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博 士 論 文

The osteogenic potential of Phosphorylated-Pullulan/β-TCP composite scaffolds and low doses of Bone Morphogenetic Protein-2 (BMP-2) in subcutaneous tissues

リン酸化プルラン/β-TCP 複合スキャホールドと低用量骨形成タンパク質 2 による骨形成

令和 5 年 3 月申請

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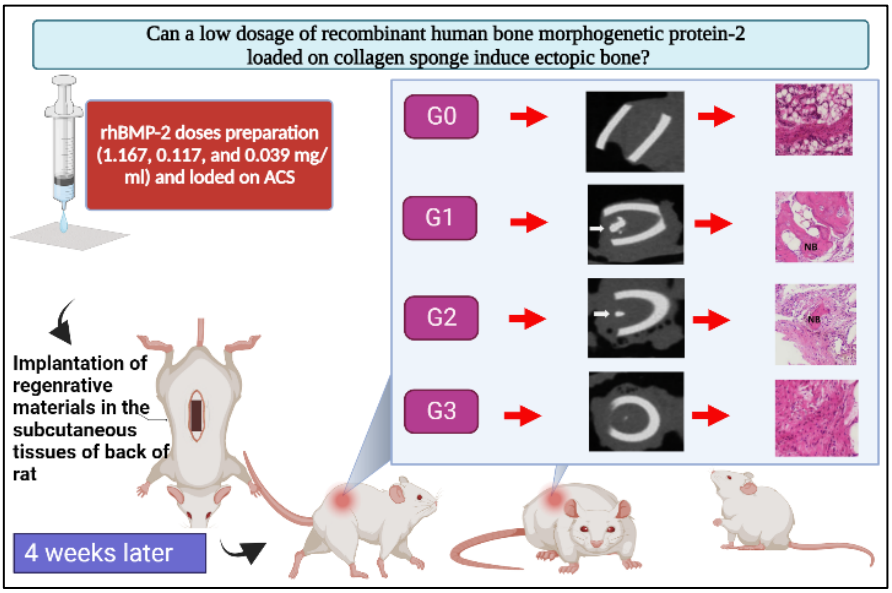
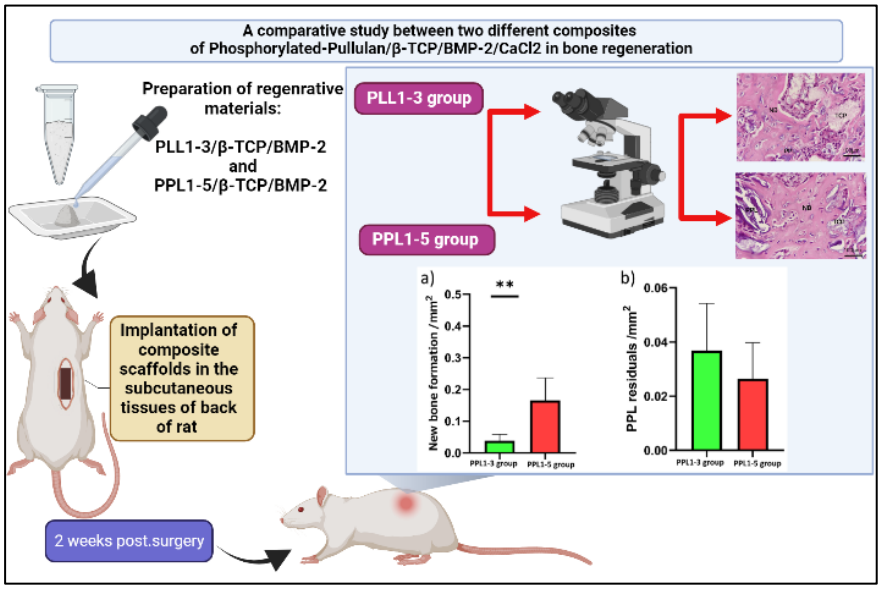
Abstract

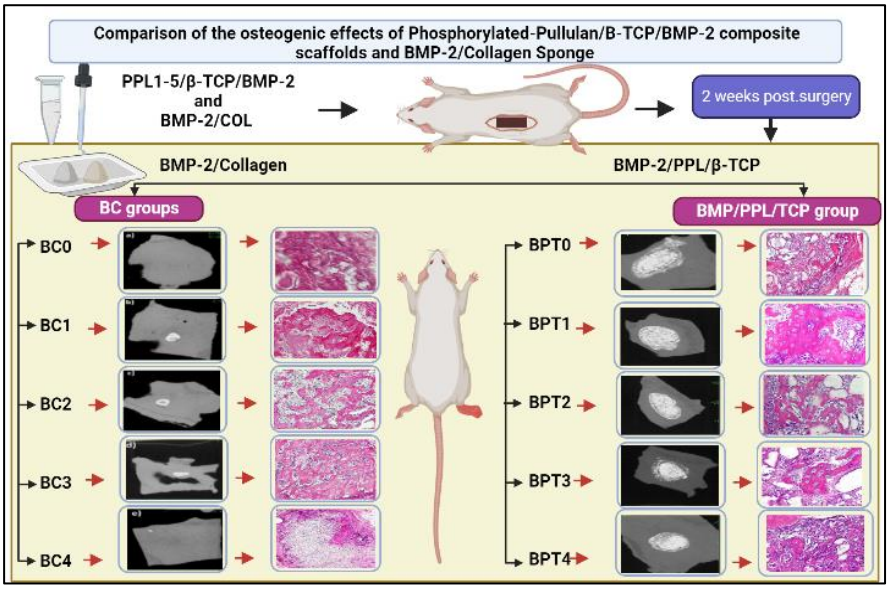
Recombinant human bone morphogenetic protein-2 (rhBMP-2) is one of the growth factors that may induce the formation of new bone. It is documented that for rhBMP-2 to be clinically effective, high doses must be administered, which may increase the cost of the treatment and its adverse effects. The aim was to determine the efficacy of low dosages of rhBMP-2 for bone regeneration using a collagen sponge as a carrier. Three doses of rhBMP-2 (1.167, 0.117, and 0.039 mg/ml) were combined with an absorbable collagen sponge (ACS) as a delivery vehicle. The rhBMP-2/ACS implants were placed in the subcutaneous tissues of a rat back. X-ray microcomputed tomography (micro-CT) and histological analysis were used to evaluate bone formation. The samples treated with 1.167 mg/ml of rhBMP-2 showed greater bone formation than the samples treated with 0.117 mg/ml of rhBMP-2 four weeks after surgery. However, there was no evidence of bone formation in the third experimental group. It was found that rhBMP-2 was osteogenic even at one-tenth of its recommended concentration, indicating its potential for clinical use at lower concentrations. (First study)



Bone engineering has been used extensively for the treatment of bone defects caused by traumatic injuries or periodontal diseases. Recently, phosphorylated Pullulan (PPL) has been discovered in the field, demonstrating high biocompatibility and an outstanding ability to induce bone formation. However, the lower the molecular weight of PPL, the faster the biodegradation rate, and that creates more space for bone formation. Hence the current study aimed to evaluate and compare histologically the osteogenic potential and biodegrading rate of two synthesized composite scaffolds of PPL/β-TCP/BMP-2 /CaCl2 with different phosphorylated pullulan molecular weights. Two mixtures of PPL MW have been created PPL1(600 000 MW of PPL) and PPL2(600 000 + 100 0000 MW of PPL). The ratio of PLL1 to PPL2 is 1:3 in (the PLL1-3 group) and 1:5 in (the PLL1-5 group). Further, both groups were subsequently mixed with β-TCP, BMP-2, and CaCl2 and implanted in the subcutaneous tissues of a rat back. Histological analysis has been performed to evaluate bone formation in vivo. Our findings revealed that both groups showed extensive areas of newly formed bone with evident marrow tissues, osteocytes, and osteoblasts at 2 weeks post-surgery. There was a statistically significant difference ($P<0.05$) between the PLL1-3 group and the PLL1-5 group with larger areas of newly formed bone associated with the PLL1-5 group. Both groups had large amounts of PPL residuals, whereas PLL1-3 had a higher amount with no significant difference. (In PPL1-3 group it was 0.036±0.017 mm², and PPL1-5 group it was 0.026±0.013 mm²) We concluded that the PPL1-5 group may be considered to efficiently enhance bone formation with a good biodegradation rate. (Second study)



The current study aimed to evaluate the osteogenic potential of the synthesized composite scaffold (PPL1-5) and rhBMP-2/ACS scaffold histologically and radiographically, as well as to reduce the BMP concentration when PPL1-5 was used as a carrier other than ACS in ectopic sites. Four doses of BMP-2 (1.167, 0.117, 0.039, and 0.023 mg/ml) were used in both experiments. The regenerative materials were implanted in the subcutaneous tissues of a rat's back. X-ray microcomputed tomography (micro-CT) and histological analysis were used to evaluate the bone formation. Bone formation was observed in 2 weeks post-surgery in all groups of samples except the samples that were treated with collagen membrane only (BC0). Moreover, the samples treated with combined BMP-2/PPL/β-TCP composite scaffolds displayed greater bone formation than the samples treated with BMP-2/ACS and the difference was statistically significant (P-values <0.05). The newly formed bone had obvious osteocytes and marrow tissues with abundant osteoblasts forming a line at the periphery. We concluded that greater bone formation was induced by the BMP/PPL/β-TCP composite scaffolds than by BMP-2/COL implant materials with less BMP-2 concentration. (Third study)



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CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

Regenerative therapy has offered a viable and safer alternative to conventional autograft for the treatment of bone defects, eliminating donor site morbidity [1,2]. Ideal requirement of bone graft includes high biocompatibility, adequate mechanical support, ostoinductive, osteoconductive and easy in handling. It should also be sterilizable and biodegradable. [3,4].

Bone substitutes can be classified into three main categories: bone grafts (autograft, allograft, xenograft), ceramics (hydroxyapatite, tricalcium phosphate, calcium sulfate) and growth factors (bone morphogenic protein, demineralized bone matrix, platelet-derived growth factor) [5].

Autograft is still considered the gold standard due to its excellent osteogenesis, osteoconductivity, osteoinductivity and absence of associated immunological complications as well as low risk of disease transmission [6,7]. It involves using bone tissues from the same individual who received the bone graft. It can be harvested from the iliac crest, the fibula, the chin, the ribs, and the mandible [8] . Nevertheless, this type of graft is associated with significant donor site morbidity and pain as well as risk of injury to blood vessels and nerves [9,10].

Allograft is a human-derived graft that is considered the main alternative of autograft. It lacks osteogenesis properties because it has no viable bone cells, like the autografts [11].There are three main forms of allografts: fresh or fresh-frozen allograft, freeze-dried bone allograft (FDBA), and demineralized freeze-dried bone allograft (DFDBA). Allografts provide excellent osteoconductive property with some osteoinductive factors in DFDBA and fresh frozen allograft [12,13]. However, such material needs extensive preparation and sterilization, which subsequently increases the material's cost [14]. At the same time, it has been associated with high risk of infectious diseases transmission such as human immunodeficiency virus (HIV) and Hepatitis B and C [15] .

Xenogeneic bone graft is obtained from a species that is genetically different to the host and most commonly bovine bone (such as Bio-Oss) It has porous in structure that resemble to the human bone [16]. Furthermore, it promotes bone regeneration through

osteoconductive process [17]. In addition, bovine xenogeneic bone substitutes have hydroxyapatite components that are comparable to those present in human bone that may improve the success rate of bone grafting procedure [18]. Nevertheless, there are some limitations related the use of xenografts include increased resorption rates, with lack of ostoinductive factors [19] and some studies have reported some graft rejections cases. [20-22].

Different Alloplastic bone substitutes have been developed to eliminate possible immunogenicity and morbidity at donor sites. Nevertheless, these artificial materials demonstrated most of the fundamental properties of bone substitutes, including biocompatibility, osteoconductive, and sometimes osteoinductivity [23,24].These materials such as hydroxyapatite (HA), tricalcium phosphate (TCP), and polymers, such as polymethylmethacrylate (PMMA) [15]. Tricalcium phosphate (CP) has been widely used for bone regeneration due to its similarity to bone composition [25]. TCP exists in three polymorphs: β-TCP at low temperatures, α- TCP and α'-TCP at high temperature. The third one is not usually applicable since it is only stable at temperatures over 1430 °C [26,27]. β-TCP is most used in clinical practice as a biodegradable material that promotes bone regeneration due to its superior stability, biocompatibility, and ease in shaping [23,28-30]. It has a solubility that is comparable to bone minerals [31] and composed of porous that facilitates, vascularization, and bone formation [26,32]. Unfortunately, it has been observed that β-TCP is brittle with low tensile and shear strength [33,34], as well as demonstrating a slow absorption rate in approximately 13 to 20 months [23,35].

Since most of bioceramics bone substitutes and synthetic cements, lack of ostoinductive properties and depend mainly on osteoconductive methods [11], utilizing of growth factors, especially recombinant bone morphogenetic protein-2 (rhBMP-2). rhBMP-2 is thought to be essential for inducing bone formation and enhancing the healing of bone defect [36]. It is associated with osteoblast proliferation, differentiation into osteogenic and chondrogenic stem cells, and migration of osteoblast progenitor cells **[37]**. It has the potential to induce bone regeneration in bony and non-bony areas, a process known as osteoinduction [38,39]. Research has shown that rhBMP-2 causes mesenchymal cells to differentiate into osteoblasts when it is implanted into soft tissues with a suitable carrier material [40-42]. However, a collagen sponge is regarded as the most common delivery vehicle for rhBMP-2 due to its excellent compatibility and biodegradability [43-45].

Binding studies showed that rhBMP-2 binding to the sponge was greater and influenced by the pH. This effect may be explained by the differences in the isoelectric points of the two proteins (collagen and rhBMP-2). Depending on the manufacturing process, collagen exhibits an isoelectric point in the neutral or slightly acidic pH range [46]. Moreover, collagen sponge has a long safety history as hemostatic agents and wound coverings , as well as it is being studied as a scaffold in the emerging field of tissue engineering [47].

On other hand, rhBMP-2 has inadequate retention on the collagen sponge and might lead to clearance of rhBMP-2 in the tissue rather than induction of surrounding mesenchymal cells [48]. An experimental study examined the retention of rhBMP-2 on an absorbable collagen sponge (ACS) and found that only 70% of rhBMP-2 was retained on the collagen on the first day of surgery and then gradually decreased till day 14 [49]. However, the more rhBMP-2 is retained, the more it is effective [50,51].

Recombinant human bone morphogenetic protein-2 (rhBMP-2) is manufactured by Medtronic (INFUSE® bone graft, Medtronic, Dublin, Ireland). The United States Food and Drug Administration (FDA) approved the use of rhBMP-2 in spine surgery, tibial fracture surgery, and dental surgery, including sinus grafting and localized alveolar ridge regeneration [52-54].

However, in 2008, the FDA published serious complications related to the use of INFUSE® bone graft in the cervical spine, including dysphagia and swelling of soft tissues [53,54]. Despite the potential of rhBMP-2 to induce new bone formation, it also enhances osteoclast activity, which may lead to bone resorption [36,55]. It has been reported that, in order for rhBMP-2 to be clinically successful, it must be administered in large dosages, which may increase the cost of the procedure and the material's side effects [56]. The amount of bone formation is highly dependent on the rhBMP-2 dosage [57]. However, overdosage of rhBMP-2 of rhBMP-2 in humans coupled with a quick release from the delivery vehicle could lead to serious complications such as ectopic bone formation within the spinal cord [58], increased bone resorption in the body due to overactivation of osteoclasts [59], formation of cyst-like bone voids, and cervical swelling [60]

In recent years, the field of bone tissue engineering has been oriented toward the use of natural polymers such as collagen and pullulan which demonstrated biocompatibility, biodegradability, and some resemblance to organic extracellular matrix [61,62]. Pullulan

is a polysaccharide made up of three glucose units that are bonded together by -(1,4) glycosidic bonds (maltotriose) and these units are bounded together by -1,6 glycosidic bonds [63]. It is naturally produced by a yeast-like fungus called Aureobasidium pullulans. [64-66] Pullulan has several applications, including those in nutrition and biomedicine. In last decade pullulan and its derivatives showed proven outcomes in drug delivery system field due to its biocompatibility, high solubility and biodegradability [67]. It could be chemically modified by a variety of chemical methods to enhance its significance in biomedicine [68]. Pullulan, on the other hand, has fundamental limitations such as brittleness, and low water resistance.

In most recent years, a group of researchers discovered a unique pullulan derivative known as phosphorylated pullulan (PPL) [69]. It has distinct ability to chemically bond with the surrounding bone tissues by substituting its phosphate groups with the hard tissue's hydroxide groups [70]. Moreover, phosphorylated pullulan has outstanding properties including high biocompatibility, superior mechanical strength, and ability to degrade biologically. This makes PPL a highly promising material for bone regeneration [71]. PPL when combined with β-TCP showed excellent results in terms of bone regeneration and mechanical compressive strength [70]. The potential to form bone and the duration of biodegradation of a material are strongly correlated, in which faster biodegradation creates earlier rooms for new bone formation. Morimoto et al, stated that the lower molecular weight of PPL, the faster the degradation leading to greater bone formation [72].

In contrast, composite materials appear to be the best for clinical applications since all the characteristics are hardly present in a single material.

1.2 Problem statement and aims of the work

1. It is documented that for rhBMP-2 to be clinically effective, high doses must be administered, which may increase the cost of the treatment and its adverse effects [56]. However, high dosages of rhBMP-2 in humans may result in serious complications and may be life-threating. Therefore, taking into consideration of safety and cost factor of rhBMP-2, we aimed to evaluate the lower limit concentration of rhBMP-2

that can be osteogenic incorporated into collagen sponge as a carrier and analyzing the residual collagen sponge. (Chapter 2)

2. It has been stated previously that the potential to form bone and the duration of biodegradation of a material are strongly correlated, in which faster biodegradation creates earlier spaces for new bone formation. The lower the molecular weight of PPL, the faster the degradation leading to greater bone formation [72]. Furthermore, in our previous study (Chapter 2), we concluded that a lower-than-recommended dose of BMP-2 (0.117 mg/ml) can be osteogenic. However, the amount of induced newly formed bone is significantly lower than that induced by the recommended dose (1.167 mg/ml). This may be attributed to the lower retention of BMP-2 on absorbable collagen sponge (ACS) cells [48,49] as well as ACS has low mechanical strength that may have a substantial impact on the osteoinduction process. Therefore, to address the limitations of BMP-2 and improve the osteogenic impact of the scaffolds we synthesized two composite scaffolds of BMP-2, β-TCP and CaCl2 with utilizing PPL of two different molecular weights as a delivery vehicle. The study aimed to evaluate and compare histologically the osteogenic potential and biodegrading rates of these two synthesized composite scaffolds of PPL/β-TCP/BMP-2/CaCl2 of different phosphorylated pullulan molecular weights. (Chapter 3)

3. In our previous study (Chapter 3), PPL1-5 group samples received PPL molecular weight (MW) mixtures, which can induce greater osteogenesis and a higher biological biodegradation rate of PPL than PPL1-3 group samples. This can be attributed to the fact that the PPL1-5 group utilized a combination of larger and smaller MW of PPL, with a higher proportion of larger MW of PPL. Moreover, higher MW of PPL promotes more bone regeneration, whereas lower MW of PPL promotes faster absorption, generating spaces for newly formed bone deposition. However, to confirm these findings further we created composite scaffolds of PPL1-5, β-TCP, and four different concentrations (1.167, 0.117, 0.039, and 0.023 mg/ml) of BMP-2. We also prepared BMP-2 in the same four concentrations utilized in the scaffolds and loaded them onto absorbable collagen sponges (ACS). The objective of this study was to examine the osteogenic potential of the synthesized composite scaffold (PPL1-5) and

rhBMP-2/ACS scaffold histologically and radiographically, as well as to minimize the BMP concentration when PPL1-5 was utilized as a carrier other than ACS. (Chapter 4)

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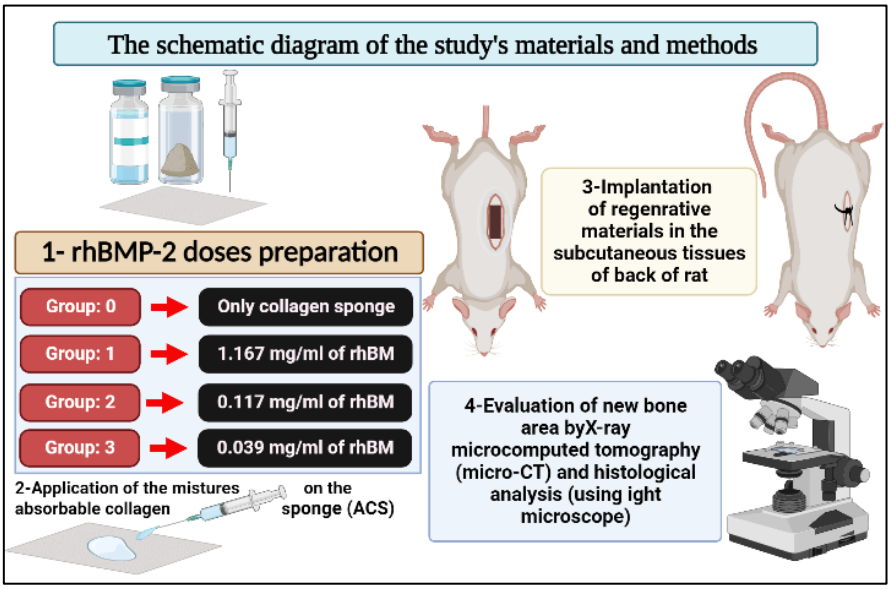
CHAPTER 2: INDUCTION OF ECTOPIC BONE BY A LOW DOSAGE OF RECOMBINANT HU-MAN BONE MORPHOGENETIC PROTEIN-2 LOADED ON COLLAGEN SPONGE INTRODUCTION

2.1 Materials and Methods



The animals were prepared following the animal experimental protocol of the institutional animal use and care regulation of Hokkaido University (Animal research committee of Hokkaido University, approval number 19-0128). Ten-week-old, male Wistar rats, weighing 180-210 gm, were selected. Ten rats were included in the study.

2.3.1 Preparation of implanting materials:

Preparation and dosage of rhBMP-2: Bone graft material was prepared from rhBMP-2 (INFUSE® Bone Graft 7510050XX Small Kit, Lot. MCM6723AAA, Medtronic, Dublin, Ireland). The rhBMP-2 was incorporated into an ACS of 1.25×5.08×0.4 cm3 in size. Furthermore, based on the manufacturer's guidelines, each collagen sponge of this standard size was injected with 0.7 ml of rhBMP-2 solution. However, in the current study, the same size of ACS was divided into 20 pieces, and thus 0.035 ml of solution was used for each piece of ACS.

Group allocation & categorization: Four groups were categorized based on the rhBMP-2 concentration. According to the manufacturer's instructions, 0.9 ml of sterile water was mixed with 1.05 mg of rhBMP-2 powder to form 1.167 mg/ml of rhBMP-2 (group 1). In Group 2, the dose of rhBMP-2 was Group 1 rhBMP-2 dose/10 or 0.117 mg/ml. In Group 3, the dose of rhBMP-2 was Group 1 rhBMP-2 dose/30 or 0.039 mg/ml. In the Control group (0), there was only the collagen sponge. After that, 0.035 ml of the mixtures was added to the 0.625×0.5×0.4 cm3 ACS. The grafting material was then placed in a 2 mm×4 mm silicon tube.

2.3.2 Surgical Procedure:

Intraperitoneal injection of a mixture of 0.3 mg/kg body weight of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol was used for general anesthesia. Dorsal skin incisions were then performed, flaps were raised, and graft materials were implanted in subcutaneous tissues. Nylon sutures (Softretch 4-0, GC, Tokyo, Japan) were used to close the incisions. The recipient sites were randomly categorized into four groups according to the dose of rhBMP-2.

2.3.3 Specimen preparation for microcomputed tomography and histological analysis:

At 1 and 4 weeks after surgery, the tissues were fixed in 10% buffered formalin and assessed by an X-ray microcomputed tomography (micro-CT) scanner (CosmoScan FX, Rigaku, Tokyo, Japan). Samples were decalcified by 10% ethylenediaminetetraacetic acid at 37°C and embedded in paraffin wax before being sliced into 5-µm-thick slices for histological analysis. The sections were stained with hematoxylin and eosin (H&E) and evaluated histologically by light microscopy (Olympus X53 camera-assisted light

microscope (Olympus Corporation, Tokyo, Japan) (camera model UC50) utilizing OLYMPUS Stream Image Analysis Software 1.9). The histomorphometry measurements for the areas of newly formed bone and residual collagen sponge in histological images were done using ImageJ software, version 1.53t (U. S. National Institutes of Health, Bethesda, MD, USA)

Statistical analysis:

The medians and interquartile ranges were calculated for all outcome parameters. In addition, the differences among the groups for newly formed bone and residual collagen sponge areas were analyzed by one-way ANOVA with the post hoc Tukey HSD test. P-values<0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA).

2.2 Results

2.4.1 Radiological findings:

After four weeks, all samples were examined by X-ray micro-CT. Group 1 and 2 samples showed well-defined opacities with irregular shapes and edges at the implanted areas, which were more obvious and larger in group 1 samples (Figure 2.1). These radiographic findings were verified by histological analysis.

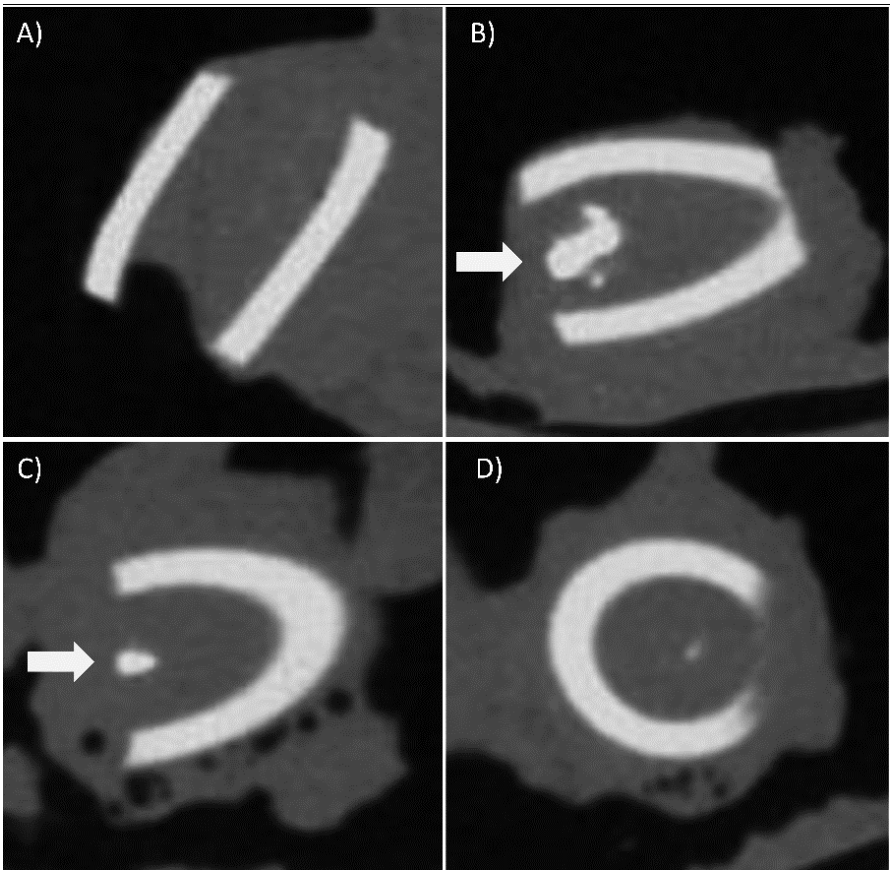


Figure 2.1. Representative microcomputed tomography images for all groups obtained after four weeks. a) Control group: Only ACS. b) Group 1: 1.67 mg/ml of rhBMP-2. c) Group 2: 0.117 mg/ml of rhBMP-2. d) Group 3: 0.039 mg/ml of rhBMP-2. A well-defined opacity (indicated by the arrow), demonstrating bone regeneration in the samples.

2.4.2 Histological findings:

All rats remained in good condition until the date of sacrifice. There was no histological evidence of osteoinduction in any of the group samples at one week. Nevertheless, all samples in the groups had extensive areas of collagen sponge and no evidence of bone formation (Figure 2.2).

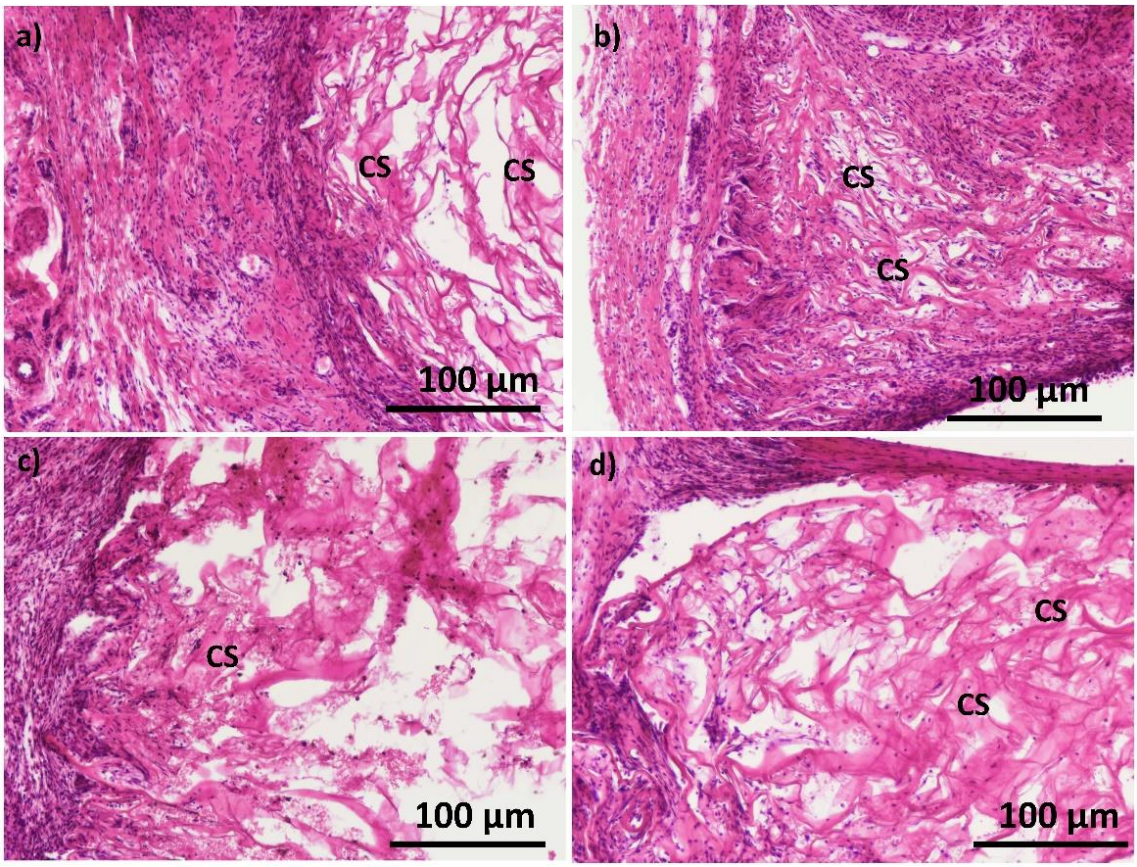


Figure 2.2. Histological findings of subcutaneous samples from all studied groups at one week. a) Control group: Only ACS. b) Group 1: 1.67 mg/ml of rhBMP-2. c) Group 2: 0.117 mg/ml of rhBMP-2. d) Group 3: 0.039 mg/ml of rhBMP-2. All groups displayed large areas of collagen sponge residual with no newly formed bone area. CS indicates collagen sponge residuals.

Control and group 3 samples showed sections of abundant connective tissue fibers in some samples with no areas of bone formation at four weeks (Figure 2.3). However, group 1 and 2 samples demonstrated bone formation within the implanted areas (Figure 2.3). Moreover, the newly formed bone in group 1 had a few osteocytes and osteoblasts. Subsequently, group 1 samples had more areas of collagen sponge than group 2 samples, whereas control group and group 3 samples showed no residual collagen sponge (Figure 2.3).

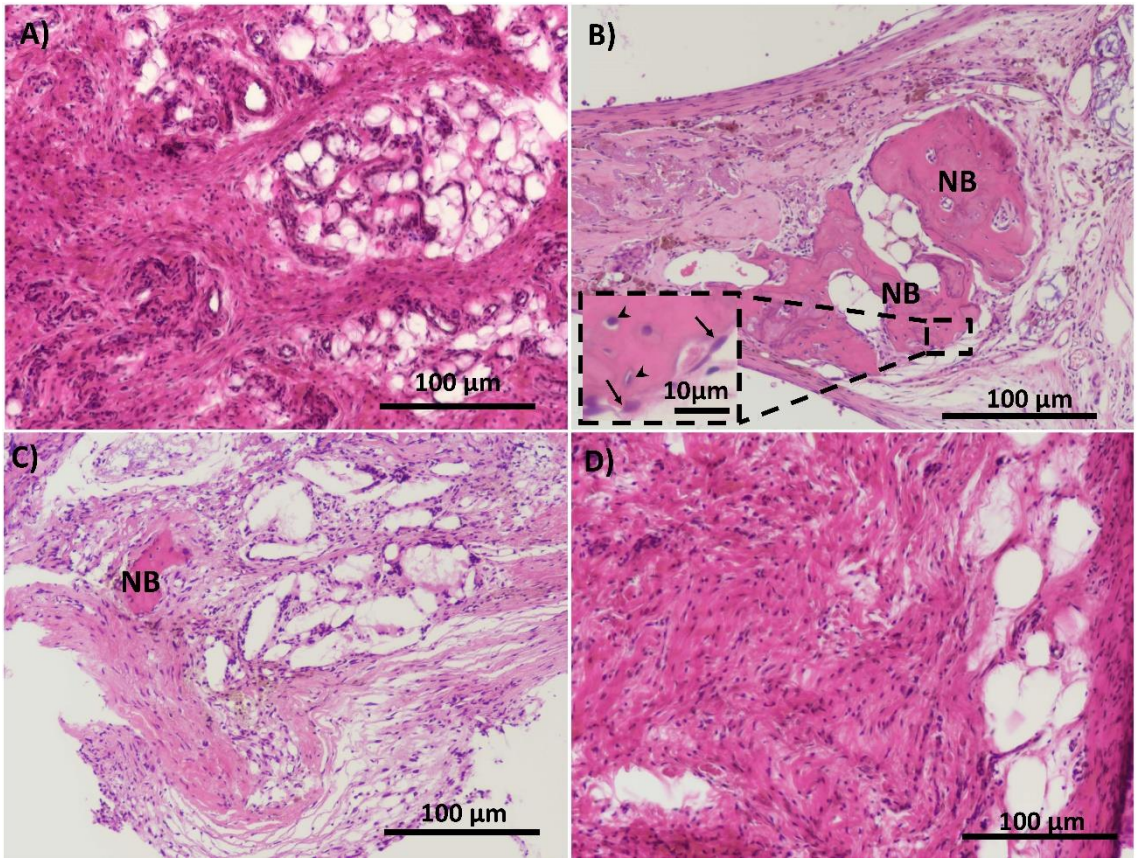


Figure 2.3. Histological findings of subcutaneous samples from all studied groups at four weeks. a) Control group: Only ACS. b) Group 1: 1.167 mg/ml of BMP-2. c) Group 2: 0.117 mg/ml of rhBMP-2 d) Group 3: 0.039mg/ml of rhBMP-2. A) Area of newly formed bone. (b) Area of collagen sponge residuals. NB indicates the newly formed bone.Notice magnified square area of the NB in (figure 3B) showing osteoblasts (arrows), and osteocytes (arrow heads).

2.4.3 Histomorphometric analysis:

The areas of collagen sponge showed no significant difference among all groups at one week ($P>0.05$) (Figure 2.4). The residual collagen sponge areas in the control group, group 1, group 2, and group 3 were 0.91±0.58 mm2, 1.55±1.42 mm^2, 0.94±0.52 mm^2, and 1.5±1.25 mm^2 respectively. The residual sponge area index at 4 weeks was 0±0 (control group and group 3), 0.16±0.10 (group 1), and 0.04±0.03 (group 2); the areas of residual collagen sponge at 4 weeks were greater in group 1 than in group 2, and the difference between the groups was significant ($P<0.05$) (Figure 4). The histomorphometric parameter of new bone area was 0±0 mm^2 in all groups at one week, as well as at 4 weeks in the control group and group 3. Nevertheless, it was 0.11±0.10 mm2 in group 1 and 0.025±0.0005 mm2 in group 2 at 4 weeks. Bone formation was significantly greater in group 1 at 4 weeks than in all other groups. Moreover, there were significantly fewer newly formed bone areas in group 2 than in group 1 ($P<0.05$).

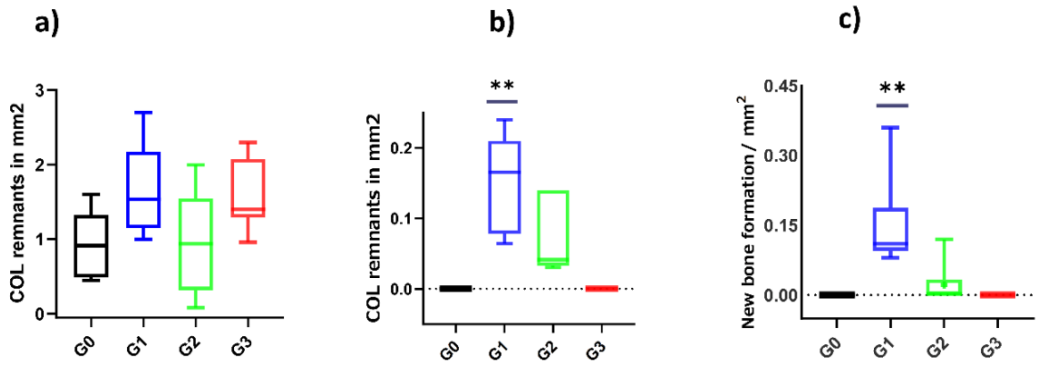


Figure 2.4. Histomorphometric analysis. a) Area of residual collagen sponge at 1 week. b) Area of collagen sponge residual at four weeks. c) Newly formed bone area at 4 weeks. **(P <0.05) Significance in each group was analyzed using one-way ANOVA with the post-hoc Tukey HSD test.

2.3 Discussion

In 2008, the FDA published serious complications related to the use of rhBMP-2 in the cervical spine, including dysphagia and swelling of soft tissues. It is important to lower the BMP-2 dosage to eliminate its potential side effects[25], and it can also reduce the cost. In the present study, no bone formation was observed at one-thirtieth of the recommended concentration of rhBMP-2. Conversely, osteogenesis was observed subcutaneously in rats at one-tenth of the recommended concentration of rhBMP-2, and the amount of osteogenesis was relatively lower compared to the ectopic bone induced by the recommended dose. With these findings, the null hypothesis must be rejected.

In the present study, osteogenesis was evaluated subcutaneously, but in clinical practice, rhBMP-2 can be used directly on bone tissues, where the bone always forms. Therefore, even at doses lower than one-tenth of the recommended rhBMP-2 dose, bone may form in the bone defects [1]. When considering the side effects of rhBMP-2, lower concentrations of material are safer, with reasonable cost. Nevertheless, the current study demonstrated that a lower-than-recommended dose can induce new bone formation.

It has been reported that rhBMP-2 when injected without a carrier will not induce bone formation [2]. Basically, the carrier's main function is to maintain the regenerative material in place, help promote bone regeneration, and be able to degrade biologically [3]. The rhBMP-2 carriers are divided into four categories: (1) natural origin such as collagen;

(2) inorganic such as hydroxyapatite; (3) synthetic such as polylactic acid; and (4) composite, which is a combination of two or more materials[3-5]. Various rhBMP-2 carriers have been tested and shown promising results[6,7].

The ACS when used as a carrier for rhBMP-2 demonstrated a high success rate in inducing ectopic bone [8]. Moreover, this type of delivery system has been widely used in recent years due to its great compatibility with rhBMP-2, potential to support newly formed bone, relatively low cost, and excellent biological degradability[8-10]. When combined with collagen, rhBMP-2 is highly compatible and can be incorporated into the collagen matrix [11]. In a rabbit ulna defect model, collagen sponge had a significantly better ability to hold rhBMP-2 in place than the buffer delivery system[12]. Kim et al. examined the bone regeneration potential of two different rhBMP-2 carriers in sinus augmentation in a rabbit model: rhBMP-2/ACS and rhBMP-2/TCP (tricalcium phosphate). The rhBMP-2/ACS group demonstrated greater and faster bone formation at 2 weeks[13]. Surprisingly, in a randomized, clinical study in which 131 patients received allograft dowels loaded with autograft bone or rhBMP-2/ACS, the rhBMP-2/ACS demonstrated significantly better results for postoperative pain[14]. However, since ACS has low mechanical strength[3], the samples were put in silicon tubes to protect them from the pressure of the surrounding tissues during the surgical procedure and the healing period. In addition, the tubes may provide additional support for newly formed bone along with the carrier.

In the present study, the absence of osteogenesis in the third group may be attributed to ACS's inability to maintain the low dose of rhBMP-2. Some studies have reported that rhBMP-2 has inadequate retention on the collagen sponge and might lead to clearance of rhBMP-2 in the tissue rather than induction of surrounding mesenchymal cells[8,15,16]. However, the leakage of such rhBMP-2 due to low retention on ACS may lead to formation of bone in undesired areas[17]. Uludag et al. examined the retention of rhBMP-2 on an absorbable collagen sponge (ACS) and found that only 70% of rhBMP-2 was retained on the collagen on the first day of surgery and then gradually decreased to day 14 [18]. Moreover, it has been previously reported that, the more rhBMP-2 is retained, the more it is effective [8,19]. In the future, finding a better carrier with higher retention

capacity of rhBMP-2 and determining a better concentration of rhBMP-2 according to osteogenic potential. Scaffolds to be included with BMP-2 which would be beneficial for improving long term outcomes, and reducing medical costs for healthcare system, as well as complications.

2.4 Conclusion

The present study demonstrated that 1.167 and 0.117 mg/ml of rhBMP-2 with ACS as the carrier induced ectopic bone formation in 4 weeks. The in vivo findings, on the other hand, confirmed the ability of ACS to retain rhBMP-2, support the newly formed bone, and show a good biodegradation rate. However, bone formation was not observed with 0.039 mg/ml of rhBMP-2. Moreover, rhBMP-2 was found to be osteogenic even at one-tenth of the recommended concentration, indicating the potential for clinical use at lower concentrations.

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CHAPTER 3: A COMPARATIVE STUDY OF TWO DIFFERENT MOLECULAR WEIGHTS OF PHOSPHORYLATED-PULLULAN COMBINED WITH B-TCP/BMP-2/CACL2 IN BONE REGENERATION

3.1 Materials and Methods

Experimental animals were performed in accordance with the animal experimental protocol of Hokkaido University's institutional animal use and care policy. (Hokkaido University's Animal Research Committee with approval number:19-0128) Ten male Wistar rats, aged ten weeks, weighing 180-210 grams, were included. n=10

3.3.1 Preparation of grafting materials:

1. Synthesis of Phosphorylated-Pullulan: Two different molecular weights of PPL (1,000,000 lot. 19XPP01) and (600,000 lot.2I02) were prepared as described previously [1]. After that two mixtures of PPL 20101B (PPL1) (600,000 MW of PPL), and 20101D (PPL2) (600,000 and 1,000,000 MW of PPL) were created.
2. Group categorization: Two groups were categorized based on the ratios of two mixtures of PPL MW (20101B, 20101D). The ratio of 20101B (PPL1) to 20101D (PPL2) was 1-3 in PPL1-3 group and 1-5 in PPL1-5 group. Latterly 50 mg of both mixtures of PPL mixed with 400mg β-TCP (Taihei Chemical Industrial Co., Ltd., Osaka, Japan), 300 µl of mg/ml BMP-2 (INFUSE® Bone Graft 7510050XX Small Kit, Lot. MCM6723AAA, Medtronic, Dublin, Ireland), and 6.082 mg of 2% CaCl2. (Sigma-Aldrich Co.)

3.3.2 Surgical Procedure:

General anesthesia was administered through intraperitoneal injection of 0.3 mg/kg body weight medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol. After that, the skin was incised, flaps were raised, and synthesized grafting materials were inserted subcutaneously. Nylon sutures (Softretch 4-0, GC, Tokyo, Japan) were utilized for suturing, and the tissues were sacrificed 2 weeks post-surgery. However, the grafted areas

have been selected randomly according to the ratio of the synthesized composites. Laterally, the tissues were fixed with 10% buffered formalin, embedded in paraffin after being dehydrated in a gradual ethanol series. Multiple sections of 5 μm thickness were sliced from the tissue samples and stained with hematoxylin-eosin to be examined histologically (Olympus X53 camera-assisted light microscope (Olympus Corporation, Tokyo, Japan) (camera model UC50) utilizing OLYMPUS Stream Image Analysis Software 1.9). The newly formed bone was evaluated histologically by light microscopy.

3.3.3 Histomorphometry measurements:

The areas of newly formed bone and residual PPL were measured using ImageJ software, version 1.53t (U. S. National Institutes of Health, Bethesda, MD, USA)

3.3.4 Statistical analysis:

The means and standard deviations among the areas all parameters have been calculated. However, the differences between the two groups were analyzed by Mann–Whitney U test using SPSS 21 (IBM, Armonk, NY). GraphPad Prism 8 software (GraphPad Software, San Diego, CA) was used to create the bar chart.

3.2 Results:

3.2.1 Histological findings:

There was no evidence of inflammation, the surgical sites healed normally, and all rats were in good condition until the time of sacrificing.

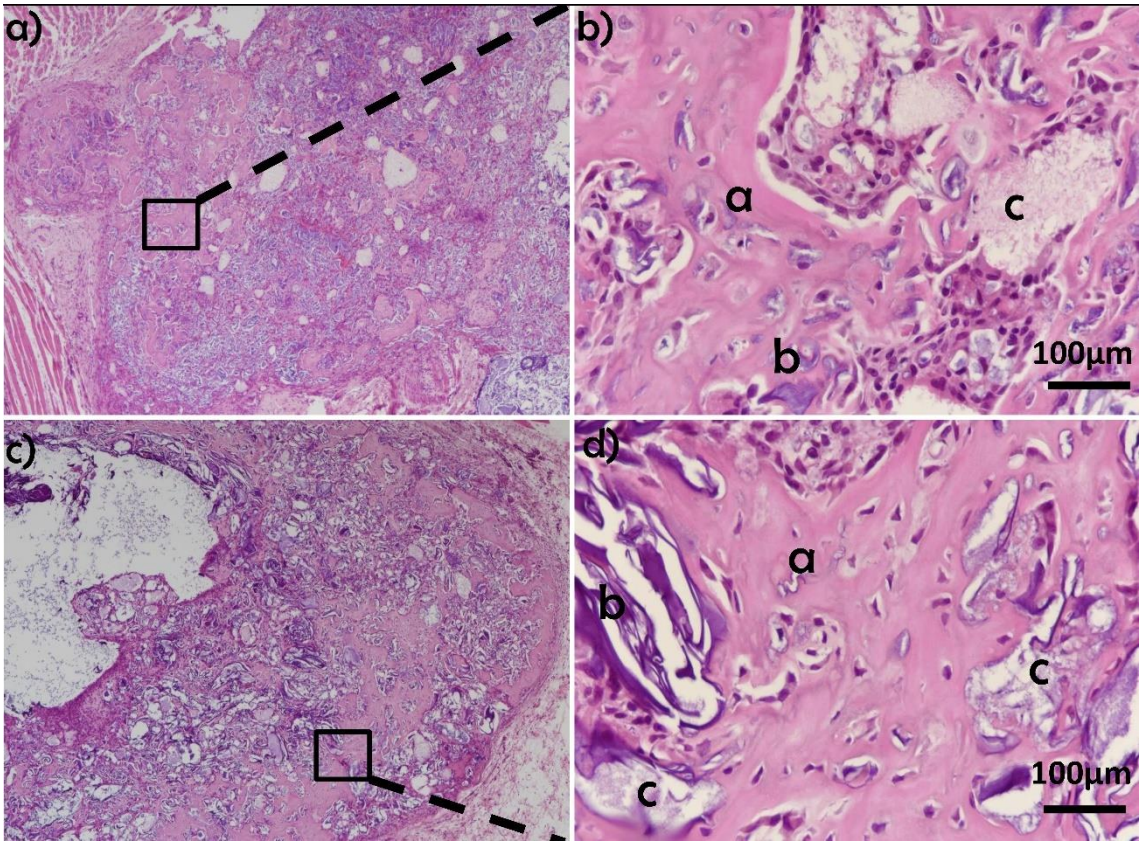

Figure 3.1. Histological findings of subcutaneous samples from both groups at two weeks. a) and b) Light and high magnification image of PPL1-3 group. c) and d) Light and high magnification image of PPL1-5 group. (a) Area of newly formed bone. (b) Area of phosphorylated-pullulan residuals. (c) Area of β-TCP residuals.

The histological examination revealed that the higher molecular weight of PPL was associated with more bone formation in that area. Two weeks post-surgery, the samples in both groups showed abundant newly formed bone especially around the β-TCP and PPL residuals (Figure 3.1). Furthermore, there were considerably more marrow tissues, osteocytes, and osteoblast with greatest number of osteoblasts found at the line around the newly formed bone and the residuals of β-TCP and PPL.

3.2.2 Histomorphometric analysis was done after two weeks post-surgically:

It revealed that the amount of new bone formation area was (0.038±0.020 mm^2) in PPL1-3 group and (0.16±0.29 mm^2) in PPL1-5 group. Bone formation in the PPL1-5 group was significantly greater than PPL1-3 group ($P<0.05$). However, the areas of residual PPL were somewhat larger in PPL1-3 than PPL1-5 with no statistically

significant difference (P<0.05). The residual PPL areas in groups PPL1-3 and PPL1-5 were 0.036±0.017 mm^2, 0.026±0.013 mm^2, respectively.

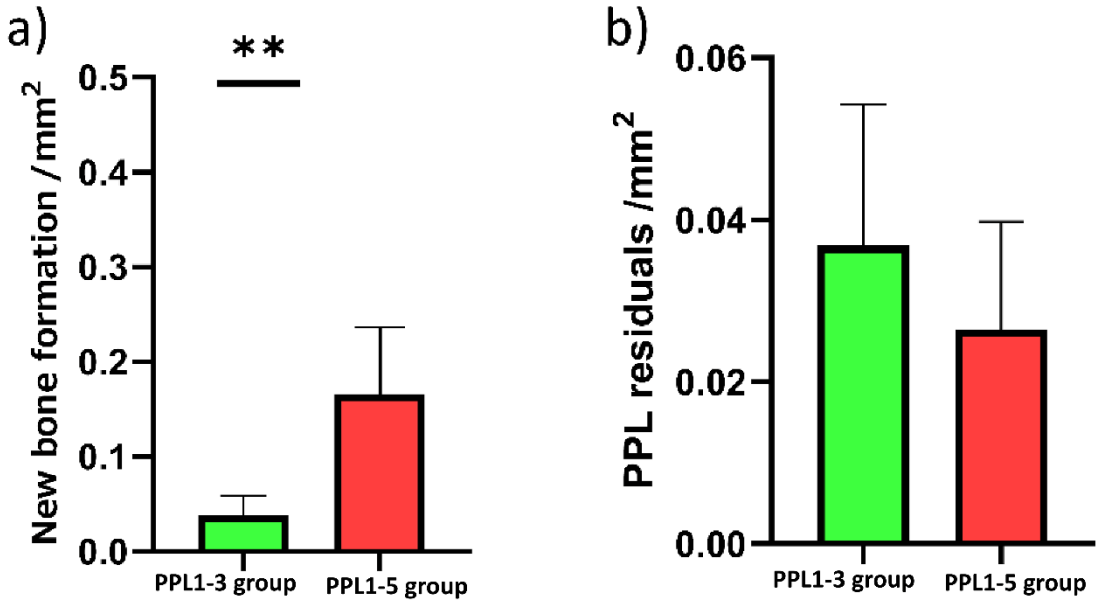

Figure 3.2. Histomorphometric analysis. a) Newly formed bone area at 2 weeks. b) Area of phosphorylated-pullulan residuals at 2 weeks. **(P <0.05) Statistical significance in each group was analyzed by Mann–Whitney U test. Performed using SPSS 21 (IBM, Armonk, NY).

3.3 Discussion

The use of autograft bone in the treatment of bone defects is sometimes not recommended due to the evident significant patient morbidity [2,3]. However, *β*-TCP has been used extensively in the field of bone regeneration due to its higher computability and resemblance to the composition of bone [4,5]. The combination of PLL and β-TCP showed excellent results in pig spinal bone defects. Histologically, the newly formed bone was detected at 4 weeks, and the trabeculae were discovered at 8 weeks. Furthermore, in the same study, the mechanical characteristics of the material were assessed using a universal testing machine (AGS-1000A, Shimadzu Co., Kyoto, Japan) to evaluate the compressive strength of the material. The PLL alone has lower compressive strength in comparison to PPL/*β*-TCP composite scaffold. However, the excellent compressive strength of PPL/*β*-TCP composite scaffold makes it a very promising material for bone regeneration [6]. Yoshida and coworkers examined PPL biocompatibility by applying it

on the pulp of rat teeth. The PPL formed osteoblast like cells indicating the high compatibility of the material [7]. In a pig model, the PLL was used to treat the titanium implant surface. There was greater bone fraction around the PPL treated implant surface than the H_2O-treated implant surface with no statistically significant difference [8,9].

In the present study we demonstrated the ability of two created composites in bone regeneration after being implanted in the subcutaneous tissues of the back of rat. We used a distinct combination of PPL, β-TCP, BMP-2, and CaCl2 with two different molecular weights of PPL. Both groups showed extensive ectopic bone formation with larger areas in PPL1-5 group. This finding can be explained by the fact that the PPL1-5 group used a mixture of larger and smaller MW PPL, with a higher proportion of the larger MW of PPL. However, the larger MW of PPL promotes greater bone formation, while on the other hand, the smaller MW of PPL encourages faster absorption, creating spaces for newly formed bone deposition [1]. Additionally, it has been well understood previously that β-TCP and BMP-2 are considered osteoinductive materials; [6,10-15] however, when combined with PPL, result in extensive bone formation ectopically. Moreover, along with the high osteogenic potential of BMP-2 and β-TCP, the high solubility of pullulan found to play an important role in enhancing the bone regeneration in the area [16]. However, phosphorylation of pullulan creates a chain by adding calcium ions, which decreased the solubility nature of the created composite [7] and that may increase the adhesiveness in return. A comparable picture was observed in a similar in vivo study in which it has been found that combining pullulan with alginate and copper enhanced bone formation in created defects in rat leges models [17]. Furthermore the same researchers made further testing for the composite and they reported that it has a good biological compatibility [18] and others claimed that this synthetic composite scaffolds have antibacterial effect against *Staphylococcus Aureus*, and may promote the development of fibroblasts and osteoblasts since they include pullulan and bioactive glass ceramics [19].

In the present study, we discovered that phosphorylated-pullulan residuals were somewhat higher in the PPL1-5 group than in the PPL1-3 group, however, the difference was not statistically significant. This can be attributed to larger MW of PPL1-3 group, in which the larger the molecular size of PPL, the slower it degraded [1]. Further studies

needed to understand the mechanism of PPL attachment bond with the surrounding bone tissues.

3.4 Conclusion

PPL1-5 group samples have received mixtures of molecular weight of PPL, which can induce greater bone formation and higher biological degradability rate of PPL than PPL1-3 group. This composite can be considered a potentially promising material in the field of bone regeneration.

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CHAPTER 4: COMPARISON OF THE OSTEOGENIC EFFECTS OF PHOSPHORYLATED-PULLULAN/B-TCP/BMP-2 COMPOSITE SCAFFOLDS AND BMP-2/COLLAGEN SPONGE IN A RAT MODEL

4.1 Materials and Methods

4.4.1 Experimental Animals:

The present study was conducted on 20 male Wistar rats (10 for each experiment) (180-210 g) aged about ten weeks. The animal experimental protocol of the institutional animal use and care regulation of Hokkaido University (Animal research committee of Hokkaido University, approval number:19-0128) was implemented during the preparation of the experiments.

4.4.2 Preparation of regenerative materials (BMP-2):

As described in the first study, INFUSE® Bone Graft system has been utilized that includes the absorbable collagen sponge and rhBMP-2 protein (INFUSE® Bone Graft 7510050XX Small Kit, Lot. MCM6723AAA, Medtronic, Dublin, Ireland). Groups categorization: five groups were categorized based on rhBMP-2 concentration. According to the manufacturer's instruction, 1.05 mg of rhBMP-2 powder was reconstituted with 9 mL of sterile water for injection [1], resulting in a standard concentration of 1.167mg/mL of rhBMP-2. (BC1 group). BC2 group: BC1 dose/10= 0.117 mg/ml of rhBMP-2. BC3 group: BC1 dose/30= 0.039 mg/ml of rhBMP-2. BC4 group: BC1 dose/50=0.023 mg/ml of rhBMP-2. Following that, 0.7 ml of the rhBMP-2 mixtures were added to 1.25× 5.08 ×0.4 cm of absorbable collagen sponge (ACS). Control group (BC0): Only ACS. (Table 4.1)

The same four concentrations of rhBMP-2 mentioned above were mixed with 50 mg of PPL1-5 (Phosphorylated-Pullulan has been synthesized according to our second study with the same molecular weight of PPL1-5) and 400 mg of β-TCP (Taihei Chemical Industrial Co., Ltd., Osaka, Japan) to create distinct composite scaffolds of different

BMP-2 doses. The volume of used biomaterials was standardized among all studied groups. The sample groups were categorized as follows: BPT0 group (Control group), BPT1, BPT2, BPT3, BPT4.

Table 4.1 Groups categorization according to rbmp-2 dose and the combination with β-TCP and PPL.

Group	rBMP-2 dose	Combination with β-TCP and PPL
BC0	0	No
BC1	1.167mg/mL	No
BC2	BC1/10= 0.117 mg/ml	No
BC3	BC1/30= 0.039 mg/ml	No
BC4	BC1 /50=0.023 mg/ml	No
BPT0	0	Yes
BPT1	1.167mg/mL	Yes
BPT2	BC1/10= 0.117 mg/ml	Yes
BPT3	BC1/30= 0.039 mg/ml	Yes
BPT4	BC1 /50=0.023 mg/ml	Yes

4.4.3 Surgical Procedure:

For general anesthesia, an intraperitoneal injection of 0.3 mg/kg body weight of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol was administered. Then, dorsal skin incisions were performed, flaps were elevated, and grafting materials were implanted in the subcutaneous tissues. The incisions were closed with nylon sutures (Softretch 4-0, GC, Tokyo, Japan). The recipient sites were divided randomly into 10 groups based on the rhBMP-2 concentration and the presence of PPL and β-TCP.

4.4.4 Specimen preparation for microcomputed tomography and histological analysis:

The rats among different studied groups were euthanized two weeks post-surgery, and the specimens were collected. Firstly the specimens subjected to a microcomputed tomography (micro-CT) scanner (CosmoScan FX, Rigaku, Tokyo, Japan) examination

followed by fixation in 10% buffered formalin for 24h at room temperature. Then the specimens were transferred to 10% ethylenediaminetetraacetic acid (EDTA) for decalcification at room temperature. The decalcified specimens were then processed for paraffin embedding and sliced at 5-µm-thick sections to be used for histological examination. Hematoxylin and eosin (H&E) were used to stain the paraffin sections and examined under a light microscope (Olympus X53 camera-assisted light microscope (Olympus Corporation, Tokyo, Japan) (camera model UC50) utilizing OLYMPUS Stream Image Analysis Software 1.9). High magnification (x200) images from H&E-stained sections were captured from three randomly selected fields of both marginal and middle zones among all studied groups and used for measuring the NFB area. ImageJ software, version 1.53t (U. S. National Institutes of Health, Bethesda, MD, USA) was used to measure the volumetric values for the areas of newly formed bone (NFB) in both the micro-CT and the HE-stained sections.

4.4.5 Statistical analysis

All outcome parameters' means, and standard errors were calculated. However, one-way ANOVA with the post-hoc Tukey HSD test was used to analyze the differences between the groups for NFB. P-values less than 0.05 were regarded as significant. Statistical analyses were performed by GraphPad Prism 8 (GraphPad Software, San Diego, CA).

4.2 Results

4.2.1 Radiological findings:

After two weeks all samples were examined by X-ray microcomputed tomography (micro-CT) (CosmoScan FX, Rigaku, Tokyo, Japan). Except for the control group, which showed no radiographic signs of bone regeneration, all BC experimental groups displayed well-defined opacities with irregular forms and margins at the implanted regions (Figure 1). However, the greatest areas of radiopacity were seen in BC1 samples and the smallest was in BC4 indicating the amount of bone formation with no statistically significant difference ($P<0.05$). (Figure 4.1) Nevertheless, in BPT experimental samples, the areas

of radiopacity were extensively larger, with the greatest quantity in BPT1 group. BPT1 group showed the largest area of NFB among all experimental groups and the difference was statistically significant (P<0.05). Furthermore, there were radiopaque spots seen in the BPT0 group confirming the amount of bone regeneration. (Figure 4.1) However, in these samples, it was challenging to distinguish between newly formed bone and the remaining regenerative materials (PPL and β-TCP) since both appeared radiopaque. These radiographic findings have been confirmed by histological analysis.

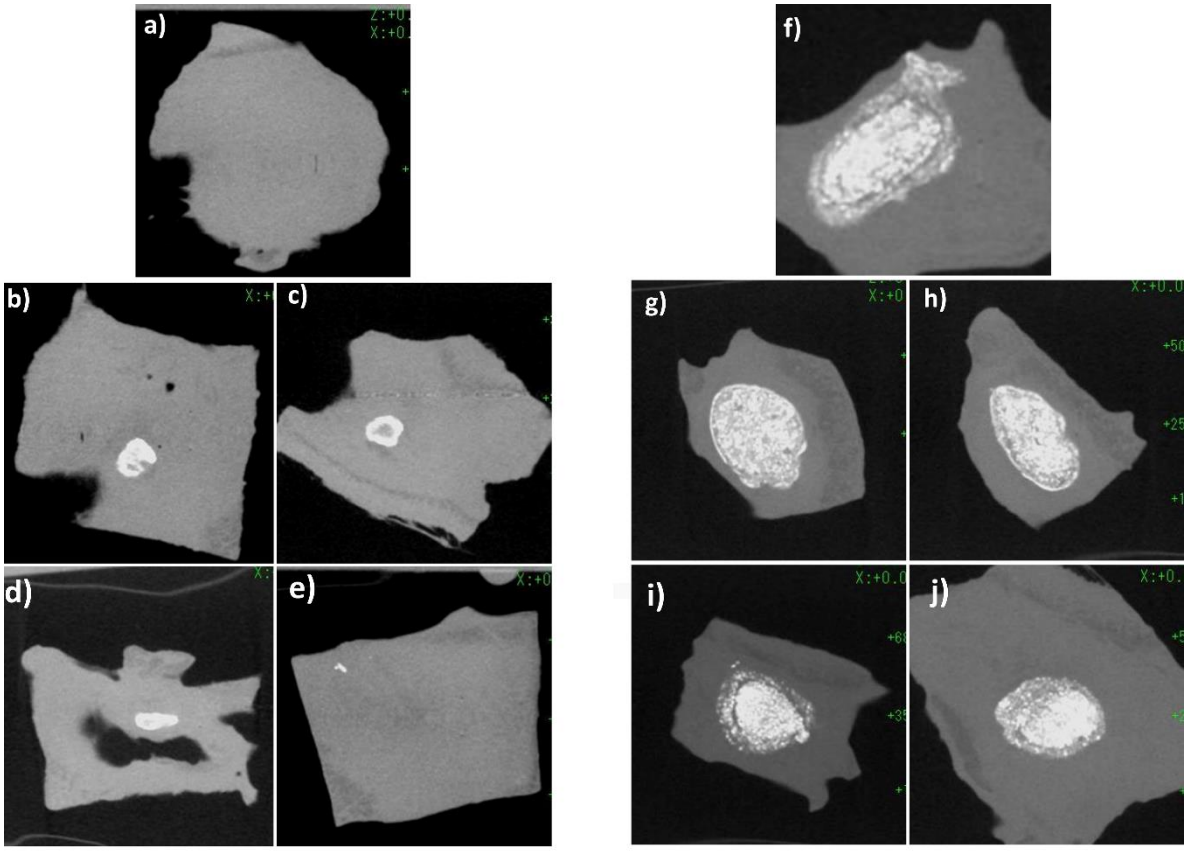

Figure 4.1. Micro-computed tomographic (μCT) images of both experiments obtained after two weeks a) Control group: Only ACS. b) BC1 group: 1.67 mg/ml of rhBMP-2. c) BC2 group: 0.117 mg/ml of rhBMP-2. d) BC3 group: 0.039 mg/ml of rhBMP-2. e) BC4 group: 0.023 mg/ml of rhBMP-2. Well-defined opacities demonstrate NFB in BC samples. However, the opacity in BPT samples demonstrate both NFB and PPL and B-TCP residuals. f) BPT0 group: combined PPL and β-TCP without BMP-2. g) BPT1 group: combined 1.167 mg/ml of BMP-2, PPL, and β-TCP. h) BPT2 group: combined 0.117 mg/ml of rhBMP-2, PPL, and β-TCP i) BPT3 group: combined 0.039 mg/ml of rhBMP-2, PPL and β-TCP j) BPT4 group: 0.023 mg/ml of rhBMP-2, PPL and β-TCP.

4.2.2 Histological findings:

The rats were in good health till the time of sacrifice. All groups of samples showed definitive histological signs of bone regeneration except BC0 (Figure 4.2). **BC0 group**:

All recipient areas were filled with connective tissue rather than newly formed bone (NFB) with few inflammatory cells (Figure 4.2). **BC1:** It showed the greatest areas of NFB among all BC groups (Figure 4.2). The center and periphery of the implanted areas had almost the same amount of NFB, with no apparent difference**.** Additionally, NFB was surrounded by a significant number of osteoblasts. A few osteocytes and marrow tissues were seen embedded in NFB. There was a limited distribution of a few collagen sponge residuals (Figure 4.2). **BC2, and BC3 groups**: NFB areas appeared to be smaller than BC1with limited osteoblasts, osteocytes, and collagen sponge residuals (Figure 4.2). **BC4 groups:** A small island of NFB with extremely limited osteocytes in the center and extremely few osteoblasts on the periphery of NFB. There was, however, a substantial quantity of collagen sponge residuals (Figure 4.2). **BPT0 group:** It demonstrated smaller areas of bone regeneration in comparison to most BPT groups. There were abundant β-TCP and PPL residuals. NFB is mostly in the margin of the specimen (Figure 4.3). **BPT1 group:** It had the largest areas of NFB among all groups and the difference was statistically significant. There were many osteoblasts surrounding the well-defined trabecular bones. Marginal NFB appears to be significantly greater than the middle with many osteocytes embedded in the surface. Moreover, these samples included a significant amount of residual PPL and β-TCP (Figure 4.3). **BPT2 and BPT3 groups:** large areas of NFB that surrounded numerous and large areas of PPL and β-TCP residuals. Many osteoblasts and osteocytes appear to be less abundant than in the BPT1 group. The marginal NFB was greater than the middle. (Figure 4.3). **BPT4 group:** Among the BPT experimental groups, it had the lowest and fewest NFB areas. NFB, however, was only present peripherally in the samples, where there were few osteoblasts and osteocytes. The NFB is surrounded by extensive areas of PPL and β-TCP residuals (Figure 4.3).

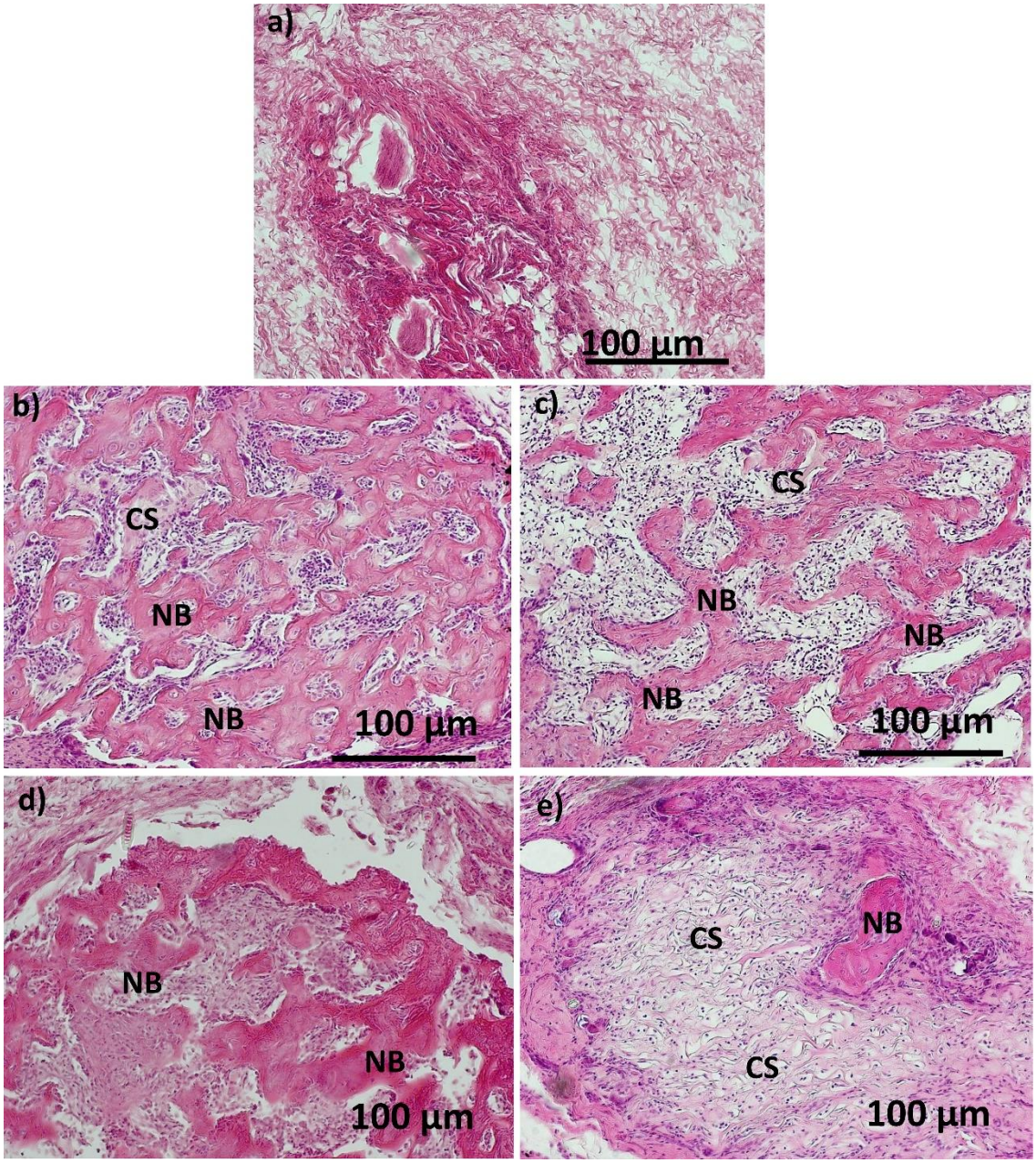

Figure 4.2. Hematoxylin and eosin histological sections of subcutaneous tissue 2 weeks after implantation. a) Control group: Only ACS (BC0). b) BC1 group: 1.67 mg/ml of rhBMP-2. c) BC2 group: 0.117 mg/ml of rhBMP-2. d) BC3 group: 0.039 mg/ml of rhBMP-2. e) BC4 group: 0.023 mg/ml of rhBMP-2. (CS) indicates collagen membrane residuals. (NB) indicates newly formed bone.

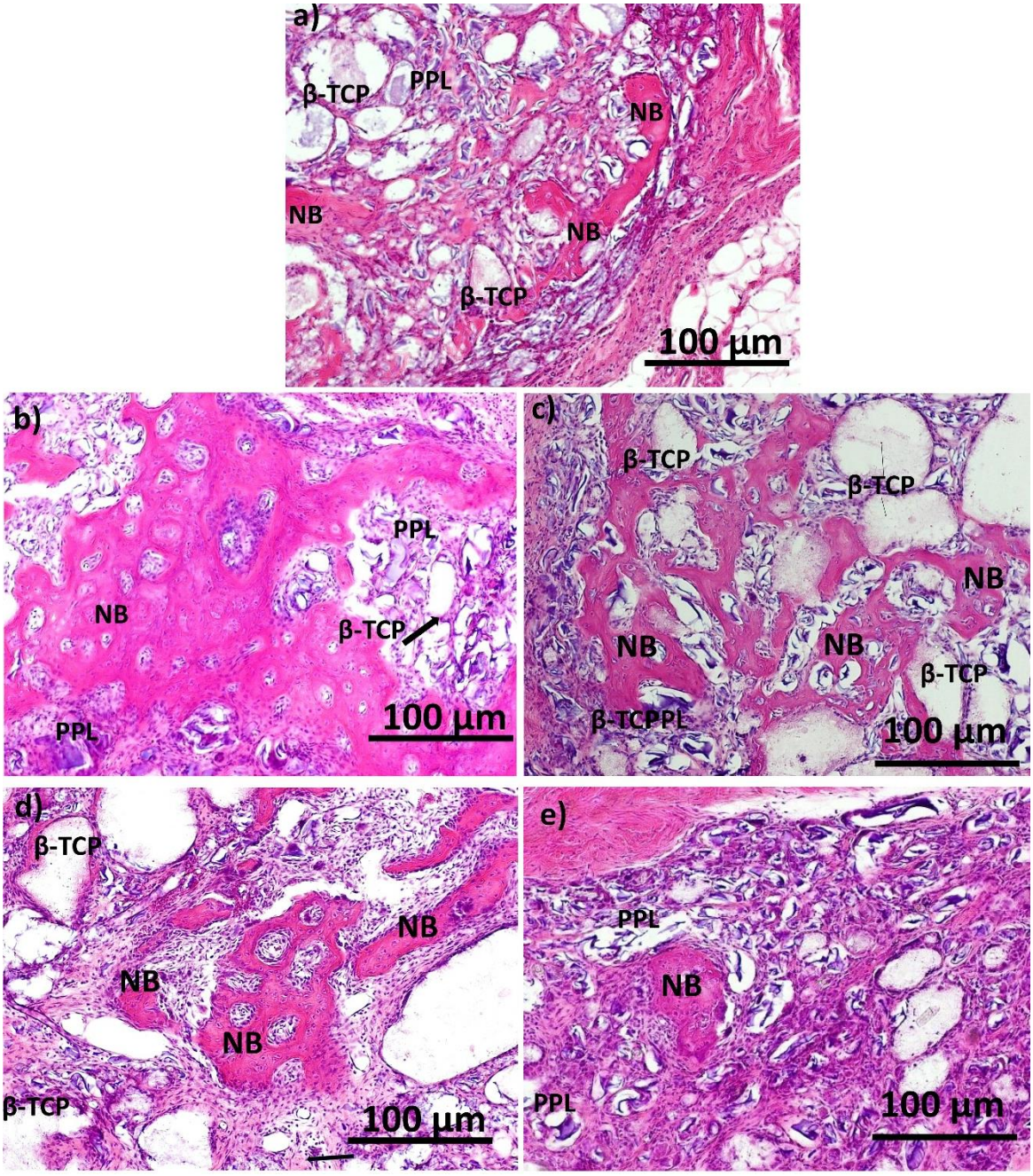

Figure 4.3. Hematoxylin and eosin histological sections of subcutaneous tissue 2 weeks after implantation. a) BPT0 group: combined PPL and β-TCP without BMP-2. b) BPT1 group: combined 1.167 mg/ml of BMP-2, PPL, and β-TCP. c) BPT2 group: combined 0.117 mg/ml of rhBMP-2, PPL, and β-TCP d) BPT3 group: combined 0.039 mg/ml of rhBMP-2, PPL, and β-TCP e) BPT4 group: 0.023 mg/ml of rhBMP-2, PPL and β-TCP.

4.2.3 Micro-Ct and histomorphometric analysis:

At two weeks, the micro-CT new bone parameters in BC0, BC1, BC2, BC3 and BC4 were 0±0 mm^2, 0.4±0.02 mm^2, 0.3±0.01 mm^2 ,0.1±0.01 mm^2, and 0.01±0.006 mm^2 respectively. However, the parameters in BPT groups were 0.4±0.03 mm^2 in BPT0, 2.0±0.1 mm^2 in BPT1, 0.6±0.02 mm^2 in BPT2 ,0.3±0.04 mm^2 in BPT3 and 0.07±0.001 mm^2 in BPT4. (Table 4.2)

The histomorphometric index of NFB at two weeks in C was 0±0 mm^2 in BC0, 0.5±0.1 mm^2 in BC1, 0.3±0.1 mm^2 in BC2, 0.2±0.1 in BC3 mm^2, 0.01±0.003 mm^2 in BC4. (Table 4.2) Nevertheless, in histomorphometric analysis of NFB was in BPT0, 0.4±0.1 mm^2 in BPT1, 2.1±0.4 mm^2, in BPT2 0.5±0.1 mm^2, in BPT3, 0.3±0.1 mm^2 in BPT4 0.02±0.003 mm^2. (Figure 4.4)

Table 4.2 Micro-Ct and histomorphometric analysis of newly formed bone at two weeks.

Group	Micro-Ct analysis of NFB	Histomorphometric analysis of NFB
BC0	0±0 mm^2	0±0 mm^2
BC1	0.4±0.02 mm^2	0.5±0.1 mm^2
BC2	0.3±0.01mm^2	0.3±0.1 mm^2
BC3	0.1±0.01 mm^2	0.2±0.1 mm^2
BC4	0.01±0.006 mm^2	0.01±0.003 mm^2
BPT0	0.4±0.03 mm^2	0.4±0.1 mm^2
BPT1	2.0±0.1 mm^2	2.1±0.4 mm^2
BPT2	0.6±0.02 mm^2	0.5±0.1 mm^2
BPT3	0.3±0.04 mm^2	0.3±0.1 mm^2
BPT4	0.07±0.001 mm^2	0.02±0.003 mm^2

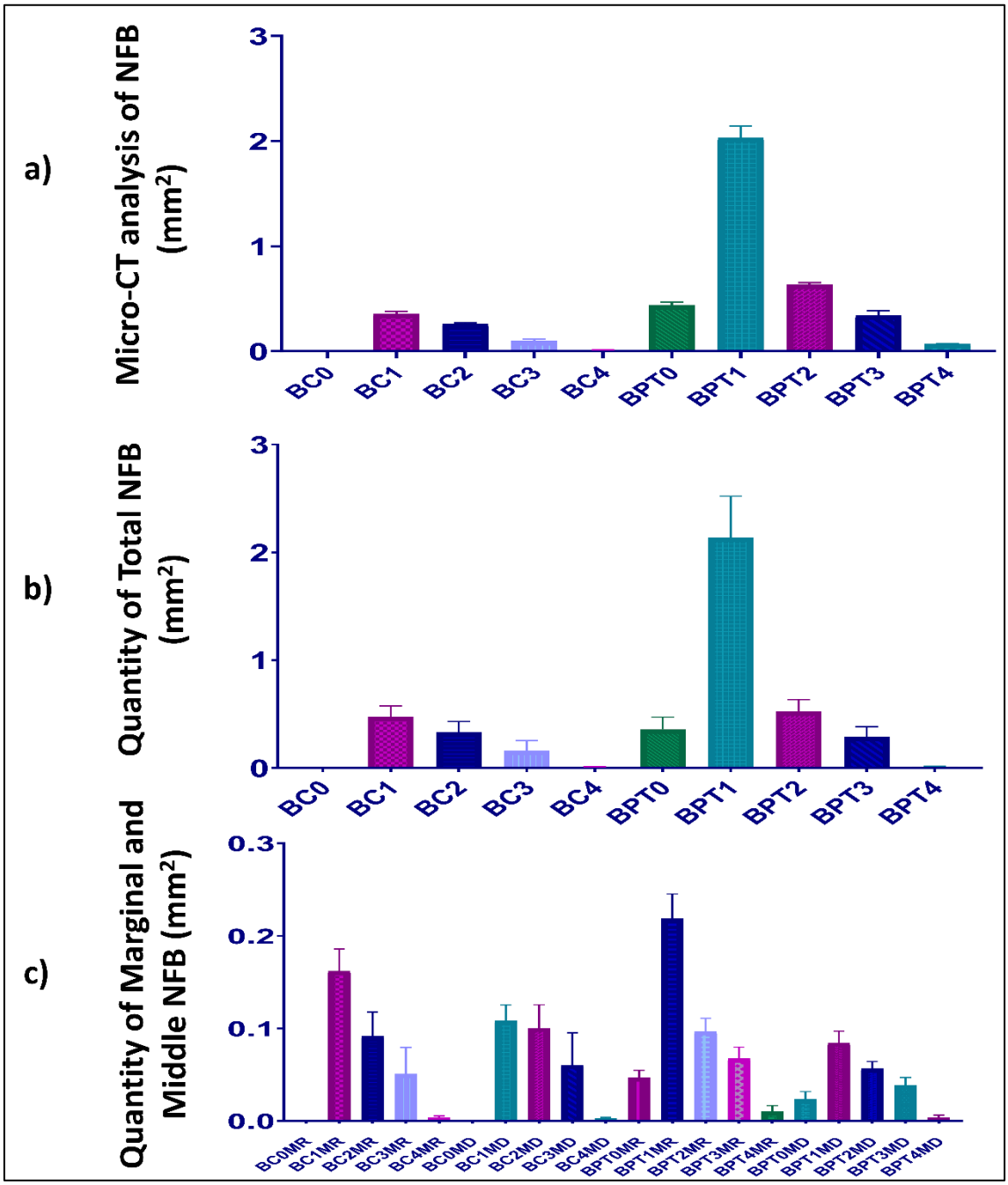

Figure 4.4. Micro-Ct and histomorphometric analyses: (a) Micro-CT analysis of newly formed bone at two weeks. (b) Histomorphometric analysis of total newly formed bone at two weeks. (c) Histomorphometric analysis of marginal and middle newly formed bone at two weeks. **($P<0.05$) Significance in each group was analyzed using one-way ANOVA with the post hoc Tukey HSD test.

4.4. Discussion

In a previous study, it was concluded that PPL/β-TCP composite scaffold showed good ability in inducing bone formation in a murine intramedullary model and suggested that this composite scaffold had an excellent adherence to the surrounding bone tissues [2]. However, in the current study we compared the osteogenic potential of BMP-2 loaded on absorbable collagen sponge (BC groups) with BMP-2/PPL/β-TCP composite scaffolds at four different BMP-2 doses. We found that the higher BMP-2 concertation the greater bone regeneration, this proved its potent ostoinductive capability. Furthermore, the samples treated with BMP-2 combined with PPL and β-TCP (BPT group) demonstrated greater bone formation than the samples treated with BMP-2 alone at the same BMP-2 doses. Interestingly we also observed that, the group of samples treated with 0.117 mg/ml of BMP-2 combined with PPL and β-TCP (BPT2 group) had more bone formation than the group treated with 1.167 mg/ml of BMP-2 alone (BC1 group). Although, the BMP-2 concentration in the BPT2 group was lower than in the BC1 group. This may be attributed to the fact that PPL and β-TCP's incorporation in composite provided greater osteogenic effect as mentioned previously [3,4]. Furthermore, the calcium and phosphate produced by β-TCP may enhance the activity of osteoblast alkaline phosphatase [5] which suggested as favorable for bone formation [6].Baheiraei et al, indicated that, the addition of β-TCP in the collagen matrix might improve the compressive strength of the composite over collagen [7].In addition, the chemical adhesion of PPL to the bone could further improve the osteogenic potential of the composite scaffold [2]. On other hand, several studies have mentioned the poor retention of BMP-2 on collagen leading to reduce its osteogenic effect [8-10] .

Numerous studies have combined BMP-2 with different osteogenic materials, which demonstrating great capacity to accelerate healing and regeneration of cartilage [11] and bone defects [12-14]. In the mouse calvarial defect model, combining BMP-2 with matrix metalloproteinase 10 (MMP10) has been shown to improve bone regeneration. MMP10 has been found to enhance BMP-2 mediated osteoinductivity and promote mineral apposition [14]. Indeed, the robust osteoinductive potential of BMP-2 encourages researchers to combine it with other regenerative materials and elements to obtain optimum results.

The osteoinductivity of BMP-2 makes it particularly effective for bone regeneration, but the effectiveness of BMP-2 may vary greatly depending on how it is delivered. BMP carriers such as ACS and fibrin glue showed relatively good bone regeneration abilities, meeting the essential conditions of localization and release control as confirmed by the experiments of Jung-Woo Nam, Hyung-Jun Kim [15]. As a BMP carrier, autoclaved autogenous bone cannot provide release control, but its space-maintenance role is remarkable. Autoclaved autogenous bone is considered an effective scaffold for large bone defects since it can provide sufficient support to the BMP/carrier complex. Therefore, in the current study use of ACS for the composite structure was justified.

With ACS, a BMP carrier derived from natural sources binds to the BMP, which is immediately incorporated by cells and reforms into bone tissue [16]. Several studies have been conducted in vivo and clinically on BMP/ACS, showing similar successful outcomes to those with autogenous bone graft [17]. ACS is still limited in clinical applications in terms of insufficient ability to maintain space, difficulty in manipulating, and immunogenicity issues. However, the xenogeneic nature of rhBMP-2/ACS was reported to produce anti-type I collagen antibodies in 20% of patients [16,17].

It has been reported that β-TCP/chitosan scaffold composite has a potent capability to promote bone marrow mesenchymal stem cells (MSCs) differentiation [18]. Additionally, a recent clinical study has shown that utilizing β-TCP in combination with polylactic acid (PLA) membrane promoted good bone regeneration in the anterior esthetic region of the maxilla.[19] In the current study, samples treated with 1.167 mg/ml of BMP-2 (BPT1 group), showed the greatest NFB among all groups and the difference was statistically significant. This can be justified by the application of recommended dose of BMP-2 along with high osteogenic effect of PPL and β-TCP. Furthermore, in the present investigation, we discovered that BPT groups had more NFB at the marginal zone, indicating that the osteogenic materials in these samples were better at maintaining the morphology of NFB than BC groups.

It has been reported that pullulan hydrogels may be enhanced by nano-crystalline hydroxyapatite (nHAp) (5 wt percent nHAp in hydrogel) and poly(3-hydroxybutyrate) (PHB) fibers and concluded that the mechanical characteristics of pullulan scaffolds increased tenfold. [20]. Cardoso et al, reported that, implants with phosphorylated

pullulan treated surface showed higher osseointegration as compared to implants treated with polyphosphoric acid [21]. Moreover, it has been claimed that phosphorylated pullulan can bind to hydroxyapatite in bone tissues by an ionic connection between phosphate functional groups and apatite calcium and that may play a crucial role in bone tissues regenerating [2]. A study, however, found that Pullulan/dextran composite scaffolds might promote early calcification and the development of bone tissue [22].

Further study is required to evaluate the adhesive property of this newly developed composite scaffold.

4.5. Conclusion

In this study, we synthesized BMP/PPL/β-TCP composite scaffolds of low BMP-2 doses to find a composite of higher osteogenic properties and lower BMP-2 dose. Our conclusion is that BPT2 scaffolds induced greater bone formation than BC1 implant material with only one-tenth of recommended doses. However, these newly developed scaffolds can be regarded as a very promising material in bone regeneration.

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CHAPTER 5: GENERAL CONCLUSION

Study 1:

The present study demonstrated that 1.167 and 0.117 mg/ml of rhBMP-2 with ACS as the carrier induced ectopic bone formation in 4 weeks. The in vivo findings, on the other hand, confirmed the ability of ACS to retain rhBMP-2, support the newly formed bone, and show a good biodegradation rate. However, bone formation was not observed with 0.039 mg/ml of rhBMP-2. Moreover, rhBMP-2 was found to be osteogenic even at one-tenth of recommended concentration, indicating the potential for clinical use at lower concentrations. Future research might focus on the development of release control delivery vehicles. Combining rhBMP-2 with various bone grafting materials improves mechanical strength and osteogenic potential while using lower rhBMP-2 concentrations.

Study 2:

This study represents a further development in the method used for Study 1. This demonstrated the same concentration of rhBMP-2 used in Study 1(1.167 mg/ml), which combined with phosphorylated pullulan and β-tricalcium phosphate. These composite scaffolds were implanted in the ectopic sites of rat model with 2 weeks of observation. We found that the mixture of 20101B (PPL1) (600,000 MW of PPL) and 20101D (PPL2) (combined 600,000 MW and 1,000,000 MW of PPL) with a ratio of 1-5 is the optimum molecular weight of PPL, which can induce greater bone formation as well as a higher biological degradability of PPL. We concluded that this composite can be considered a potentially promising material in the field of bone regeneration. In the future experiment, the optimum doses of BMP-2 and the optimum amount of β-tricalcium that induce greater bone formation with no side effects might be determined.

Study 3:

This study also reflects an advancement in the approach utilized in Study 1 and 2, in which four different rhBMP-2 doses combined with phosphorylated pullulan and β-tricalcium phosphate. However, we synthesized BMP/PPL/β-TCP composite scaffolds of low BMP-2 doses to find a composite of higher osteogenic properties with lower BMP-2

dose. Our conclusion is that BPT2 scaffolds induced greater bone formation than BC1 implant material with only one-tenth of recommended doses. These newly developed scaffolds can be regarded as a very promising material in bone regeneration. Further studies are required to examine the adhesive behavior of this recently developed composite scaffold.

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