Exploring and evaluating scaffolds for in-vivo application of TAT-BMP

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Exploring and evaluating scaffolds for in-vivo application of TAT-BMP

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감사의 글

이제 논문을 마무리하면서 감사의 글을 쓰게 되니 지나온 대학원 생활이 주마등처럼 떠오릅니다. 직장과 학교에서 시간에 쫓겨 가면서 무던히도 힘겨워 했던 제 모습, 우여곡절에 지지와 격려를 아끼지 않으셨던 지인 분들에 대한 감사한 마음과 논문을 무사히 마무리하게 된 안도감이 교차합니다.

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쉽지 않은 길 하고자 한다면서 열심히 하라고 끝까지 묵묵히 이끌어주시고 격려해주신 김정국 원장님께 고개 숙여 깊이 감사 드립니다. 오랜 만남은 아니지만 대선배로써 학문적인 깨우침 뿐 아니라 사회 선배로서의 조언과 격려를 아끼지 않으시고 배움에 대해 노력하는 자세와 자긍심을 일깨워 주신 김정문 선배님께 평생 보은의 마음으로 살아가겠습니다. 함께한 긴 시간 속에 단단한 가르침으로 늘 마음의 위안과 끊임없는 용기를 주시고 항상 저를 걱정해 주시고 세심한 배려해주시는 손규호 박사님께 무한한 은혜 가슴에 담겠습니다. 언제나 든든한 버팀목처럼 모든 걸 감수하고 이른 아침 챙겨준 아내와 든든하고 사랑스러운 보배 두 아들 민환, 우준에게도 사랑과 고마운 마음을 전합니

다. 하늘에서 끝까지 절 응원해 주실 어머님과 늘 곁에서 믿어주시고 격려해 주신 아버님께 가없는 사랑의 마음을 드립니다. 항상 묵묵히 지켜봐 주시고 격려해 주신 장인 어른과 장모님께 깊은 감사 드립니다.

마지막으로 지면을 통해서 일일이 언급하지 못했지만 그 동안 저를 아끼고 지지해주신 모든 분들께 다시 한번 깊은 감사의 마음 오랫동안 간직하겠습니다.

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ABSTRACT

Exploring and evaluating scaffolds for in-vivo application of TAT-BMP

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(Directed by Professor Jong In Yook, D.D.S., Ph.D.)

Although the need for bone regeneration is gradually increasing due to aging, clinical modality is not yet definite. Exogenous growth factors, like bone morphogenetic proteins (BMPs) should have sufficient bioactivity and be in harmony with the carrier system by controlled release of biological factors with mechanical support. Recombinant human BMP-2 (rhBMP-2) are currently used in clinics, but their efficacy remains obscure.

Contrary to soluble hydrophilic rhBMP-2, inactive form TAT-BMP-2 fusion protein has a hydrophobic nature that requires an appropriate scaffold system toward in vivo application. In this regard, this study aims to explore the most proper scaffold among collagen, fibrin and hydrogel, which all have in-vivo biocompatibility, biodegradability by evaluating bone regeneration efficacy when combined with TAT-BMP-2 in the critical sized defect of a rabbit skull. Also, chemical residues of the most excellent scaffold,

hydrogel, were detected by several methods to proceed into preclinical study. The results are as follows:

1. The bone regeneration efficacy of hydrogel and collagen were in themselves comparable.
2. 5μg of the TAT-BMP-2 and hydrogel composite induced 50% more efficient bone regeneration than blank, collagen and fibrin. Also it exhibited the most superior bone mineral density.
3. Fibrin seemed to inhibit osteoinduction. Furthermore, the TAT-BMP-2 fibrin composite did not demonstrate significant bone regeneration compared to control or other experimental groups.
4. The concentration dependency of the TAT-BMP-2 and hydrogel composite in bone regeneration needs further investigation.
5. The elution buffer of hydrogel contained EDC and TCEP, which needs to be removed by serial dialysis.

These results indicated that TAT-BMP-2 hydrogel composite showed the most effective in vivo mode of action to deliver secretory growth factors. Further treatments to remove the chemical residues in hydrogel should be done to move forward clinical application.

Key words: PTD (Protein Transduction Domian), HIV-1 TAT, BMP (Bone Morphogenetic Protein), hydrogel

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I. INTRODUCTION

As one of the transforming growth factor beta superfamily, bone morphogenetic proteins (BMPs) are renowned for their role in osteoinduction. So far, approximately twenty different members of the BMP super group have been identified. However, only a subset is able to promote osteoinduction, including BMP-2 through-7 and BMP-9 (Abe, 2006; El-Amin et al., 2010; Termaat et al., 2005). The intracellular BMP signal transduction starts from the formation of type I,II membrane receptor heteromeric complex and the activation of cytosolic serine/threonine kinase. The phosphorylation of series of SMAD protein binds with SMAD4 which is able to bind with several kinds of phosphorylated SMAD (Massague et al., 1992; Wrana et al., 1994). Besides osteoinductivity, BMPs have a wide range of effects in regulating differentiation of

mesenchymal stem cells, apoptosis and synthesis of extracellular matrix via correlation of Smad with TFG-β, Wnt, Jak-STAT signaling (Reddi, 2001).

Since discovering the role of BMPs in bone regeneration (Urist, 1965), unceasing efforts and trials are being done to elute and deliver BMPs. Until now, there are two ways of eluting BMPs. One is by decalcifying with acid and the other is using gene recombination technique by developed molecular biology. The first method produces a demineralized bone matrix by removing inorganic minerals with exposure to acid, leaving behind an organic collagenous matrix and non-collagenous proteins including growth factors (Dinopoulos and Giannoudis, 2006). But this method has shortcomings in the purity of the eluted protein and the extraction efficiency (Wozney, 2002).

The latter method is currently the most popular way to synthesize BMPs by using viruses, animal cells, like CHO cells and bacteria. In case of using a virus system, trials either direct delivering the virus by the adenovirus system or combining transformed cells with carriers and/or scaffolds that have been challenged. However, the future of this gene delivering method remains unclear due to their mutagenic potential in normal cells as well as its uncontrollable gene expression (Winn et al., 2000). Recombinant human BMPs (rhBMPs), which are composed of secreted mature protein produced by transformed mammalian cells, are acquirable in regenerative medicine markets, as they have received FDA approval. But rhBMPs have mutually dependent economic and biological shortcomings: they have expensive production costs and overdose risks due to their low efficiency (Aspenberg and Turek, 1996). Additionally, rhBMPs obtained from bacteria are good economically, but still have biological problems due to low efficiency because post-translational modification processes are deficient (Itoh et al., 1999).

To amplify the regeneration efficacy of exogenous BMPs, the protein transduction domain (PTD), which delivers passenger protein directly to intracellular space, can be considered (Joliot and Prochiantz, 2004; Kabouridis, 2003). The function of PTDs was discovered from the human immunodeficiency virus (HIV)-1 trans-activator of transcription (TAT) protein that enters into the cytoplasm in a concentration-dependent manner without going through any receptor systems (Frankel and Pabo, 1988; Green and Loewenstein, 1988). The HIV-1 TAT fusion protein consists of 86 amino acids, including two essential exons, to replicate HIV. Eleven of the amino acids (RKKRRQRRR) are known to regulate unrestricted protein transduction (Schwarze et al., 2000).

Although TAT fusion proteins have been recognized to be transported into cytosol through cell membranes, regardless of any receptors, it has been reported that the TAT fusion protein transport system follows a temperature dependent endocytic pathway due to decreased transportion efficacy at 4℃ (Fittipaldi et al., 2003; Wadia et al., 2004). Also, transported fusion proteins, with the assistance of endosome, have difficulties in restructuring for activation on account of encircling lipid bilayer (Takenobu et al., 2002). The N-terminal HA2 peptide of influenza virus haemagglutinin-2, which is able to escape from the endosome is inserted into the TAT fusion protein to solve the endosome-related problems and to increase the efficiency of fusion protein (Michiue et al., 2005).

While several approaches make steady progress to deliver full-length proteins, DNA, liposomes and nano particles through TAT peptides, the advantages are well known in converting bacteria originating as inactive proteins into active proteins, not regarding cell type, cell membrane, and protein size, showing little effects in immune response. Until

now, the application of TAT fusion proteins is limited in the area of intracellular transcription factors, not being extended in the area of secreted protein.

Recently, Kim N.H. designed the TAT-BMP-2 fusion protein and demonstrated its cellular nature and in-vitro activity by increased alkaline phosphatase activity, RUNX2 transcriptional activity, and von Kossa staining (Kim, 2007). Also, Son K. validated superior in-vivo activity of the TAT-BMP-2 hydrogel composite in the cylindrical defect model in femur of beagle (Son, 2012).

The discovery of BMPs has raised hope that bone could be regenerated by the local application of BMPs. Previously, reduced efficacy observed in large animal models using BMPs delivered in formulation buffer solutions may be due to the combination of a reduced pool of available responsive stem cells and insufficient retention of the BMPs at the repair site to stimulate an appropriate increase in the number of responsive cells. In contrast, BMPs, combined with injectable or implantable carriers with longer residence time than formulation buffer solutions, accelerate bone healing, tendon/ligament healing, and cartilage healing in numerous studies in both small and large animal models, as well as in people (Seeherman and Wozney, 2005).

However, a major limitation to growth factor therapy is that enormous quantities of growth factors, largely exceeding physiological levels, are needed to induce the formation of bone. For example, 3.5 mg of recombinant BMP-7 used for the treatment of bone defects corresponds to 2-fold of the entire amount of BMP-7 found in a human being (Bishop and Einhorn, 2007; Lienemann et al., 2012)

It is clear that better delivery strategies must be developed to improve growth factor therapy. Not surprisingly, the development and application of novel biomaterials for the

controlled delivery of growth factors have stimulated considerable interest in the tissue engineering community (Bessa et al., 2008b; Kanayama et al., 2006; Laurencin et al., 1996). In particular, biomimetic scaffolds emerged, whose design was inspired by the role of the natural extracellular matrix (ECM) in regulating tissue regeneration (Garrison et al., 2007; Schmidmaier et al., 2007). Such scaffolds are conceived to take advantage of the bodies' inherent capacity to heal, essentially using the body as bioreactor to regenerate bone.

Generally, the major categories of carrier materials include: (1) natural origin polymers such as collagen and, hyaluronans; (2) inorganic materials and ceramics/cements such as hydroxyapatite, tricalcium phosphates, and sulphates as well as bio glasses and metals; (3) synthetic biodegradable polymers such as poly(lactic acid) (PLA), polyglycolide (PLG) and their copolymers, poly L-lactic acid (PLLA), poly D,L-lactide-co-gycolic acid (PLGA) and poly e-caprolactone (PCL); and (4) composites which are combinations that take advantage of each material class as well as other biomolecules(Fei et al., 2008).

As a natural polymer, collagen is a popular choice for bone tissue regeneration applications due to its biocompatibility, ease of degradation, and interaction with other bioactive molecules. In fact, the only rhBMP-2 containing FDA-approved product for clinical use in spinal fusions, tibial shaft fractures, and oral surgeries is comprised of an absorbable collagen sponge (ACS). Despite the proven clinical efficacy of collagen carriers, it is known that delivery of rhBMPs from these matrices have a number of disadvantages. Of the existing problems, the most prominent include the lack of

mechanical strength and unpredictable biodegradability of the collagen matrix (Tan et al., 2007).

Fibrin, derived from blood clots, can be formulated in an adhesive glue-type delivery vehicle for BMPs (Seeherman and Wozney, 2005). Fibrin is a material that can be rapidly invaded, remodeled, and replaced by cell-associated proteolytic activity (Murphy and Gavrilovic, 1999). Although there are conflicting results concerning the use of fibrin gel for in-vivo bone regeneration applications (Patel et al., 2006), it has been shown that in combination with heparin-functionalized nanoparticles and rhBMP-2, fibrin gel promotes significant improvement and effective bone regeneration in a rat calvarial critical size defect (Chung et al., 2007). Further, covalently conjugating heparin to fibrin has been shown to significantly enhance bone formation in comparison to rhBMP-2 and free heparin loaded in fibrin matrices (Yang et al., 2010).

Hyaluronic acid (HA) is another naturally -occurring biopolymer, which plays a significant role in wound healing. HA and its derivatives have been largely studied in biomedical and tissue engineering applications as gels, sponges, and pads, and as a viscous gel injected percutaneous in ophthalmic surgery (Bessa et al., 2008a; O'Regan et al., 1994). HA also has an osteoinductive action itself where it has been shown to result in improved bone formation in mandibular defects in comparison to collagen sponges when both carriers were used to deliver BMP-2 in rats as well as in a human clinical trial (Arosarena and Collins, 2005). That is probably due to that HA-based delivery vehicles might possess the capacity to retain more BMPs than collagen (Kim and Valentini, 2002). In addition, hyaluronans seem to stimulate the proliferation of bone marrow stromal cells and the expression of osteocalcin, as well as enhance ALP activity and interact positively

with BMPs to generate direct and specific cellular effects. This increased affinity is attributed to HA being anionic, thus forming ionic bonds with the cationic BMPs significant for potential future clinical applications (Peng et al., 2008).

Unlike rhBMPs, which are eluted in hydrophilic form in formulation buffer solutions, TAT-BMP is hydrophobic, which means TAT-BMP has different physical and chemical properties in comparison with rhBMPs. Thus, finding proper scaffold systems combined with TAT-BMP, is very important for further in-vivo experiments and clinical application. Above all, such biomaterial that can be maintained for several weeks with controlled degradation in living tissues to establish sustained release of TAT-BMP-2 is needed.

In this regard, this study aims to evaluate the in-vivo bone regeneration efficacy of TAT-BMP-2 by combining it with collagen, fibrin, and hydrogel to search for the most appropriate scaffold system.

II. MATERIALS AND METHODS

1. TAT-BMP-2 protein purification

The TAT-BMP-2 vector was same as previously reported (Kim, 2007) (Figure 1). Recombinant protein was expressed through BL21 (DE3) Escherichia. coli (Invitrogen, U.S.A). The pTAT-BMP-2 vector was transformed to bacteria and streaked on a plate that contained 100μg/ml ampicillin. After incubating for over 16 hours, bacteria was collected by scraping and inoculated to 400ml 2 x YT (Q-Biogen, France) LB media, which contains 100μg/ml ampicillin (Sigma, U.S.A). Isopropyl-β-D- thiogalactoside (Sigma, U.S.A) was added to reach a final concentration of 0.4mM when the value of OD600 was between 0.5 and 0.7. After four more hours of cultivation, centrifugation was done for 30 minutes under 5500rpm. Soluble lysis buffer (50mM Tris, pH8.0, 300mM NaCl, 20mM imidazole, pH8.0) 10ml was added to precipitation for resuspension, then sonication (amplitude 90, 60 seconds, two times) was done. Triton X- 100 was added to reach a final concentration at 0.5 percent, and then centrifugation was done for 20 minutes under 4℃, 12000rpm. To denature the expressed protein, 10ml of inclusion body solution (8M urea, 50mM tris, pH8.0, 100mM NaCl) was added and dissolved for one hour at room temperature, and then centrifugation was done for 15 minutes under 15000rpm. Supernatant reacted to 1ml of nickel-nitrilotriacetic acid (Ni- NTA) agarose (Qiagen, Germany) for two hours at 4℃, which was substituted with a binding buffer (8M urea, 50mM tris, pH8.0, 100mM NaCl, 20mM imidazole,

pH8.0). Reacted Ni-NTA agarose was washed three times with 10ml of binding buffer and mixed with 500nM, 1mM imidazole binding buffer respectively for 20 minutes at 4℃. Afterward, centrifugation was done for one minute under 1000rpm to purify TAT-BMP-2.

In this experiment, the TAT-BMP-2 protein was eluted by ATGen(ATGen, Korea) with purifying proteins and detoxifying endotoxins. The endotoxin level of TAT-BMP-2 was measured with Charles-River's endotoxin detection and microbial identification kit (Charles River laboratories, USA)

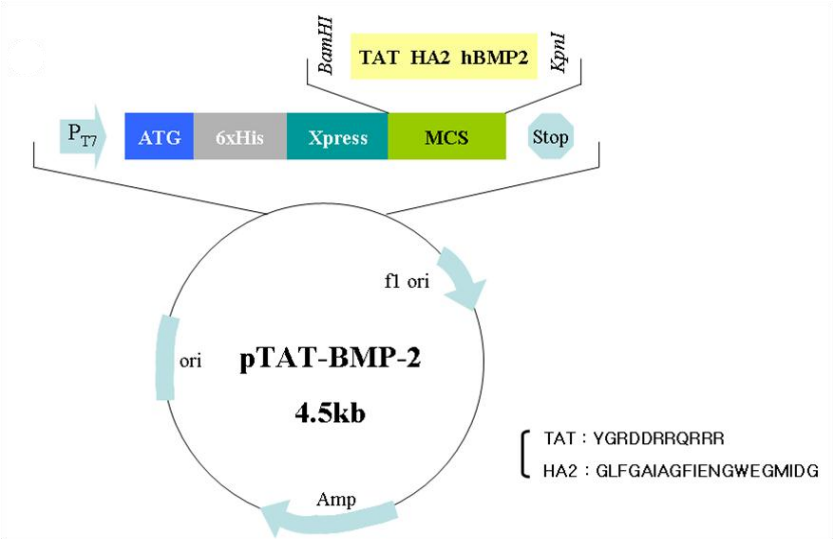


Figure 1. TAT-BMP-2 vector map.

2. Scaffolds for in-vivo experiment

2.1. Collagen

Collagen was offered from RMS (Sewon Cellontech, Korea) in an injectable style. RMS collagen has a highly purified triple helix of three collagen chains, like natural collagen, constituting most of human body parts. It can provide a stable scaffolding structure, induce cell migration with enriched sites for adherent cells, optimize cell conduction into the scaffold, and provide an advanced remodeling phase. The TAT-BMP-2 protein was uniformly mixed before animal experimentation and reloaded in a syringe.

2.2. Fibrin

A Greenplast fibrin kit was purchased from Greencross (Greencross, Korea). The TAT-BMP-2 protein was mixed into the aprotinin, fibrinogen mixture while minimally adding thrombin and calcium chloride. Gelation procedures were done on the polyvinyl siloxane mold, which mimicked real defect shape.

2.3. Hydrogel

Hydrogel was supplied by Seoul National University of Science and Technology (SNUT), and its gelation procedure was similar with fibrin on the customized putty mold. Hyaluronic acid-apidic acid dihydrazide (HA-ADH) was synthesized by adding apicidic acid dihydazide (ADH), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide (EDC) in

hyaluronic acid (HA). Hyaluronic gel was acquired through mixing HA-ADH-acrylic acid and HA-ADH-Tris(2-carboxyethyl)phosphine hydrochloride (TCEP).

In addition, ^{1}H Nuclear magnetic resonance spectrometer (NMR), High performance liquid chromatography (HPLC) and Gas chromatography/Mass spectrometry (GC/MS) analysis were done to detect the chemical residue of hyaluronic gel by eluting unreacted compounds in distilled water for 24 hours. ^{1}H NMR was done in SNUT, and HPLC, GC/MS were done in the Korea Institute of Basic Science.

3. Animal experiment

3.1. Designing critical sized defect in rabbit skull

In-vivo experiment was done in the calvaria of a New Zealand rabbit on account of its several advantages, ease of operation and maintenance, and the existence of previous study models. Double cylindrical defects with 8mm diameter were considered to be the best model for this experiment while considering published reports (Kim et al., 2005; Lin et al., 2012). The observation periods were determined as 4 and 8 weeks by reflecting on the rabbit's healing capacity (Sohn et al., 2010).

3.2. Designing experimental group

A total of 24 rabbits were divided into three groups following the type of inserted biomaterial, collagen, fibrin, and hydrogel. Two rabbits of each group were sacrificed at 4 weeks to check the intermediate process. The defects of these rabbits were classified as "blank" which had nothing in space, "scaffold" which had one of those biomaterials, and "TAT-BMP-2 with scaffold". The concentration of delivered TAT-BMP-2 was 5μg/mL while the single defect of each group was filled with 2μg/mL TAT-BMP-2 to find the possibility of concentration dependent osteoinduction (Table 1).

Table 1. Groups in animal experiment.

	4 weeks			8 weeks		
	Animal number	Defect1(R)	Defect2(L)	Animal number	Defect1(R)	Defect2(L)
Group1 Hydrogel	1	Hg	Hg+BMP5ug	3	Blank	Hg+BMP2ug
	2	Blank	Hg+BMP5ug	4~8	Hg	Hg+BMP5ug
Group2 Collagen	9	Co	Co+BMP5ug	11	Blank	Co+BMP2ug
	10	Blank	Co+BMP5ug	12~16	Co	Co+BMP5ug
Group3 Fibrin	17	Fb	Fb+BMP5ug	19	Blank	Fb+BMP2ug
	18	Blank	Fb+BMP5ug	20~24	Fb	Fb+BMP5ug

3.3. Surgical procedures

Twenty-four rabbits (male weighing 2.5kg each) were purchased and fed in separate cages with free access to food and water. Animals were anesthetized using an intramuscular 1mL injection of Zoletil and Narcoxyl (3:2 ratio) with additional intravenous 0.5mL injection of same drugs. The surgical site was shaved and disinfected with 10 percent Betadine. A linear incision was made along the midline and the skin layer was reflected first. After dissecting the subcutaneous layer, the fresh periosteal layer was exposed and an additional incision was laid on the bone table. After elevating periosteum

with careful intention, two 8mm diameter cylindrical defects were made in both sides of the calvarium using a trephine bur while irrigating with saline. After copious saline irrigation to the defect site, the implant was placed into the defect, and layer-by-layer suture was performed (Figure 2). The periosteum was closed over the defect site using 4-0 vicryl, and the skin layer was closed with 3-0 Maxon. Intramuscular injection of Zoletil and intravenous injection of chloropotassium were done to sacrifice the animal on each prepared time point. After resecting the operated field by disc bur with saline irrigation, it was saved in 10% formalin.

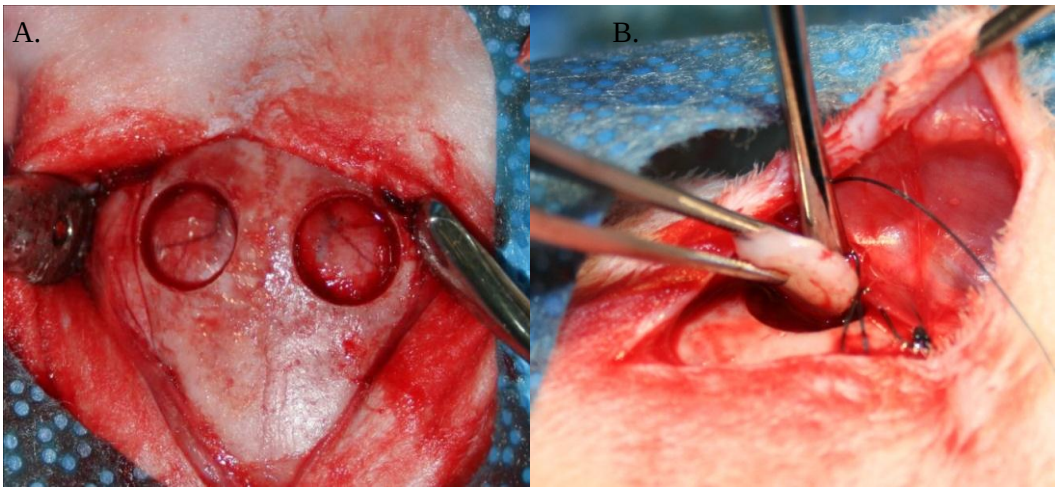


Figure 2. Surgical procedures. A. Two defects with 8mm diameter were made without damaging brain. B. Implant materials were laid in the defect while suturing on periosteum.

4. Micro CT analysis

Micro-computed tomography (micro CT) analysis was performed using Skyscan 1173 (Skyscan, Belgium). Acquired images were processed in Bitmap format and read by CTAnlyzer (Skyscan, Belgium) to observe three- dimensional structure. Bone density and volume were calculated and the spectrum of bone density was acquired by the software

5. Histological analysis

The bone pieces were then decalcified in 10 percent EDTA, pH 7 at room temperature, dehydrated in 70 percent ethanol, and embedded in paraffin. The paraffin-embedded sections (5 micrometers) were stained with hematoxylin eosin and viewed using microscopy. Digital images from stained sections were processed with SPOT version 4.5 (Diagnostic instrument Inc., U.S.A).

6. Statistical analysis

The bone mineral density and volume of regenerated bone were obtained and compared with each other. Statistical analysis was processed with SPSS 12.0 (SPSS, U.S.A) using t test. Critical p-value was 0.05.

III. RESULTS

1. Purity and endotoxin level of TAT-BMP-2 protein

The TAT-BMP-2 protein was eluted and purified by ATGen. The purity of sample 1 was 85% and its endotoxin level was 0.17EU/microgram while that of sample 2 was 90%, 1.6EU/microgram. The endotoxin level was decreased by serial dialysis and elution. Sample 1 was chosen to use in animal experiment due to its low toxicity

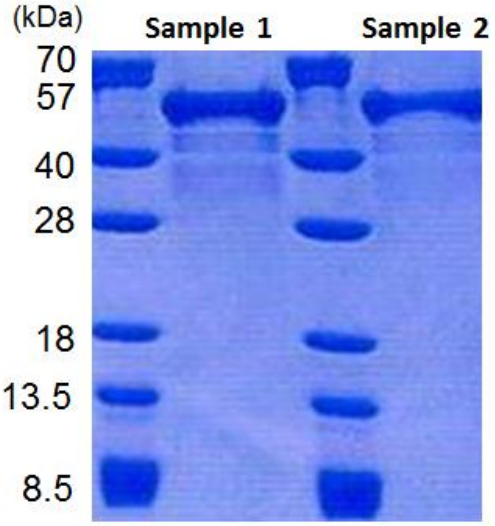


Figure 3. Gel image of eluted TAT-BMP-2 protein.

2. Micro CT analysis of regenerated bone

2.1. Control defects with no treatment

2.1.1. Four weeks result of control defects

There were three blank defects at 4 weeks which had no implanted biomaterials in the cylindrical defects, while their bone regeneration pattern was relatively constant without docking regenerated bone from the margin. Regeneration was seen mainly on the floor of the defect following the cerebral ventricle and on the margin of the defect toward the core of the defect (Figure 4).

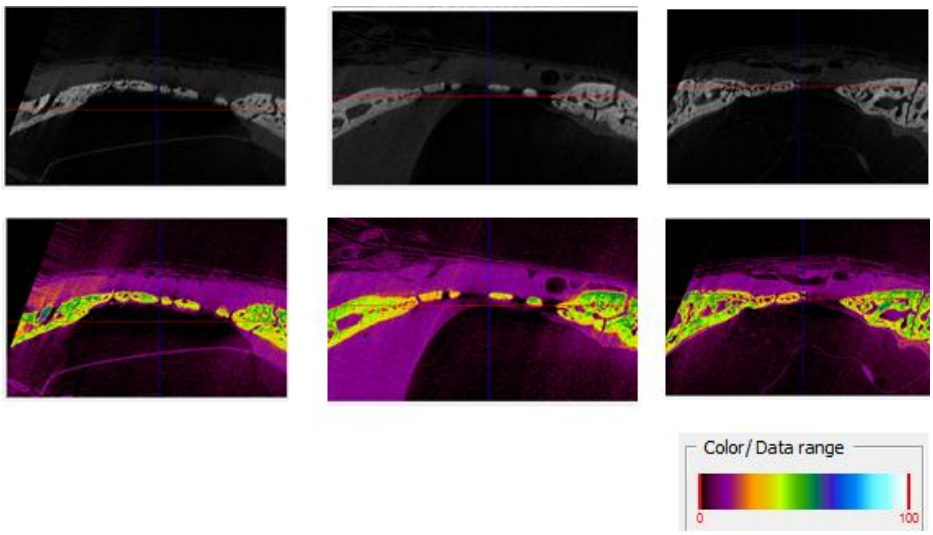


Figure 4. Micro CT images of three control defects at 4 weeks. The upper panel is of raw CT images, the lower panel is a view of the density gradient. Color key on right bottom represents the bone density.

2.1.2. Eight weeks result of control defects

There were three blank defects at 8 weeks, which showed comparably constant bone regeneration patterns. The amount of regenerated bone was little more than that of the 4 week samples, however, the docking of regenerated bone at the middle of the defect was not accomplished yet (Figure 5).

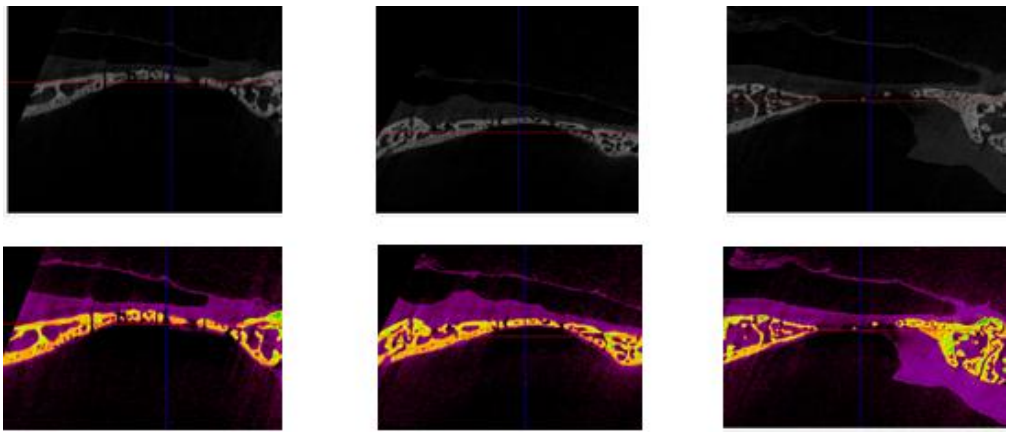


Figure 5. Micro CT images of three control defects at 8 weeks.

2.2. Defects filled with collagen

2.2.1. Four weeks result of collagen applied defects

There were no differences between one collagen applied defect and two collagen+TAT-BMP-2 5μg delivered defects. They all did not show docking of regenerated bone alike blank defects (Figure 6).

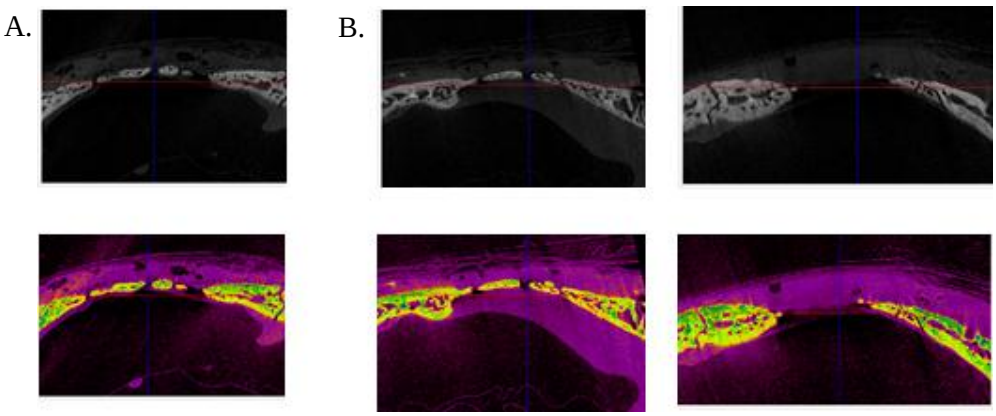
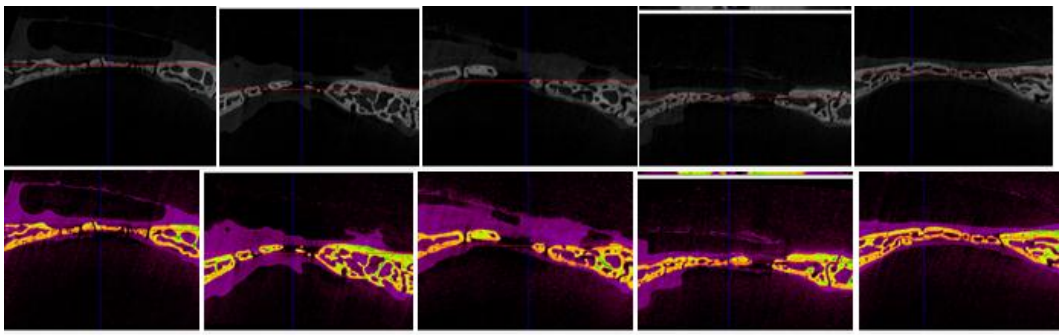


Figure 6. Micro CT images of collagen applied defects at 4 weeks. A. Collagen alone applied defect. B. Collagen+TAT-BMP-2 5μg applied defects.

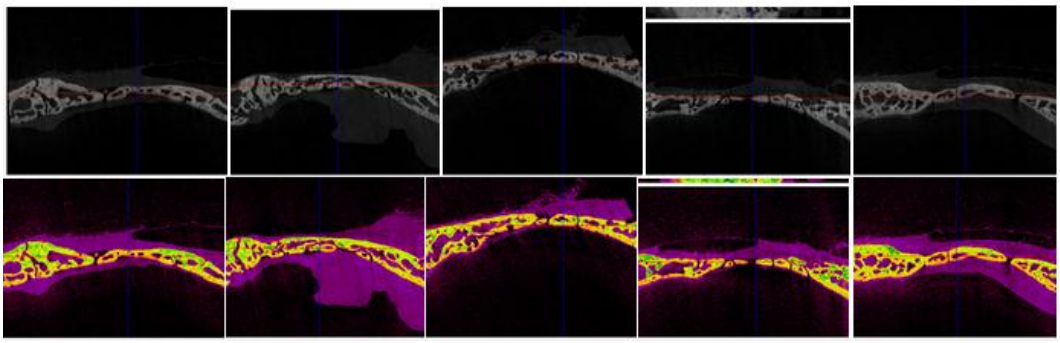
2.2.2. Eight weeks result of collagen applied defects

The TAT-BMP-2 applied defects demonstrated little more bone regeneration than collagen only applied defects due to their increased docking tendency. There was no significant difference in the result of bone regeneration following concentration of TAT-BMP-2 between 2μg and 5μg (Figure 7).

A.



B.



C.

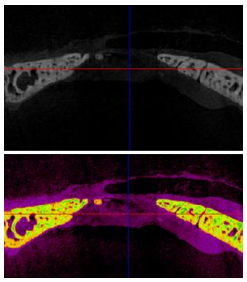


Figure 7. Micro CT images of collagen applied defects at 8 weeks. A. Collagen alone applied defects. B. Collagen+TAT-BMP-2 5μg applied defects. C. Collagen+TAT-BMP-2 2μg applied defect.

2.3. Defects filled with fibrin

2.3.1. Four weeks result of fibrin applied defects

There was almost no bone regeneration in the defects filled with fibrin. In spite of their biocompatibility and volume-maintaining capacity, almost no bone was grown from the margin of the defect. Although a little scattered bone regeneration was seen in defects with TAT-BMP-2 5μg delivered space, the effect of TAT-BMP-2 was not enough to induce active bone regeneration (Figure 8).

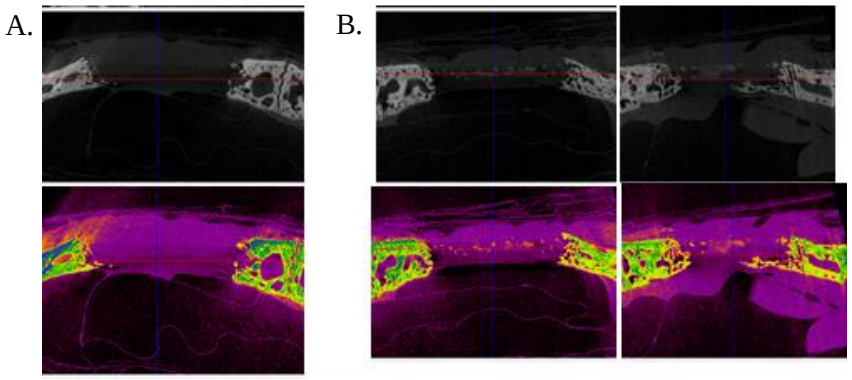
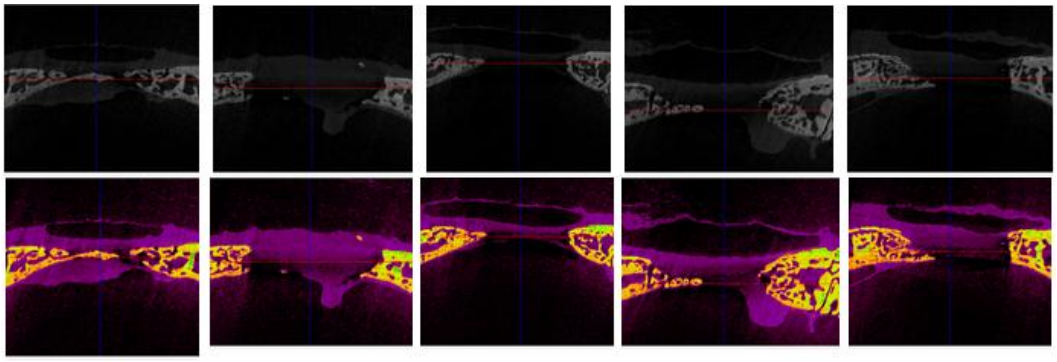


Figure 8. Micro CT images of fibrin applied defects at 4 weeks. A. Fibrin alone applied defect. B. Fibrin+TAT-BMP-2 5μg applied defects.

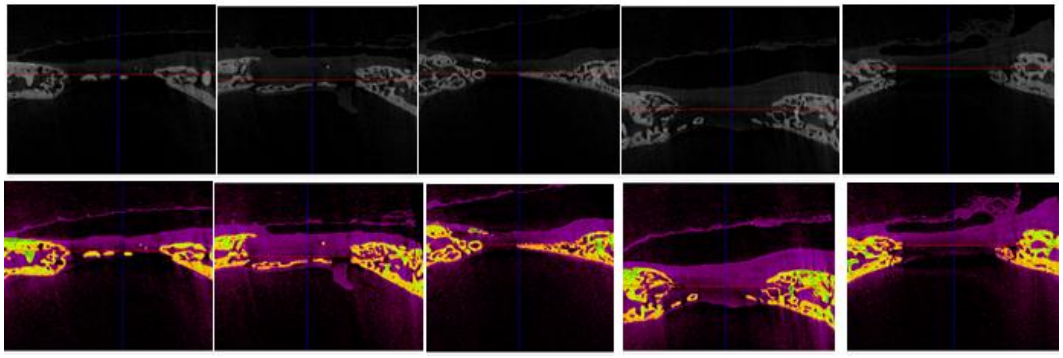
2.3.2. Eight weeks result of fibrin applied defects

Like the results of the 4 week sample, little regeneration pattern was seen in the fibrin applied defects at 8 weeks, showing some creeping bone growth patterns through the bottom of the defect. Though TAT-BMP-2 induced a little more bone regeneration than the fibrin only applied specimen, its amount was small and the structure of regenerated bone was irregular and scattered. There was also no significant difference in the result of bone regeneration following concentration of TAT-BMP-2 between 2μg and 5μg (Figure 9).

A.



B.



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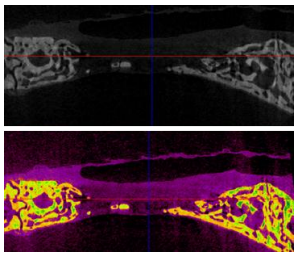


Figure 9. Micro CT images of fibrin applied defects at 8 weeks. A. Fibrin alone applied defect. B. Fibrin+TAT-BMP-2 5μg applied defects. C. Fibrin+TAT-BMP-2 2μg applied defect.

2.4. Defects filled with hydrogel

2.4.1. Four weeks result of hydrogel-applied defects

Notably, all three hydrogel scaffolds were expanded by hydration enough to elevate the periosteal and skin layer. In the defect with hydrogel-only applied, bone regeneration was observed following the cerebral ventricle with a creeping growth pattern, while the others with TAT-BMP-2 demonstrated more efficient regeneration not only at the bottom of the defect, but also around the periostium (Figure 10).

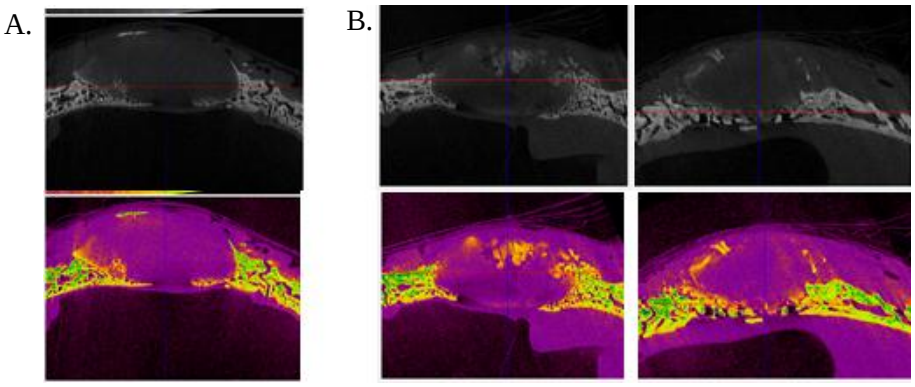
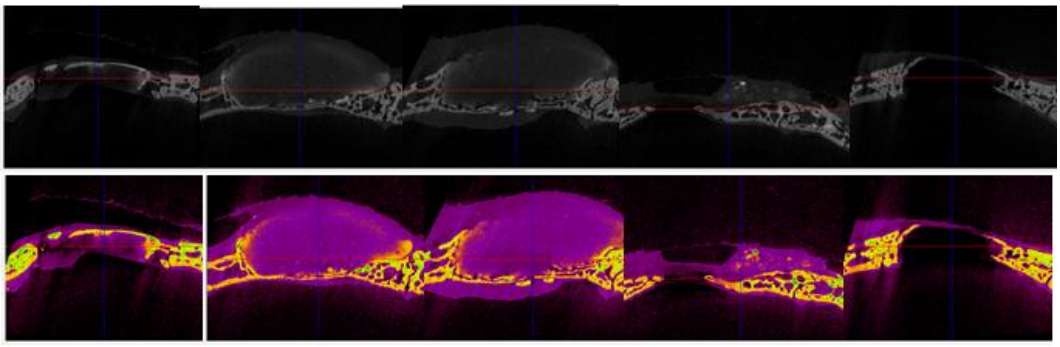


Figure 10. Micro CT images of hydrogel applied defects at 4 weeks. A. Hydrogel alone applied defect. B. Hydrogel+TAT-BMP-2 5μg applied defects.

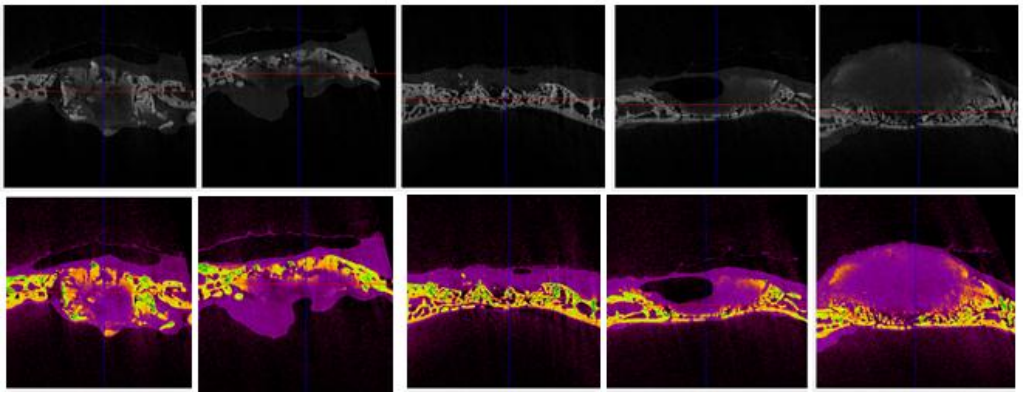
2.4.2. Eight weeks result of hydrogel-applied defects

Two of five defects filled with hydrogel only showed sustaining expanded hydrogel structure while the others showed almost resorption of scaffolds. The bone regeneration pattern of hydrogel-applied defects was going along with the cerebral ventricle accomplishing thin continuity of regenerated bone. TAT-BMP-2 induced more abundant bone regeneration, substituting much of the hydrogel space with mature bone structure. Specially, there was a significant difference in the result of bone regeneration following concentration of TAT-BMP-2 between 2μg and 5μg. The former induced bone regeneration of almost twice the efficiency (Figure 11).

A.



B.



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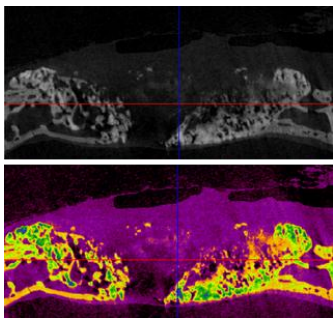

Figure 11. Micro CT images of hydrogel applied defects at 8 weeks. A. Hydrogel alone applied defect. B. Hydrogel+TAT-BMP-2 5μg applied defects. C. Hydrogel+TAT-BMP-2 2μg applied defect.

2.5. Quantified analysis of regenerated bone

2.5.1. Regenerated bone volume

At 4 weeks, regenerated bone volume was almost 50% superior in 5μg of TAT-BMP-2 and hydrogel composite than the other scaffold systems. In particular, the fibrin-applied defect showed an inferior regeneration to the blank defects. At 8 weeks, the regenerated bone volume was still almost 50% superior in 5μg TAT-BMP-2 and hydrogel composite than other scaffold systems while TAT-BMP-2 2μg applied defect demonstrated the most excellent regeneration efficiency (Figure 12).

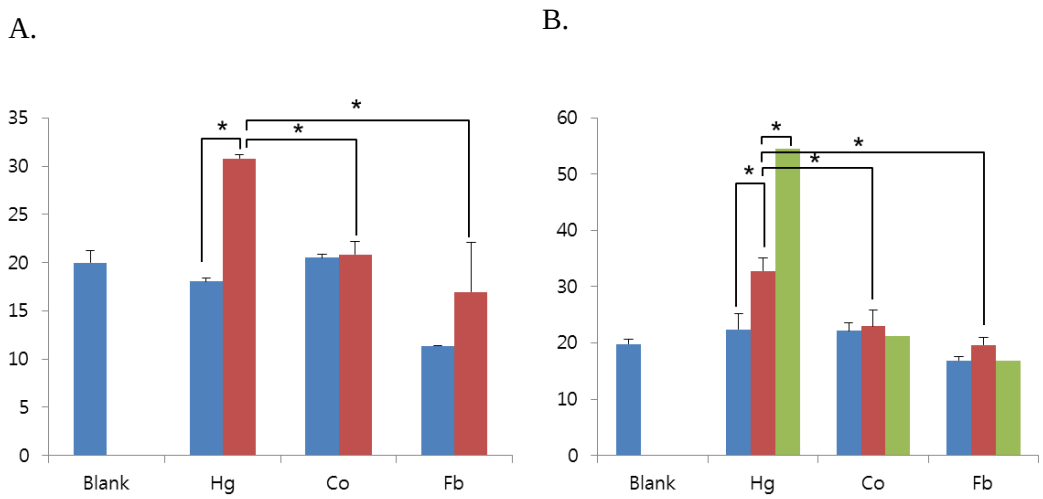

Figure 12. Quantified volume of regenerated bone. A. Result at 4 weeks, B. Result at 8 weeks. Blue bar means control and red bar is scaffold+TAT-BMP-2 5μg, green bar is scaffold+TAT-BMP-2 2μg.

2.5.2. Mineral density of regenerated bone

The mineral density of regenerated bone showed similar results with the volume of regenerated bone, indicating TAT-BMP-2 with hydrogel composite induced the most superior regeneration process (Figure 13).

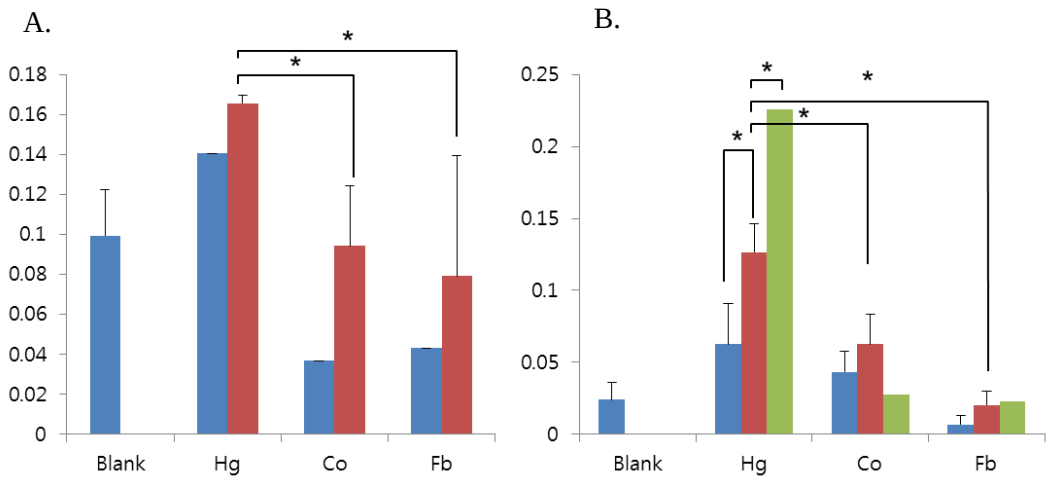

Figure 13. Quantified mineral density of regenerated bone. A. Result at 4 weeks, B. Result at 8 weeks. Blue bar means control and red bar is scaffold+TAT-BMP-2 5μg, green bar is scaffold+TAT-BMP-2 2μg.

3. Histological analysis

Decalcified hematoxylin-eosin stained slides demonstrated almost same result compared with micro CT. But there was a noticeable finding in histological analysis. In case of fibrin, the scaffold was stained red by eosin, which caused confusion regarding the red area of mature bone tissue. However, the high magnitude view proved its structure different from mature bone, being consistent with the view of micro CT images. Most of the space was filled with fibrous tissue to which fibrin was applied (Figure 14).

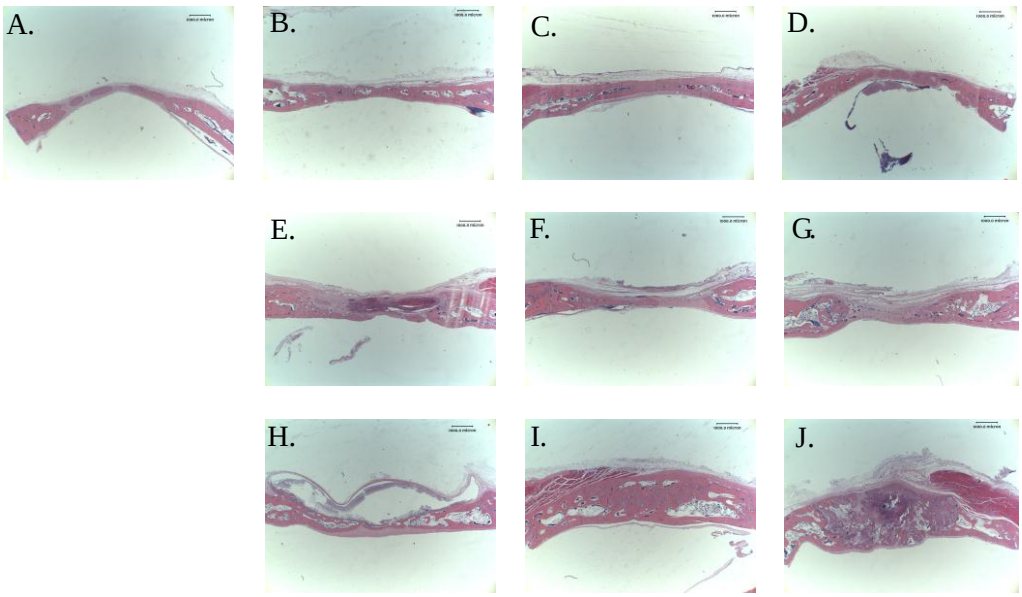

Figure 14. Histologic images of specimen. (X40), A. Blank. B. Collagen, C. Collgen+TAT-BMP-2 5μg, D. Collgen+TAT-BMP-2 2μg, E. Fibrin, F. Fibrin+TAT-BMP-2 5μg, G. Fibrin+TAT-BMP-2 2μg H. Hydrogel, I. Hydrogel+TAT-BMP-2 5μg, J. Hydrogel+TAT-BMP-2 2μg

4. Chemical residue analysis of hydrogel

Following the animal study, which showed TAT-BMP-2 hydrogel composite as the most proper drug-delivering system in the point of bone regeneration by secreted growth factors, further investigation of hydrogel itself was needed before applying it to further animal studies and preclinical trials. Several studies were performed to detect possible toxic chemical residues in the elution buffer of hydrogel.

4.1. ^{1}H NMR analysis

In ^{1}H NMR analysis, the possible residence of Acrylic acid, ADH, EDC, TCEP, HA-AC, and HA-TCEP in elution buffer of hydrogel were checked. Following the final results by ^{1}H NMR, hydrogel was proven to not involve any chemical residues that are harmful to humans when compared its own peaks with that of standard chemicals (Figure 15).

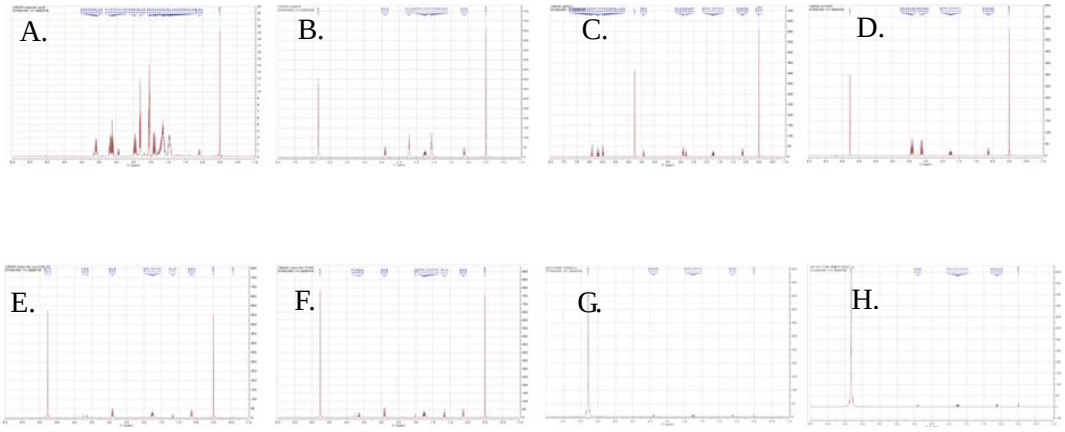

Figure 15. NMR spectrum of standard chemicals and hydrogel sample. A. Acrylic acid, B. ADH, C. EDC, D. TCEP, E. low HA-AC, F. low HA-TCEP, G. DDW, H. Hydrogel sample.

4.2. HPLC analysis

HPLC was done to detect Acrylic acid and ADH. The sample of hydrogel elution buffer did not contain those chemicals following the results of HPLC (Figure 16).

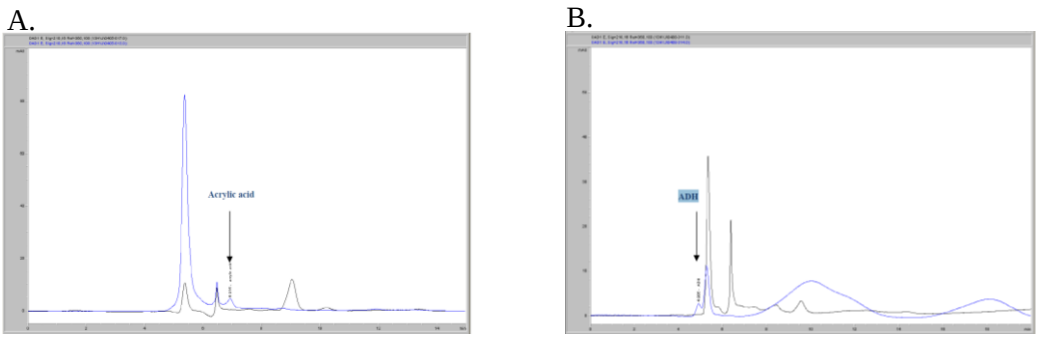

Figure 16. HPLC result of hydrogel sample. A. Comparing with standard Acrylic acid. B. Comparing with standard ADH. The blue curve means standard while the black curve represents the result of the sample.

4.3. GC/MS analysis

When GC/MS was performed to elucidate the residence of EDC and TCEP in the hydrogel elution buffer, EDC 34ppm, TCEP 574ppm were detected (Figure 17) (Table 2,3,4).

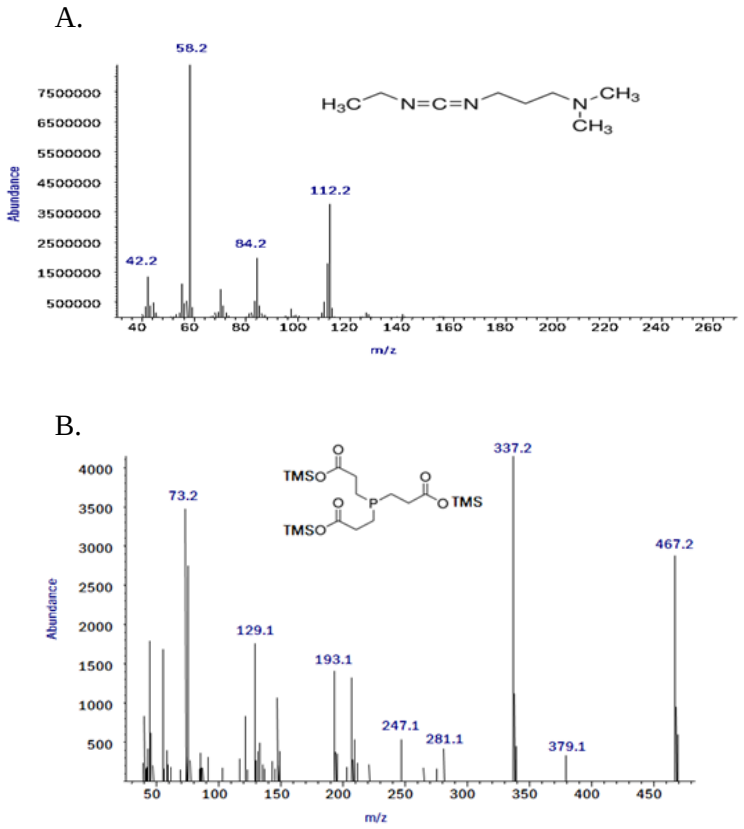

Figure 17. GC/MS results of standard chemicals. A. EDC, B. TCEP

Table 2. Retention time and characteristic ions.

Compound	Retention time (min)	Characteristic ions (m/z)	
		Quantification ion	Confirmation ion
EDC	8.98	58	112
TCEP	22.97	337	467

Table 3. Equation of calibration curve.

Compound	Equation	Linear coefficient
EDC	y=15179x+1455.2	0.9981
TCEP	y=315.81x-314.1	0.9996

Table 4. Quantification of compound in hydrogel samples

Compound	LD* (μg)	Hydrogel sample	
		Area	Concentration (μg/mL)
EDC	0.01	6650	0.34
TCEP	0.05	1499	5.74

* LD : lowest detected amount in calibration curve

IV. DISCUSSION

The BMPs are pleiotropic growth factors playing a pivotal role in a wide range of physiological processes including osteogenesis, adipogenesis, angiogenesis, renal regeneration, and stem cell differentiation (Lo et al., 2012). The MoA (mode of action) of current BMPs and cytokines, regardless of whether they are produced from mammalian cells or bacterial expression, are inevitably soluble maintaining proper tertiary protein structure to directly bind its cognate receptors. However, considering physiologic regeneration processes in clinical settings, the cytokine or growth factors are required for several weeks to support a regeneration cascade. For instance, the half-life of soluble BMP-2 is about 6.7 min in primates due to the enzymatic degradation and rapid clearance rate by body fluids (Poynton and Lane, 2002; Seeherman and Wozney, 2005; Seeherman et al., 2004). To increase the effectiveness with a sustained release of soluble growth factors, numerous studies have suggested variable biocompatible carriers, or scaffolds, though the materials for overcoming the initial burst release of pharmacokinetic profiles and proteolytic degradation during the regeneration process are very limited. Supra-physiological concentrations resulting from imperfect release kinetics of BMPs where 30% of the encapsulate is lost in the initial bust phase (Geiger et al., 2003) are additionally being related to severe clinical complications, including generalized hematomas in soft tissue and para-implant bone resorption (Mroz et al., 2010).

The aim of this study was to find the most appropriate biocompatible, biodegradable scaffold system that delivers hydrophobic TAT-BMP-2 safely in-vivo. Collagen is a

widely used biomaterial that shows superior biocompatibility. But, collagen is unable to maintain its volume for more than 2 weeks in-vivo regardless of its form (Seeherman and Wozney, 2005). Though several products that deliver rhBMP-2 in-vivo are applied mainly with collagen, collagen's burst releasing pharmacokinetics make it impossible to sustain a long enough period for releasing growth factors in a controllable manner. In this study, which observed 4- and 8-week periods after graft surgery, the collagen-applied defects, whether being combined with TAT-BMP-2 or not, did not show any significant difference when compared with blank defects.

Fibrin is mainly used for hemostasis during the last step of open surgery to avoid congestion by micro bleeding. Several in-vivo studies demonstrated successful bone regeneration by using fibrin as a scaffold system (Chung et al., 2007). However, although it has good biocompatibility, fibrin did not induce bone regeneration in this experiment even when it was combine with TAT-BMP-2. Though the exact reason of this phenomenon is unclear, it is certain that at least TAT-BMP-2 does not get along with fibrin to deliver the growth factor in-vivo.

Hyaluronic acid(HA) was chosen as the base matrix for delivering growth factors due to its demonstrated potential as a scaffold material. HA is a naturally occurring, hydrophilic, non-immunogenic glycosaminoglycan. HA is concerned with several biologic processes such as morphogenesis, scar healing processes, wound healing, and the early stage of bone healing process (Gandhi et al., 2006; Gruber et al., 2006; Lu et al., 2008; Lynch et al., 2008). Like other biocompatible and degradable scaffolds such as fibrin and collagen, HA is composed of naturally derived materials. In this study, HA was investigated for the scaffold of TAT-BMP-2 in-vivo, and exhibited excellent efficacy in bone regeneration showing

50% superior results than hydrogel-alone-applied results. In particular, several kinds of hydrogel are able to be manufactured by diverse recipes and are also controllable for releasing bound growth factors during degradation. Following the integrated information from animal experiments and analysis of hydrogel property, hydrogel is the most appropriate scaffold to deliver TAT-BMP-2 in-vivo. However, detected chemical residues such as EDC and TCEP need to be removed by a more intensive serial dialysis, and more works related in the safety of hydrogel should be done in next study.

A proper scaffold, such as hydrogel, provides significant advantages when compared to the current delivery of secreted growth factors, especially in clinical settings. First, our strategy provides the sustained delivery of secreted growth factors, as well as overcomes the initial burst release of secreted growth factors, because the release of TAT-fusion polypeptides to the patient's own progenitor cells is only dependent on the absorption or degradation of the scaffold materials.

Second, maintaining biological activity of the secreted protein during the regeneration period, usually for several weeks, is the challenging issue in clinical trials. Our PTD-mediated approach with denatured proteins does not require the consideration for maintaining a biological activity with a proper tertiary structure and loss of biological activity due to proteolytic degradation during the regeneration period, providing diverse options for clinical application. Indeed, many of the embedded polymer biomaterials have been developed to allow injectable application and to overcome initial bursts of BMP soaked collagen sponges or ceramics. However, it should be noted that these methods involve exposing soluble growth factors to solvents and acidic conditions that disrupt the protein structure and biological activity.

Third, the current growth factors, even with a well-designed carrier, are required for supraphysiological protein loading of the proteins due to their pharmacokinetics. The overdoses give rise complications such as edema, angiogenesis, and bone formation on an undesired site. For instance, mg scale of BMP-2, which is about one million times found endogenously, is often applied to induce osteogenic effects in mammals, suggesting that only a few fractions of applied BMPs can reach their biological functions. In our model, we showed successful osteogenic effects with μg scale of TAT-BMP-2, indicating that an indirect delivery strategy is more potent by about 1,000 times when compared to conventional soluble growth factors, especially in-vivo.

Forth, the cost-effectiveness became an emerging issue in growth factor therapy. Although bacterial recombinant proteins provide economic advantages when compared to purification procedures from the mammalian cell culture, high-level expression of the protein in E. coli results in accumulating the major fraction of expressed protein as insoluble aggregates in inclusion bodies. Concerning the procedures for the renaturation of inclusion bodies, refolding, purification, and maintaining tertiary structure make up the major costs of the soluble recombinant proteins, while our PTD-mediated denatured protein does not required those processes. The high cost-effectiveness would be more meaningful on trials for large internal organs, such as the kidney, liver and lungs in humans.

Lastly, due to a novel MoA of our technique, the platform is easily extended to other secreted growth factors. As the EMT controlled by TGF-β and Wnt/Snail axis involved in many types of degenerative disorder, chronic inflammatory disease, and cancer, our platform can be adopted easily to deliver antagonists of TGF-β and Wnt signaling.

V. CONCLUSION

1. The bone regeneration efficacy of hydrogel and collagen were in themselves comparable.

2. 5μg of the TAT-BMP-2 and hydrogel composite induced 50% more efficient bone regeneration than blank, collagen and fibrin. Also it exhibited the most superior bone mineral density.

3. Fibrin seemed to inhibit osteoinduction. Furthermore, the TAT-BMP-2 fibrin composite did not demonstrate significant bone regeneration compared to control or other experimental groups.

4. The concentration dependency of the TAT-BMP-2 and hydrogel composite in bone regeneration needs further investigation.

5. The elution buffer of hydrogel contained EDC and TCEP, which needs to be removed by serial dialysis.

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ABSTRACT (IN KOREAN)

TAT-BMP의 생체 내 적용을 위한 전달체 발굴 및 평가

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노령화 사회로 진행됨에 따라 골 조직 재생 술식의 필요성은 점차 증가하고 있으나 현재까지 명확한 해결책은 부족한 실정이다. 외인성 골 형성 유도물질이 효과적으로 작용하기 위해서는 충분한 생체활성을 지녀야 하며 재료의 방출을 조절할 수 있는 전달체와 생물학적, 화학적, 기계적 조화를 이루어야 한다. 생물학적 골 재생을 위해 재조합골형성단백질 (rhBMP)가 임상적으로 시도되고 있으나. 낮은 효율성을 보이는 것으로 보고되고 있다.

TAT-BMP는 기존의 수용성 BMP-2와는 달리 hydrophobic한 특성을 가지기 때문에 전임상 단계에서의 생체 내 적용 및 나아가 임상 적용을 위해서 적절한 전달체와 결합하는 것이 중요하다. 이에 본 연구에서는 생체적합성과 생분해성이 뛰어난 세 가지 전달체 (콜라겐, 피브린, 하이드로겔)를 TAT-BMP와 결합하여 가토 두개골에서의 골 재생 효과를 관찰하였다. 또한 골 재생 효과가 가장 우수하였던 하이드로겔의 임상 적용을 위해 잔류화합물을 평가하였으며 다음과 같은 결과를 얻었다.

1. 전달체만 적용하였을 때 하이드로겔과 콜라겐은 비슷한 골 재생 효과를 나타낸다.
2. TAT-BMP와 결합하였을 때 하이드로겔의 골 재생 효과 및 골 성숙도가 다른 전달체에 비해 50% 가량 우수한 것으로 관찰되었다.
3. 피브린은 전달체만 적용할 경우 골 재생을 억제하는 것으로 보이며 TAT-BMP와 결합하여도 뚜렷한 골 형성 유도능을 보이지 못한다.
4. TAT-BMP와 하이드로겔 복합체의 농도 의존성 골 형성 능력에 대해서는 추가적인 연구가 필요하다.
5. 하이드로겔의 용출액에 남아있는 잔류 화합물을 분석했을 때 EDC, TCEP가 미량 검출 되었다.

이상의 결과에서 TAT-BMP와 하이드로겔 복합체가 생체 내에서 가장 효과적인 골 재생을 유도하는 것을 알 수 있으며, 임상 적용을 위해서는 추가적인 투석 과정 등을 통해 잔류 화합물을 제거할 필요가 있을 것으로 사료된다.

핵심어 : 단백질 전달영역, HIV-1 TAT, 골형성단백질, 하이드로겔