviation (C) mRNA expression of different osteoblastic markers in BM-MSC. The highest expression was observed for type III collagen (Col III), osteocalcin, and osteoprotegerin in cells grown on Col/nHAp/BMP-2. The levels of type I collagen (Col I), osteopontin, osteonectin, and BMP-2 expression were higher in BM-MSCs on Col/nHAp and Col/nHAp/BMP-2 than for those with Col enrichment.



**Fig 6. Implant installation on rabbit tibia.** Photograph(A) and histologic image(B) of uncoated titanium implant (left) and Col/nHAp/BMP-2 coated implant (right). (C-E) Bone implant contact, new bone area and removal torque. At 4 weeks after implant installation on rabbit tibia, implants coated with Col/nHAp/BMP-2 complex showed increased mean values in all 3 factors with significant improvement in new bone area. The values presented are the mean  $\pm$  standard deviation. n=6

# 콜라겐하이드로젤 / 나노수산화인회석 / 2형골형성단백질 복합체 코팅이 임플란트 골유착에 미치는 영향

서울대학교 대학원 치의학과 구강악안면외과학 전공

( 지도교수 이종호 )

방 강 미

## 연구배경 및 목적

본 연구의 목적은 콜라겐 하이드로젤 (Col)/나노하이드록시 아파타이트 (nHAp)/ 2형골형성 단백질 (BMP-2) (Col/nHAp/BMP-2) 복합체를 제작하여 그 효과를 검증하고, 이 복합체를 타이타늄 표면에 코팅했을 때의 골유도 효과와 임플란트 표면에 코팅했을 때 생체 내 골유합능을 확인하는 것이다.

## 재료 및 방법

1형아텔로콜라겐 파우더, 콘드로이틴-6-설페이트, 나노수산화인회석, 2형골형성단백질을 0.001N의 염산에 용해시켜 Col/nHAp/BMP-2 복합체를 제작하였다. 마우스 피하에 Col/nHAp/BMP-2 복합체를 주입하고 4주 후에 희생하여 골형성 마커의 발현을 관찰하였고, 이 복합체가 코팅된 타이타늄 디스크의 표면 거칠기와 표면 친수성, BMP-2의 방출정도를 측정하였다. 또한 코팅된 디스크에 골수유래중간엽줄기세포를 배양하여 골형성 마커의 발현을 분석하였고, Col/nHAp/BMP-2복합체를 타이타늄 임플란트에 코팅하여 토끼 양쪽 경골에 식립하고, 4주 후 골-임플란트 접촉율, 신생골 형성면적, 제거토크를 평가하였다.

## 결과

마우스 피하 주입 실험에서 Col/nHAp/BMP-2 복합체는 osteonectin과 CD31의 증가된 발현을 보였다. 복합체를 코팅한 타이타늄 디스크는 친수성이 증가하고 BMP-2의 방출을 5일간 지속시켰다. BM-MSC를 배양했을 때, 코팅한 표면에 있던 중간엽줄기세포가 줄기세포 특성을 크게 상실하였고, 가장 높은 골형성세포 마커의 발현을 보였다. 토끼 경골 임플란트 식립 모델에서 Col/nHAp/BMP-2 복합체를 코팅한 임플란트는 유의할 만한 신생골 형성의 증가를 보였으며, 골-임플란트 접촉율과 제거토크를 향상시켰다.

## 결론

본 연구를 통하여 콜라겐하이드로젤/나노하이드록시아파타이트/BMP-2 복합체는 골형성 마커의 발현을 유도하고, 타이타늄 표면 특성을 개선시키고, 주변 세포의 골모세포로의 분화를 유도하였다. 이 복합체를 임플란트 표면에 코팅 하였을 때, 전반적으로 골유착과 관련된 수치의 증가를 보여주었으며, 특히 유의하게 증가된 신생골형성능을 보여주었다. 콜라겐하이드로젤/나노하이드록시아파타이트/BMP-2 복합체를 이용한 치과 임플란트 표면 코팅 기술은 골유착을 개선시킬 수 있는 가능성이 있다고 하겠다.

주요어 : 임플란트 표면개질, 치과 임플란트, 골유착, 2형 골형성단백질,  
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학 번 : 2009-31117

