Enhancement of Osseointegration of Artificial Ligament by Nano-Hydroxyapatite and Bone Morphogenic Protein-2 into the Rabbit Femur

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The MTT assay showed that the cell proliferation on hydroxyapatite (HAp) and HAp/bone morphogenic protein (BMP) coated group was better than the control and BMP coated groups at 5 days. And after 7 days of culture, the mRNA expression levels of type I collagen, osteonectin, osteopontin, bonesialoprotein, BMP-2, alkaline phosphatase (ALP) and Runx-2 in the HAp/BMP coated group were significantly higher than the other groups. Also, in this group showed the most significant induction of osteogenic gene expression compared to mesenchymal stem cells (MSCs) grown on the other groups. In addition, the cells in the HAp/BMP coated group delivered higher levels of ALP than the other three groups. Also, silk scaffolds were implanted as artificial ligaments in knees of rabbits, and they were harvested 1 and 3 months after implantation. On gross examination, HE staining showed that new bone tissue formation was more observed in the HAp/BMP coated group 3 weeks postoperatively. And masson staining showed that in the HAp/BMP coated group, the silk fibers were encircled by osteoblast, chondrocyte, and collagen. Furthermore, the analysis showed that the width of the graft-bone interface in the HAp and HAp/BMP coated group was narrower than that in the other two groups 3 weeks postoperatively. So, it is concluded that BMP incorporated HAp coated silk scaffold can be enhanced osseointegration and osteogenesis in bone tunnel. As a result, these experimental designs have been demonstrated to be effective in the acceleration of graft-to-bone healing by increasing new bone or fibrocartilage formation at the interface between graft and bone.

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INTRODUCTION

Ruptured anterior cruciate ligament (ACL) are usually surgically replaced, resulting in less than perfectly satisfying outcomes [1]. The problems associated with an autograft include lengthy rehabilitation as well as persistent patellar pain and allografts carry the risk of disease transmission, and their procurement is difficult and costly [2,3].

To overcome the problems associated with autografts and allografts, a synthetic ligament was developed and implanted

into patients with ligament injuries. Permanent synthetic ligament prostheses may show satisfactory performance in the short-term, but they tend to break down and fail in the long-term [4-6]. The synthesis ligament, since it is hydrophobic, seems to have poor integration into the surrounding bone. It has been reported that the healing potential of grafts in a bone tunnel is extremely poor since a layer of fibrovascular scar tissue develops in the interface between the ligament graft and the bone tunnel [7-10]. Also, Gao et al. [10] reported that ligament reconstruction using the artificial ligament were noted to exhibit graft failure at the bone tunnel, and they found that an interposed layer of fibrous scar tissue appears at the graft-bone interface at the second arthroscopic revision surgery. Despite numerous studies, there is not optimized for adequate technique and methods about increasing of osseointegration or osteogenesis on graft-bone interface until now.

In order to promote osseointegration of artificial ligament, various methods had been tried to enhance new bone growth

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and formation at the artificial ligament-bone tunnel interface region, such as the surface modification by coating with various bioactive materials. So, several approaches have been investigated to augment graft-to bone healing, with coating of hyaluronic acid [9], hydroxyapatite (HAp) [11,12], bioactive glass (BG) [13], hydroxypropylcellulose [14], and bone morphogenetic protein-2 (BMP-2) [15,16].

Li et al. [11] reported that HAp coating on the synthesis artificial ligament surface has a positive effect in the induction of artificial ligament osseointegration within the bone tunnel using a rabbit extra-articular tendon-bone healing model. The other investigator reported that BG coating on the synthesis artificial ligament surface resulted in more BMP-2 and VEGF expression, and BG-coated graft induced great new bone formation between the graft and host bone [13]. Also, Pan et al. [15] reported that BMP accelerated osseointegration of tendon graft for ACL reconstruction and they showed the BMP composite increased the new cartilage and bone formation in the interface graft-tunnel during the whole healing process and biomechanically, the ultimate failure load was increased.

So, we hypothesize that HAp combined with BMP-2 will have a synergic effect in improving grafted silk scaffolds to bone for osteogenesis compare to using HAp or BMP-2 alone. In this study, HAp was deposited on the silk scaffold using soaking technique and BMP was coated by air drying methods. And osteoinductivity of the HAp and/or BMP-coated silk scaffold were assessed by culturing human mesenchymal stem cells (MSCs) *in vitro*. Also, advantages of HAp combined with BMP coating on silk scaffold were evaluated histologically compare silk scaffold-bone interface healing between HAp, BMP-2, and HAp combined with BMP into femoral of rabbit during the early stage.

MATERIALS AND METHODS

Preparation of silk scaffold and nano-hydroxyapatite coating

Raw silk fibers (*Bombyx mori*) were purchased from Won Corporation (Seoul, Korea) and silk tubes were made using a braiding machine. Scaffolds were prepared by removing the sericin (a glue-like protein that coats the native silk fiber) using an aqueous solution containing 0.02 M Na₂CO₃ and 0.3% Ivory® detergent [1].

The HAp solution was made with 0.15 g of HAp powder (Bio-Alpha Inc., Seongnam, Korea) and 10 mL phosphate-buffered saline (PBS) and stirred for five minutes. And silk scaffolds immersed into the HAp solution for 60 min and then they were dried in air for 24 hours. The silk scaffolds and HAp coated silk scaffolds were sterilized with γ -irradiation at 15 KGy. After

γ -irradiation, 25 μ g of BMP-2 was coated by air-drying method. The dimensions of the scaffold used in this study were “30 mm length and 3 mm width”.

Culture of human bone marrow mesenchymal stem cells

Human BM-MSCs (hBM-MSCs) were purchased from Lonza (Basel, Switzerland) and maintained in culture in Dulbecco's modified eagle's medium (low glucose DMEM, Welgene, Daejeon, Korea) with 10% fetal bovine serum (FBS, Lonza, Basel, Switzerland), 1% penicillin/streptomycin (PS, Welgene, Daejeon, Korea), 25 μ M L-ascorbate 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA) in 37°C incubator at 5% CO₂ humidified atmosphere. BM-MSCs were used from passages 4 to 6 with similar results obtained throughout.

The various coated silk scaffolds were placed in a modified teflon chamber to increase the cell-matrix interaction [18]. The chamber contained 100 mm dish, each 3 mm wide×5 mm deep×30 mm long. The scaffolds were inoculated with 500 μ L of the cell suspension at a concentration of 2.5×10^5 cells by direct pipetting, incubated for 1 h at 37°C, and 500 μ L of media was added.

Proliferation assay of hBM-MSCs

Cell proliferation were measured by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay. For the MTT assay, cells cultured in a well plate, and each well supplemented with MTT (3 mg/mL) (n=3) and the plates were incubated in the dark at 37°C in an atmosphere containing 5% CO₂ for 90 min and the supernatant was aspirated. And then dimethylsulfoxide (DMSO) was added and the well plate was shaken slowly for 30 min. The absorption was measured at 570 nm.

Scanning electron microscopy

For scanning electron microscopy (SEM, JSM-840A; Jeol Inc., Tokyo, Japan), scaffolds were fixed in a mixture of 4% glutaraldehyde and 2% formaldehyde in phosphate buffer (0.2 M mixture of monosodium phosphate and dipotassium phosphate, pH 7.4) for 2 hours at room temperature. After thoroughly rinsing with 0.175 M phosphate buffer, specimens were immersed in 2% osmium tetroxide buffered with 0.2 M phosphate for 2 hours, dehydrated with hexamethyldisilazane (HMDS; Bio-Rad, Somerset, UK), sputter-coated with gold-palladium (E-5400; Bio-Rad, Somerset, UK), and observed by SEM at 15 kV.

Osteogenic Differentiation of hBM-MSCs

Osteogenic differentiation medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Welgene, Daejeon, Korea)

with 10% fetal bovine serum (FBS, Lonza, Basel, Switzerland), 1% penicillin/streptomycin (PS, Welgene, Daejeon, Korea), 10 mM β -glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 50 μ M L-ascorbate 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), and 100nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). The medium was changed 2–3 days.

Alkaline phosphatase assay

Alkaline phosphatase deposition was measured using a SensoLyte[®] pNPP Alkaline Phosphatase Assay Kit (ANASPEC, Fremont, CA, USA). Briefly, each of cell cultured scaffolds was drop into 200 μ L of lysis buffer and then it was vortexing during 10 min. And then 50 μ L of extraction solution was mixed with pNPP substrate solution. The mixtures were incubated for 30 min and stop solution was added. The absorption was measured at 405 nm.

Lactate dehydrogenase assay

Lactate dehydrogenase assay (LDH) activity was measured using an LDH-LQ kit (Asan Pharmaceutical Inc., Seoul, Korea). Briefly, after 7 days of culture, 20- μ L aliquots of medium and 50- μ L of working solution were mixed and incubated in darkness at room temperature for 30 min. The reaction was terminated by adding stop solution (1 N HCl), and the absorbance was measured at 570 nm.

Reverse transcription polymerase chain reaction analysis

Total RNA of cells was isolated using 500 μ L TRIzol (Sigma, St. Louis, MO, USA). Subsequently, 100 μ L of chloroform was added and the solution was mixed and incubated for 3 min. After centrifugation at 12000 rpm, 4°C for 15 min, the upper phase was transferred into a new tube and 500 μ L of isopropanol was added. After incubation period for 10 min and another centrifugation step at 14000 rpm, 4°C for 10 min, the supernatant was discarded. The pellet was washed with 1 mL of 70% ethanol and centrifuged at 4°C for 5 min. The supernatant was discarded, and the pellet was dried. After the addition of 20 μ L of DEPC-

water, the pellet was dissolved for 10 min on ice. The amount and purity of total RNA were determined by measuring using a nanodrop. Reverse transcriptase (RT) reactions were used to synthesize cDNA from 1 μ g of total RNA using an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA). RT-PCR was routinely performed and the sample was cooled to -20°C and stored until further analysis. For PCR, the primers were purchased from Bioneer (Bioneer Co., Daejeon, Korea) and primer sequence was shown at Table 1.

Western blotting analysis

After 7 days of cell culture, cells were lysed with RIPA buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS (Sigma), protease inhibitors (Complete[™], Roche, Mannheim, Germany). 30 μ g of protein was then separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes, which were then blocked with 5% skim milk in PBS containing 0.2% Tween 20. Immunoblotting analysis was performed according to the manufacturer's instructions with primary antibodies anti-bonesialoprotein (BSP), anti-BMP-2, anti-osteopontin, anti-osteonection, anti-collagen I and anti-actin. Blots were incubated with the primary antibodies at a dilution of 1:5000, and then further incubated with horseradish peroxidase-conjugated secondary antibody.

Statistical analysis

The data for MSC growth, ALP, and LDH release were evaluated statistically using Student t-test. The data are represented as the mean $\pm$ SD. *p* values <0.05 were considered significant.

Animal experiments

The Animal welfare committee of the Catholic University of Korea approved the experimental protocol (CUMC 2011-0041-02) and twenty four of mature female New Zealand white rabbits weighing 2.5 kg were used. The rabbits used in the experiments were adult rabbits without any special history prior to the experiments. The rabbits were anesthetized by intramuscu-

Table 1. Primers used for RT-qPCR

Genes	Upstream primer sequence	Downstream primer sequence
GAPDH	5'-ACC ACA GTC CAT GCC ATC AC-3'	5'-TCC ACC ACC CTG TTG CTG TA-3'
Collagen I	5'-GAA AAC ATC CCA GCC AAG AA-3'	5'-CAG GTT GCC AGT CTC CTC AT-3'
Osteonectin	5'-CCA GAA CCA CCA CTG CAA AC-3'	5'-GGC AGG AAG AGT CGA AGG TC-3'
Osteopontin	5'-TCG CAG ACC TGA CAT CCA GT-3'	5'-TCG GAA TGC TCA TTG CTC TC-3'
BMP-2	5'-GTC CAG CTG TAA GAG ACA CC-3'	5'-GTA CTA GCG ACA CCC ACA AC-3'
Runx-2	5'-CTC ACT ACC ACA CCT ACC TG-3'	5'-TCA ATA TGG TCG CCA AAC AGA TTC-3'
BSP	5'-CAC AGC CTC ATC TTC ATG G-3'	5'-GCA TCT CAT AGT GCA TCT GG-3'

BMP-2: bone morphogenic protein-2, Runx-2: runt-related transcription factor-2, BSP: bone sialoprotein

lar injection of 1 mg/kg of Zoletil® (Virbac SA, Paris, France) mixed with Rompun® (Bayer Korea Ltd., Seoul, Korea) at a 1:1 ratio. Following anesthesia, skin disinfection and incision, a 2.5-mm-diameter bone tunnel was drilled in the proximal femoral distal epiphysis.

The legs of the rabbits were randomly divided into four ex-

perimental groups. The first group (control) received uncoated silk scaffold, the second group (BMP) received silk scaffold coated with BMP-2 (25 µg each scaffold), the third group (HAp) received HAp coated silk scaffold and the forth group (HAp/BMP) received HAp and BMP-coated silk scaffold with the same level of BMPs.

Figure 1. Gross appearance of the silk scaffold (control), BMP-coated silk scaffold (BMP), HAp-coated silk scaffold (HAp), and HAp- and BMP-coated silk scaffold (HAp/BMP). BMP: bone morphogenic protein, HAp: hydroxyapatite.

Histological and immunohistochemical analysis

The rabbits were sacrificed and examined 1 and 3 weeks after implantation. Immediately after sacrifice, the graft-bone complex specimens were fixed in 10% formalin for 72 h, then decalcified in ethylenediaminetetraacetic acid solution and then embedded in paraffin wax.

The HE and masson's trichrome (MT) staining were performed to assess for inflammation, cell migration, synthesis of collagen, and new bone formation in the implanted scaffolds. The horizontal cross-section was performed in the center of the scaffolds. The MT staining was performed on 4–5 µm thick serial sections mounted on poly L-lysine coated slides. The sections were deparaffinized, and mordanted in Bouin's solution, microwaved 1 min, and allowed to stand 15 min. After wash-

Figure 2. Scanning electron microscopy observation of the silk scaffold (A), BMP-coated silk scaffold (B), HAp-coated silk scaffold (C), and HAp- and BMP-coated silk scaffold (D). Surfaces of the silk fibres without HAp coating are quite smooth and clear (A and B). With HAp coating, some of the nano-hydroxyapatite particles are deposited on the fibre surface, with a size from 200 to 1000 nm in diameter. Bar=100 µm. HAp: hydroxyapatite, BMP: bone morphogenic protein.

ing with distilled water for 5 min, they were treated with Weigert's working hematoxylin for 10 min, rinsed in distilled water for 5 min, soaked in Biebrich scarlet solution, and treated with phosphotungstic/phosphomolybdic acid. The sections were transferred and maintained in aniline blue solution for 5 min. Finally, they were washed with 1% acetic acid for 1 min and followed by distilled water.

For immunohistochemical staining, these section were incubated with the respective monoclonal antibody of anti-osteonectin (1:500 dilution, AB 1858, Chemicon, Carlsbad, CA, USA) for 24 hours, and developed using EnVision Plus reagent (Dako, Carpinteria, CA, USA), diaminobenzidine as chromagen, and Mayer's hematoxylin as counterstain. The slides were counterstained with Mayer's hematoxylin, rinsed in tap water, and then mounted.

The relative staining intensity was scored arbitrarily on a light microscopy image as follows: no or weak staining (-), low intensity (1+), moderate intensity (2+), and strong intensity (3+).

RESULTS

Gross and SEM observation

Figure 1 shown gross appearance of the silk scaffold (control), BMP coated silk scaffold (BMP), HAp coated silk scaffold (HAp), and HAp and BMP coated silk scaffold (HAp/BMP). We could not found the difference of the each scaffolds by observation by eye.

In the SEM observation, the silk threads remained smooth and uniform, and no cracks could be identified. However, after surface modification with HAp coating, a few of crystal particles were deposited on the silk fibre surface. The HAp particles had approximately a size from 200 to 1000 nm (Fig. 2).

After 2 days of seeding on various surface modified silk scaffolds, it was found that there was few of the bead-like MSC was attached on the silk thread which was no modified silk thread surface (Fig. 3A) and a few of cells were observe in the BMP coated silk scaffold (Fig. 3B). However, MSCs were fully spread throughout HAp coated silk scaffold surface (Fig. 3C) and MSCs attached and were well spread on the surface of the HAp/BMP coated silk scaffold (Fig. 3D).

Figure 3. Scanning electron microscopy observation of the silk scaffold (A), BMP-coated silk scaffold (B), HAp-coated silk scaffold (C), and HAp- and BMP-coated silk scaffold (D) after 2 days of MSC culture. Original magnification $\times 500$. BMP: bone morphogenic protein, HAp: hydroxyapatite, MSC: mesenchymal stem cell.

EDX analysis

The chemical composition of the ligaments was further investigated by EDX, and Figure 3 represents a typical spectrum corresponding to the depositing inorganic crust of surface modified silk scaffold. The electron diffraction patterns has shown peak of calcium and phosphate (Fig. 4).

The EDX showed further intensity increases in the Ca and P peaks compared to non-coated scaffold (Fig. 4C and D). So, we could know that HAp was coated on the silk scaffold. In body, bone mineral Ca/P atomic ratio is 1.65 which is amorphous condition [17]. At this study, the Ca/P ratio in the inorganic layer was 3.36 ± 0.5 (HAp coated silk) and 2.36 ± 0.65 (HAp/BMP coated silk) (Table 2). This EDX results indicate that the coated HAp on the silk scaffold is not amorphous calcium phosphate but crystallized HAp.

Proliferation of MSC cells on scaffolds

A general trend of cell proliferation was observed with time during the 5 days of culture, as determined by MTT analysis (Fig. 5). The initial seeding cell number was the same (2.5×10^5

cells) in each group. After 5 days of culture, the cell number was estimated to be 3.2×10^5 cells in the silk scaffold, 4.0×10^5 cells in the BMP coated silk scaffold, 5.1×10^5 cells in the HAp coated silk scaffold and 4.8×10^5 cells in the HAp/BMP coated silk scaffold. The cell density on the HAp coated silk scaffold increased significantly by an average 60% compared to the silk scaffold. As a result, bioactive matrix played a role in increased cell proliferation.

In vitro differentiation of MSC cells on scaffolds

The mRNA expression levels of osteogenesis-related genes from the hBM-MSCs following the induction of osteogenic differentiation for 7 days is shown in Figure 6. At 7 days, the mRNA expression levels of type I collagen (2.7 vs. 1.0), osteonectin (5.0 vs. 3.0), osteopontin (6.1 vs. 5.0), BSP (5.1 vs. 2.7), BMP-2 (5.8 vs. 1.8), ALP (6.2 vs. 3.3) and Runx-2 (5.2 vs. 2.5) in the BMP coated group were increase than those in the control group. And, the mRNA expression levels of type I collagen (5.6 vs. 1.0), osteonectin (7.2 vs. 3.0), osteopontin (10.1 vs. 5.0), BSP (7.8 vs. 2.7), BMP-2 (3.8 vs. 1.8), ALP (7.1 vs. 3.3) and

Figure 4. Energy-dispersive X-ray spectrum pattern of the surface of silk scaffold. (A) Silk scaffold (control), (B) BMP-coated silk scaffold (BMP-2), (C) HAp-coated silk scaffold (HAp), (D) HAp- and BMP-coated silk scaffold (HAp/BMP). BMP: bone morphogenic protein, HAp: hydroxyapatite.

Table 2. The results of energy dispersive X-ray spectrometer analysis

Element	Control [wt.%]	BMP-2 [wt.%]	HAp [wt.%]	HAp/BMP [wt.%]
Carbon	51.19	49.47	36.32	38.81
Nitrogen	15.95	16.63	10.52	13.39
Oxygen	32.21	33.22	47.70	39.53
Calcium	0.36	0.43	4.20	5.98
Phosphorus	0.29	0.25	1.25	2.30
Total	100	100	100	100

BMP-2: bone morphogenic protein-2, HAp: hydroxyapatite

Figure 5. Measurement of MSC proliferation with MTT assay at 5 days after cell culture. Silk scaffold (control), BMP-coated silk scaffold (BMP-2), HAp-coated silk scaffold (HAp), HAp- and BMP-coated silk scaffold (HAp/BMP). $p < 0.05$. MSC: mesenchymal stem cell, MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, BMP: bone morphogenic protein, HAp: hydroxyapatite.

Runx-2 (9.7 vs. 2.5) in the HAp coated group were higher than those in the control group. Furthermore, the mRNA expression levels of type I collagen (9.5 vs. 1.0), osteonectin (9.0 vs. 3.0), osteopontin (9.0 vs. 5.0), BSP (8.0 vs. 2.7), BMP-2 (9.5 vs. 1.8), ALP (8.3 vs. 3.3) and Runx-2 (9.2 vs. 2.5) in the HAp/BMP coated group were significantly higher than those in the control group.

However, there were no significant differences in the mRNA expression levels of osteonectin, osteopontin, BSP, ALP, and Runx-2 between the HAp coated group and HAp/BMP coated group.

As a results, MSCs cultured on HAp/BMP coated silk scaffolds showed significantly higher mRNA expression (3-fold increase) compared to control group and HAp/BMP coated silk scaffolds was more efficient than only HAp or BMP coated silk scaffold.

Western blot analysis

To examine osteogenic differentiation, osteogenesis-related

proteins were detected by Western blot analysis at day 7 of induction. As shown in Figure 7, the expression levels of osteogenesis-related proteins (BSP, BMP-2) were increased in the HAp and/or BMP coated silk scaffold group. MSCs cultured on BMP coated silk scaffolds showed higher gene expression for osteonectin, BSP, BMP-2, and ALP compared to MSCs cultured on silk scaffolds. Also, MSCs cultured on HAp coated silk scaffolds showed significantly higher gene expression for collagen type I, osteonectin, BMP-2 and bone sialoprotein compared to MSCs cultured on silk scaffolds. And the combination of HAp and BMP coating group showed the most significant induction of osteogenic gene expression compared to MSCs grown on the other groups. Especially, osteonectin was strongly expressed in the HAp/BMP coated silk scaffold group. (Fig. 7).

And, ALP is an early mineralization-related protein marker for the osteogenesis of osteoblasts. So, cellular differentiation was measured by evaluating the ALP activity with cell lysis. Figure 8 shows the results of the ALP activity for all samples. After 7 days of culture, the cells in the HAp/BMP coated group showed a much higher level of ALP activity than in the control group and the differences between the BMP coated group and the HAp coated group were insignificant. These results suggest that BMP incorporation in HAp increases ALP activity compared to BMP or HAp alone in hBM-MSCs during osteogenesis.

Also, LDH analysis was performed on day 7 to evaluate membrane change of cells caused by differentiation (Fig. 9). Our results showed that LDH release increased HAp coating and HAp/BMP coating groups compared to control groups ($p < 0.05$), however no significant difference in the LDH release between HAp coating and HAp/BMP coating groups. LDH is a cytoplasmic catalytic enzyme related to the reversible conversion between pyruvic and lactic acid and it is released through the cell membrane. Therefore, we hypothesis that LDH release induced the change of cell membrane by differentiation.

Histological analysis after grafting *in vivo*

The dog was anesthetized and the femur was exposed by making a sterile incision in the skin along the midline of the

Figure 6. RT-PCR analysis shows mRNA expression levels of osteogenic marker after cell differentiation culture at 7 days. Compared to the control, the MSCs on nHAp and/or BMP-2 coated scaffold had significant higher levels of collagen type I, osteonectin, osteopontin, BSP, BMP-2, ALP, and Runx-2, gene expression (two-fold more than increase). RT-PCR: reverse transcription polymerase chain reaction, MSCs: mesenchymal stem cells, nHAp: nano hydroxyapatite, BMP-2: bone morphogenic protein-2, BSP: bone sialoprotein, ALP: alkaline phosphatase.

Figure 7. Differentiation of MSC with osteogenic media on various surface modification of silk scaffolds. Compared to the other groups where MSCs were seeded on nHAp/BMP-2 had highest coll I, osteonectin, and BMP-2 protein expression at 7 days after culturing. MSC: mesenchymal stem cell, nHAp: nano hydroxyapatite, BMP-2: bone morphogenic protein-2.

knee joint and hole was drilled on femur, and the silk scaffold was inserted into hole in the knee (Fig. 10). All animals survived during the operation and the postoperative period without any complications. Thus, 24 rabbits completed the study and were euthanized at 1 and 3 weeks after surgery. All the cross-section of slides was performed in the center of grafted scaffolds and each three slides were observed.

After 1 week implantation, many of the blood vessels were observed in the most of the grafted scaffolds, and the monocyte and neutrophil were invaded into the graft-bone interface. And no immune reaction osteolysis was observed, but at this time, mild inflammatory responses were observed in the all implanted group. So, we could know that acute inflammatory reaction did not induced by silk, BMP and HAp (Fig. 11A-D). After 3 week implantation, in the middle zone of HAp or/and BMP coated silk scaffold become filled with fibroblast-like cell, lymphocyte, monocyte, and collagen fibers, but few of the cells penetrated into the interior of the silk scaffold (control). And grafted scaffolds were filled with loose fibrous tissue and growing ECM islands within BMP and/or HAp coated silk scaffolds. But blood vessels were reduced at 3 weeks in the tunnel zone and silk scaffold (Fig. 11E-H).

Within the same period, the safranin-O staining was performed. In the coated group, there was formation of new cartilage (red color) in comparison with the control group and this cartilage was primarily composed of round chondrocytes, and lacunae or concave areas were observed (Fig. 12A-D).

Also, immunohistological results of osteonectin staining (brown color) showed that the BMP coated group and HAp/BMP coated group had more positive stained than control group at the nearby new formed cartilage like tissue area (Fig. 12E-H). This relative staining intensity was evaluated by light microscopy (Table 3).

And MT staining showed new collagen fiber in the around area of silk scaffold. And gap of silk fibers and original bone of tunnel margin (Fig. 13), and osteoblast around of silk scaffold thread the HAp coated and HAp/BMP coated group (Fig. 13G

and H). In the control group, collagen fibers moderately formed and infiltrated into the silk scaffold, while in the HAp and/or BMP coated silk scaffold group 3 weeks after surgery, there were more collagen bundle formed, and the silk were more

Figure 8. Effect of BMP and/or HAp stimulus on the ALP release of MSCs at 7 days (n=3). Silk scaffold (control), BMP-coated silk scaffold (BMP-2), HAp-coated silk scaffold (HAp), HAp- and BMP-coated silk scaffold (HAp/BMP). * $p<0.05$, ** $p>0.05$. BMP: bone morphogenic protein, HAp: hydroxyapatite, ALP: alkaline phosphatase, MSCs: mesenchymal stem cells.

Figure 9. Effect of BMP and/or HAp stimulus on the LDH release rate of MSCs at 7 days (n=3). Silk scaffold (control), BMP-coated silk scaffold (BMP-2), HAp-coated silk scaffold (HAp), HAp- and BMP-coated silk scaffold (HAp/BMP). * $p<0.05$, ** $p>0.05$. BMP: bone morphogenic protein, HAp: hydroxyapatite, LDH: lactate dehydrogenase.

Figure 10. Procedure for the implantation of the silk scaffolds in the knee joint of a rabbits. The dog was anesthetized (A) and the femur was exposed by making a sterile incision in the skin along the midline of the knee joint and hole (2 mm) was drilled on femur (B), and the silk scaffold was inserted into hole in the knee (C and D).

encircled by ECM, and neoformative bone-like tissue protruding into the interface of the graft and host bone was observed (Fig. 13L). However, the orientations of the newly formed ECM and collagen were not enough.

DISCUSSION

Many artificial ligaments of different materials such as carbon fibers, polypropylene, dacron and polyester have been utilized, but most of the artificial ligament presented serious drawbacks, such as cross-infections, immunological responses, tunnels osteolysis, foreign-body synovitis, chronic effusions, recurrent instability and knee osteoarthritis [20]. Especially, the

reason for failure might be related to the the graft anchorage of the bone tunnel zone of the artificial ligament [8]. Fibrous scar tissue formation has been shown to be an incomplete osteointegration of graft and this immature granulation fibrous layer is extremely weak and will significantly influence the graft stability in the bone tunnel [7-9,11,14,18,19].

The research using silk materials were satisfactory in terms of both biocompatibility and physical properties, but the silk material alone did not achieve sufficient attachment or growth of cells. So, some investigator tried using an adhesion sequence or protein and found that RGD-binded silk matrices supported the improved attachment of bone marrow stromal and ACL cells and showed higher cell growth [20,21]. But such a surface

Figure 11. Histological characterization of silk scaffold-bone interface between the host bone and the graft at 1 (A-D) and 3 (E-H) weeks after surgery. A fibrous scar tissue band is finally established at the interface of the graft and host bone (H&E staining). Original magnification: $\times 20$; Scale bar: 500 μm . HB: host bone. IF: interface, G: graft.

Figure 12. Histological observation at 3 weeks: Sagittal sections stained with safranin-O (A-D) and immunohistochemical staining for osteonectin in the regenerated tissue of the graft-tunnel zone (E-H). control group (A), BMP-coated group (B), HAp-coated group (C), HAp/BMP group (D). Red color indicates newly formed cartilage. Original magnification: A-D $\times 20$, E-H $\times 200$; Scale bar: A-D 500 μm , E-H 100 μm . BMP: bone morphogenic protein, HAp: hydroxyapatite.

modification did not increasing of graft and bone tunnel integration and osteogenesis.

In this study, we used for HAp and BMP for increasing of osteogenesis in silk scaffold. HAp, as the main component of skeletal bone, has been widely studied for its strong osteoconductive capacity. So, it has been successfully applied to treat bone defects or articular cartilage defects and to induce bone ingrowth into and/or onto the soft tissue or metal implants in large experimental animals [22-25]. Additionally, it has been reported that HAp powder can promote tendon osseointegration through increasing new bone formation in a tendon-to-bone healing model [14]. Also, a critical element in the bone formation is BMP-2, which has an essential role in promoting bone formation *in vitro* and *in vivo* [26]. BMPs induce bone formation by recruiting osteoprogenitor cells and inducing osteogenic differentiation [27]. Also, Some BMPs are known to facilitate cartilage regeneration, preserve cartilage, and promote chondrogenic

differentiation of bone marrow stem cells [28]. So, the application of BMP-2 for the repair and regeneration of large bone defects or non-union bone fractures has been extensively studied. But some of the investigator reveal that high doses of growth factor lead to side effects such as excessive bone formation, downregulation of receptor and immune response [27,29,30].

At this study, we had designed and investigated the synergic effect of HAp impregnated with BMP-2 on the osteoblast differentiation *in vitro* from BM-MSC and osteointegration and osteogenesis in the rabbit model.

Many other investigator have been tried such a synergic effects of osteogenesis and chondrogenesis with composite-bioactive materials. The BG-coated BMP-2 were shown to exert a synergistic effect with the highest osteogenic gene expression levels and ALP activity. And Lu et al. [26] reported that BMP-2 coating on HAp/ β -tricalcium phosphate scaffold exerted a synergistic effect promoting osteogenic related gene expression

Table 3. The relative staining intensity score of safranin-O and osteonectin

Staining	Control	BMP-2	HAp	HAp/BMP
Safranin-O	+	++	++	+++
Osteonectin	+	++	++	++

BMP-2: bone morphogenic protein-2, HAp: hydroxyapatite

Figure 13. Collagen fiber status of the control group (A), BMP-coated group (B), HAp-coated group (C), HAp/BMP group (D) at 1 weeks (A-D) and 3 weeks (E-L) after surgery. The figure shows the formation and infiltration of the collagen fibers and bone formation at the interface of the graft and the host bone (Masson staining). Original magnification: A-H $\times 20$, I-L $\times 100$; Scale bar: A-H 500 μ m, I-L 200 μ m. BMP: bone morphogenic protein, HAp: hydroxyapatite.

levels and alkaline phosphatase activity in adipose tissue-derived mesenchymal stem cells. Also, Maehara et al. [29] reported that porous HAp scaffold can be used as a suitable material for use as a bone void filler and as a carrier of BMPs. Additionally, they showed that HAp scaffold impregnated with a FGF-2 promoted subchondral bone and cartilage regeneration.

The HAp-BMP binding may be attributed to the electrostatic interaction between the positively charged amino acid residues in BMP-2 and the negatively charged sites of the apatite particles [27].

This results of our experiments indicated that the expression levels of collagen I, osteonectin, BSP, BMP, and ALP were slightly increased in the BMP coated group than control group. However the osteoblast markers, osteopontin, osteonectin, and ALP were highly expressed, also the expression levels of major bone matrix protein genes, such as collagen I, BMP-2, and BSP had shown significantly increased in the HAp coated and HAp/BMP coated silk scaffold group than control group.

BSP is a highly post-translationally modified acidic phosphorus protein normally expressed in bone and it is a late stage marker of osteoblast differentiation and an early stage marker of matrix mineralization [31,32]. And osteopontin is expressed throughout matrix maturation, followed first by BSP, which characterizes the post-proliferate phase and collagen I is the most abundant protein in the bone matrix and is an early marker of osteoblastic differentiation and the major organic component of the mineralized bone matrix. McKee et al. reported that during bone wound healing, the osteopontin plays important roles in the clearance of bone debris by macrophage phagocytosis and the formation of a new bone at the margins of the bone defects [33]. Also, runx-2 is involved in the production of bone matrix proteins as it is able to upregulate the expression of major bone matrix protein genes leading to an increase in immature osteoblasts from stem cells [34,36].

As results of this study, the representative osteogenesis-related genes were highly expressed in the HAp/BMP coated silk scaffold group compare to BMP or HAp coated and control group. So, we demonstrated that the BMP-2 and HAp silk scaffolds had a synergistic effect in inducing osteogenic differentiation in BM-MSCs *in vitro*.

After implantation, BMP-2 recruits osteoprogenitor cells to the intended area and induces their differentiation into osteoblastic cells. The graft-bone interface of the control group was filled with granulation tissue, and a thick fibrous scar tissue was formed at the graft-bone interface. But in the group with HAp or BMP coating, the graft-bone interface also appeared somewhat messy with fibrous tissue. At the middle site in the HAp/BMP coated group, interface width appeared much narrower and host bone grew onto to the graft. Also, the HAp

binding system for BMP-2 showed much higher cell migration, chondrogenesis and bone formation compared with uncoated silk scaffolds. There are also osteoblasts infiltration, which induce the formation of bone or fibrocartilage at the interface. This cartilage matrix will be replaced by the new bone through endochondral ossification and integrated with newly formed bone extending from the transverse process.

Also, the immunohistochemical staining of osteonectin shows the HAp and/or BMP coated group had more positive stained than control group. And, a lot of the osteoblast or chondrocyte observed in HAp and HAp/BMP coated groups. The osteonectin is an acidic calcium-binding protein and plays roles in bone mineralization and cell-matrix interaction and it was reported that osteonectin is synthesized by in case of hypertrophic chondrocyte and accumulated in the mineralized zone in cartilage [7,37]. So, we thought that HAp and HAp/BMP can induced mineralization.

As results of this study showed possibility of solved the problem of graft-tunnel integration in ligament reconstruction with the HAp and growth factor modified scaffolds. The HAp/BMP coated silk scaffold was not optimal for osteointegration and osteogenesis of ligament reconstruction, but the HAp-BMP did increase the rates of cell migration and new bone formation compared with silk scaffold. Moreover, these matrix induced chondrogenesis, which is essential for the initial phase regeneration of a new bone. Our future work will focus on optimization of both growth factor concentration and HAp coating methods.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

The Animal welfare committee of the Catholic University of Korea approved the experimental protocol (CUMC 2011-0041-02).

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