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Original article

RhBMP-2 and -7 combined with absorbable collagen sponge carrier enhance ectopic bone formation: An *in vivo* bioassay

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Background: Recombinant human bone morphogenetic proteins (rhBMPs) have been characterized especially chondrogenic and osteogenic activity both *in vitro* and *in vivo* studies. However, delivery of more than one growth factor by sustained release carrier to orthopedic site has yet been questionable in terms of efficacy and synergism.
Objective: Evaluate osteoinductivity and synergistic effect of rhBMP-2 and -7 using absorbable collagen sponge (ACS) carrier system *in vivo*.
Methods: cDNA of BMP-2 and -7 active domains were cloned and expressed in *Escherichia coli* BL21 Star™ (DE3) using pRSETc expression system. Then, the purified rhBMPs were loaded onto ACS and evaluated by *in vivo* rat subcutaneous bioassay. Two and eight weeks postoperatively, all treated groups were histologically verified for evidence of new bone formation and neovascularization by hematoxylin-eosin staining and light microscopy.
Results: The Wistar rat treated with rhBMP-2 or -7/ACS exhibited new bone formation, compared to ACS control. The group treated with ACS supplemented with both rhBMP-2 and -7 significantly showed the osteoid matrix very well-organized into trabeculae-like structure with significant blood vessel invasion.
Conclusion: The osteogenic induction of rhBMPs was combined with ACS carrier in the *in vivo* bioassay. In addition, the combination of both two potent recombinant osteoinductive cytokines, rhBMP-2 and -7, with ACS carrier demonstrated synergistic effect and might be a more promising and effective choice for therapeutic applications.

Keywords: Absorbable collagen sponge, bone morphogenetic protein, ectopic bone formation, rat subcutaneous model

Bone morphogenetic proteins (BMPs), belonging to the protein super-family of transforming growth factor-β (TGF-β), remain being hot spots for tissue engineering research. BMPs play multifunctional roles in the regulation of growth, differentiation, and apoptosis of various cell types. They exhibit important functions especially during embryonic development and tissue morphogenesis, including chondrogenesis and osteogenesis [1-3]. Many studies concerning osteoinductive property of over 20 BMPs characterized were conducted, but only a few members have been verified to have potential osteoinductive property. The BMP-2 and BMP-7, also known as osteogenic protein-1 (OP-1), have been extensively demonstrated in the osteogenic activity both *in vitro* and *in vivo* bioassay [4, 5]. The benefit of BMPs in induction of mesenchymal stem cells to undergo osteoblastic differentiation have led to the application of recombinant BMPs for promoting bone healing and treatment of bone defects. BMP-2 and BMP-7 have been approved by the FDA as therapeutic protein targets for clinical applications, i.e. long bone fractures and spinal fusion, due to their potent osteoinductive activity.

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To date, recombinant biotechnology has been applied for simple and high-yielded production of the attractive proteins including BMPs. Both BMP-2 and -7 have been commercially cloned and expressed to meet a large therapeutic demand. Traditionally, clinical achievement in the use of rhBMPs also requires the biomaterial which functions as a sustained-release and biocompatible matrix carrier. This requires the capability of carrier to either hold or protect the growth factor from degradation at the site of implantation. Nowadays, absorbable collagen sponge (ACS) has been considered as the qualified candidate for both experimental and clinical studies [6, 7]. Many studies have been reported on evaluating the efficacy of BMPs in conjunction with ACS for local repair of bone defects and *de novo* ectopic bone formation. However, the formulation using the ACS combined with both BMP-2 and -7 has not yet been elucidated.

In this study, we postulated that the combination of rhBMP-2 and -7 might synergistically induce ectopic bone formation when combined to ACS. To prove this hypothesis, we examined the osteogenic induction of ACS combined with either rhBMPs, compared to only ACS and ACS with either rhBMP alone in the *in vivo* rat subcutaneous implant model.

Materials and methods

Animals

Forty male Wistar rats weighing 200-250 g were purchased from National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. Rats were individually housed in stainless steel cages under diurnal lighting cycles at 23±2 °C and fed standard rat laboratory food and water *ad libitum*. The animal experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) and approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University.

Construction of pRSETc/BMP-2 and pRSETc/BMP-7 expression vectors

An osteosarcoma-derived cell line, Saos-2 (American Type Culture Collection, Manassas, USA), was selected as a cell source for BMP expression. Total RNA was extracted from osteosarcoma cells using the RNeasy Mini Kit (Qiagen, Valencia, USA) according to the protocols provided by the manufacturer. The coding region of both mature BMP-2 and -7 were amplified by RT-PCR using total osteosarcoma RNA as a template. The following primer pairs: bmp2F (5' GGATCCAAGCCAAACA CAAACAG 3') and bmp2R (5' AAGCTTAGCGA CACCCACAACCCTC 3') for BMP-2; bmp7F (5' CTGCAGCTGTCCACGGGGAGCAAACAG 3') and bmp7R (5' AAGCTTAGTGGCAGCCACAGG CCCG 3') for BMP-7, were used for cDNA amplification. The underlined nucleotides for cloning purposes were represented as BamHI and HindIII restriction sites for BMP-2 and PstI and HindIII restriction sites for BMP-7 in the forward and reverse primers respectively. The amplicon corresponding mature BMP-2 domain was initially cloned into pGEM T-easy vector (Promega, Madison, USA). After positive clone selection, DNA fragments encoding mature domain of BMP-2 and -7 were digested and sub-cloned in frame downstream of N-terminal 6x histidine (His) tag in a pRSETc expression vector (Invitrogen, Carlsbad, USA), resulting in the construct pRSETc/BMP-2 and pRSETc/BMP-7 respectively. Construction of expression plasmids as described above was done in *Escherichia coli* strain DH5α and verified for correct insertion by automated DNA sequencing analyses.

Expression of rhBMP-2, -7 proteins

The recombinant plasmid pRSETc/BMP-2 and pRSETc/BMP-7 were individually transformed into *E. coli* BL21 Star™ (DE3) (Invitrogen, Carlsbad, USA) for expression experiments. A freshly transformed colony of *E.coli* was inoculated into Luria Bertani (LB) broth containing 100 μg/mL ampicillin and incubated overnight at 37°C with 200 rpm. The overnight starter was diluted 1:100 in LB-ampicillin and incubated at 37°C until an OD_{600} value reached approximately 0.4-0.6. Then, the culture was induced for protein expression by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and allowed to grow at 18°C with 180 rpm for an additional 18 hours. The bacterial cells were harvested by centrifugation at 4,000xg for 10 minutes at 4°C. The pellet was washed twice with phosphate buffered saline (PBS) and kept frozen at -80°C until use.

Purification of rhBMP-2, -7 inclusion bodies

The frozen pellet was resuspended in PBS supplemented with lysozyme to a final concentration of 0.5 mg/mL and incubated on ice for 30 minutes prior to sonication four times with a burst duration of

15 seconds each. Briefly, the pellet fraction containing rhBMP inclusion bodies was washed in wash buffer, pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, 0.5% Triton X-100), centrifuged (12,000xg at 4°C for 15 minutes), and resuspended in buffer B, pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 8 M urea) at 4°C for 30 minutes with gentle agitation. After centrifugation to remove insoluble material (12,000xg at 4°C for 20 minutes), the supernatant was collected as urea-solubilized fraction.

The proteins of interest were purified under denaturing condition using Ni^{2+}-nitrilotriacetic acid (Ni-NTA) spin kit (Qiagen, Valencia, USA). Briefly, the solubilized fraction was applied onto a column pre-equilibrated with buffer B. The column was washed thrice with buffer C, pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole and 8 M urea). Finally, the bound His-tagged rhBMP was eluted with buffer E, pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole and 8 M urea). Refolding using the SnakeSkin® dialysis tubing (Pierce, Rockford, USA) was performed in a stepwise manner as follows: 100 volumes of the dialysate 1 (50 mM NaH_2PO_4, pH 8.0, 100 mM NaCl, 0.1 mM dithiotreitol (DTT) and 5 M urea), 100 volumes of the dialysate 2 (50 mM NaH_2PO_4, pH 8.0, 125 mM NaCl and 2.5 M urea), and 100 volumes of the dialysate 3 (50 mM NaH_2PO_4, pH 8.0 and 150 mM NaCl), for three hours, respectively. The dialysis process was all carried out at 4°C. Then, the refolded proteins were applied to HiTrap™ heparin-sepharose columns (GE Healthcare, Uppsala, Sweden) as previously described [11] for dimer purification. Aliquots of the refolded dimer were used for protein quantitation by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, USA) using bovine serum albumin as a standard.

Absorbable collagen sponges preparation

Absorbable collagen sponge was prepared from pepsin-treated type I collagen. Briefly, pepsin-treated type I collagen was dissolved in 0.05 mM acetic acid at final concentration of 0.8% (w/v). Then, 1 mL of collagen solution was poured into wells and frozen at -20°C for 24 hours. The frozen collagen was lyophilized and then incubated at 37°C and 80°C under vacuum condition for at least three hours and 24 hours, respectively. The sponge was cut into small pieces with 6 mm diameter and 2 mm thickness using biopsy punch and finally UV-irradiated for two hours.

In vivo rhBMPs/ACS implantation

The rhBMP-2 and -7 solution were individually loaded onto collagen sponges with 5 μg of total protein amount for rhBMP-2/ACS and rhBMP-7/ACS and 2.5 μg of each for rhBMP-2 and -7/ACS. The pieces of collagen sponges were then incubated for two hours at 37°C prior to the operation. The rats were anesthesized, shaved and disinfected with povidone iodine at dorsal surface. The incisions were vertically performed at the paravertebral back skin to implant the sponges at the subcutaneous tissues. Two and eight weeks postoperatively, the implants were removed from the sacrifices, fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin prior to sectioning. The 5 μm thick sections were stained by conventional hematoxylin-eosin (H&E) method and verified by light microscopy.

Results

Expression of rhBMP-2 and -7

Complete cDNA fragments encoding mature domain of BMP-2 and -7 were successfully amplified via RT-PCR using RNA template from osteosarcoma cell. After verifying inframe insertion by DNA sequencing, rhBMP cDNAs were subcloned from pGEM T-easy vector into pRSETc expression vector and induced by 0.5 mM IPTG to express heterologous protein in *E. coli* BL21 Star™ (DE3) under T7 RNA polymerase/promoter system. The insoluble pellets were obtained after sonication and solubilized in denaturing condition. The solubilized fraction was then loaded into Ni-NTA spin column for first-step His-tag purification. The purified sample was stepwise dialyzed for excessive urea removal and *in vitro* refolding. Finally, monomers and polymers were excluded from active dimers by heparin affinity chromatography with NaCl gradient elution.

In vivo rat subcutaneous model

The numbers of Wistar rats giving positive result of new bone formation are shown in **Table 1.**

At two weeks, a large number of inflammatory cells, especially both polymorphonuclear and mononuclear cells, were markedly infiltrated in ACS control (**Fig. 1**), particularly in the periphery of the implant but new blood vessel invasion was limited.

Unsurprisingly, ectopic bone formation was evident in ACS implants soaked with rhBMP(s). For rhBMP-2/ACS (**Fig. 2**) and rhBMP-7/ACS (**Fig. 3**), newly synthesized collagen was extensively well-organized

into osteoid matrix islets with osteoblast lining at the periphery of the implant without evidence of significant adverse reaction. Considering rhBMP-7/ACS, angiogenesis was not pronounced, compared to rhBMP-2/ACS experiments.

Table 1. The numbers of Wistar rats giving positive result of new bone formation to total number of enrolled rats.

	ACS control	rhBMP-2/ACS	rhBMP-7/ACS	rhBMP-2 and -7/ACS
2 weeks	0:10	7:9*	9:9*	10:10
8 weeks	nd	nd	nd	nd

* After postoperative two weeks, two rats were excluded due to death from surgical complications. nd = not detectable (for positive histological findings in all rats).

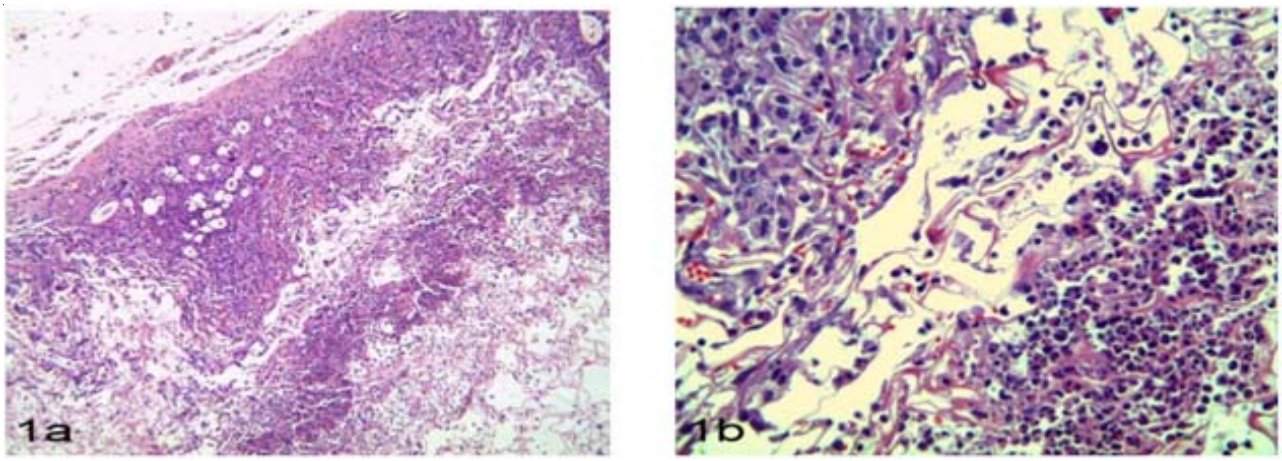

Fig. 1 H&E section photomicrograph of rat subcutaneous tissue implanted by ACS. Original magnification: x 100 (**a**), x 400 (**b**).

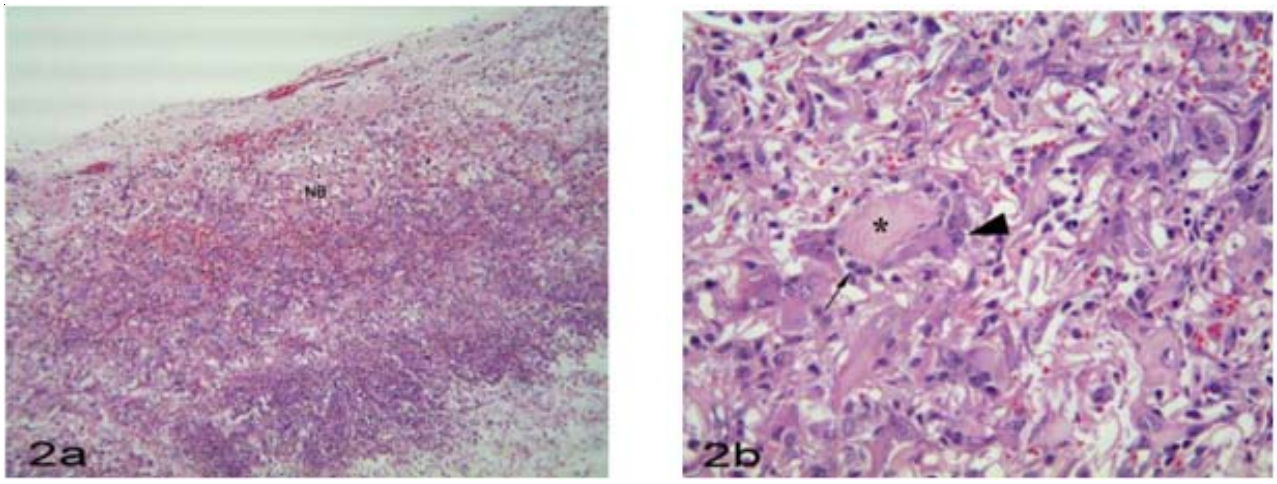

Fig. 2 H&E section photomicrograph at ACS supplemented with rhBMP-2 at 2 weeks. NB = new bone, arrow head = multinucleate giant cell, arrow = osteoblast lining, star = osteoid matrix. Original magnification: x 100 (**a**), x 400 (**b**).

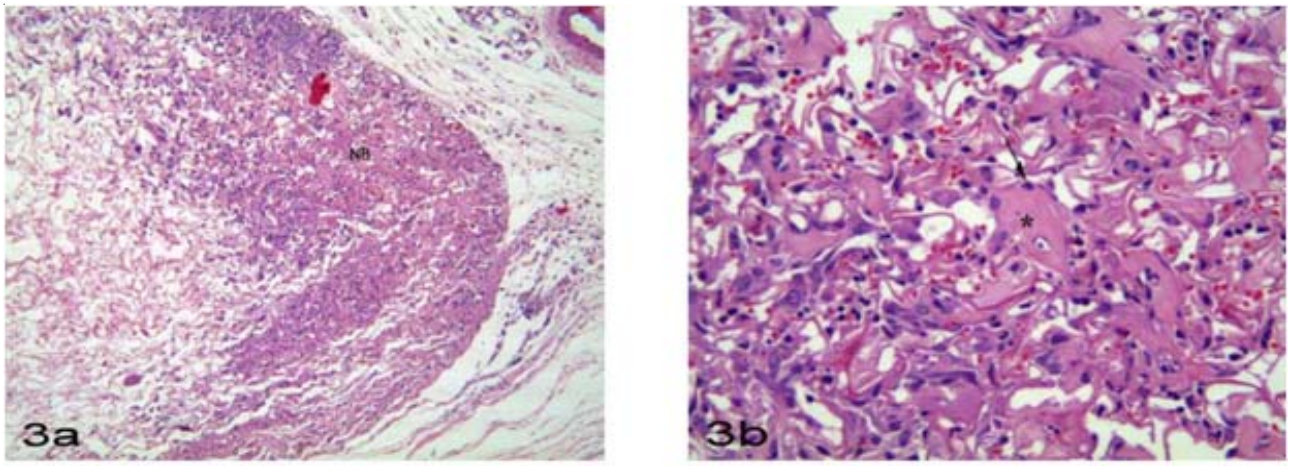

Fig. 3 H&E section photomicrograph at ACS supplemented with rhBMP-7 at 2 weeks. NB = new bone, arrow = osteoblast lining, star = osteoid matrix. Original magnification: x 100 (**a**), x 400 (**b**).

In case of rhBMP-2 and -7/ACS (**Fig. 4**), osteoid matrix, observed at the edge of implants, was more extensively well-organized into trabeculae-like structure with osteoblast lining than other experimental groups. Some osteoblasts were embedded in small lacunae of osteoid matrix. In addition, multinucleated giant cell could be found along the rim indicating the evidence of remodeling. Neovascularization was also extensively pronounced like the rhBMP-2/ACS group, without significant inflammation. At eight weeks, neither residual collagen sponge nor the newly formed bone was observed due to possible degradation.

Discussion

BMP-2 and -7 are the most potent osteoinductive cytokines belonging to TGF-β super family. Extensive studies have reported that these BMPs signaling play the key role in regulation of cartilage and bone formation [1, 2]. Another striking feature was that the solved crystal structure of BMP-2 at 2.7 angstrom resolution has been shown to be very similar to that of BMP-7. Therefore, this structural similarity reflected their resembled biological role [8, 9].

At present, both BMP-2 and -7 are widely applied in both *in vitro* and *in vivo* researches as well as

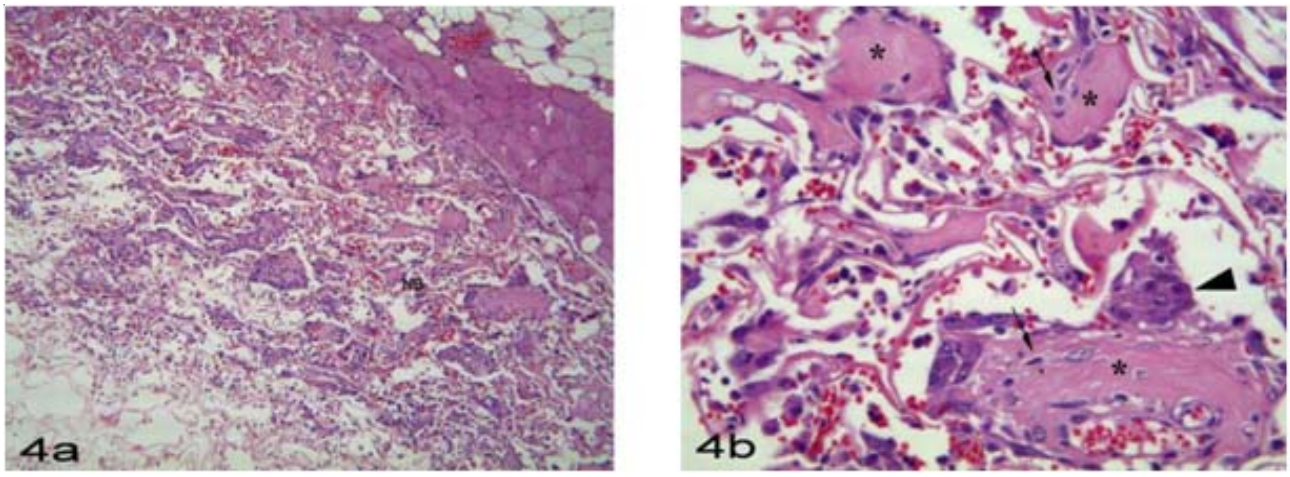

Fig. 4 H&E section photomicrograph at ACS supplemented with rhBMP-2 and -7 at 2 weeks. NB = new bone, arrowhead = multinucleated giant cell, star = osteoid matrix, arrow = osteocyte in lacunae. Original magnification: x 100 (**a**), x 400 (**b**).

clinical therapeutic purposes. Therefore, recombinant DNA and protein purification technology were required for producing these therapeutic cytokines. The expression of heterologous forms of the early BMPs was accomplished using mammalian cell lines, most particularly Chinese hamster ovary cells [4, 10]. However, several methods had been successfully developed for producing fully functional BMPs in *E. coli* expression system [11-13].

To produce active rhBMPs, transformants harboring pRSETc/BMP-2 and -7 were induced by IPTG to express the protein of interest. Expectedly, highly-yielded insoluble BMPs were obtained. We have attempted to adjust growth temperature, IPTG concentration, and medium component, but the solubility was not yet improved (data not shown). A possible explanation for these findings was that our eukaryotic proteins were expressed in prokaryotic system lacking post-translational modifications such as glycosylation and disulfide bond formation, essential for fully functional and correctly folded dimeric BMPs. Although some studies had attempts to insert periplasmic localizing sequence at N-terminal for enhancing disulfide bond formation and proper conformation at periplasmic space, those expressed proteins were yet in form of inclusion bodies [13]. However, our heterologous dimeric BMPs after two -step purification had retained biological activities when co-implanted with absorbable collagen sponge in rat subcutaneous tissue.

We could successfully demonstrate the effect of rhBMP-2 and -7 in combination with collagen sponge carrier to *in vivo* ectopic bone formation in the rat subcutaneous model. The rat subcutaneous bone formation assay has been the standard method used to evaluate the osteoinductive potential of BMPs. Implantation of purified recombinant BMP with collagen matrix in subcutaneous sites in rats induces a sequence of cellular events that eventually results in the formation of new bone. Although this subcutaneous model would remarkably show the new bone formation in amount and maturation aspect less than the calvarial defect model, this ectopic model could presumably exclude the osteogenic effects of several growth factors from orthopedic site.

Bone has very high potential to self-repair and regenerate a new bone identical to the native tissue. Thus, to evaluate the potency of rhBMPs when combined with ACS, the subcutaneous model was conducted to enable the exclusion of the intrinsic effect from surrounding native bone and clarify the hypothesis. Nevertheless, the growth factor concentration for optimal osteoinduction had been yet a controversial issue. Visser et al. [14] demonstrated the achievement of new bone formation at rhBMP-2 concentration of 0.5 .00g. Kubler et al. [15], in contrast, claimed that higher dose (40 .00g) was required for more effective osteoinduction in ectopic sites. In the present study, 5 µg of total purified protein, determined as the optimal dose of rhBMP(s) in each group, yielded a satisfactory result. Obviously, newly ectopic bone in subcutaneous tissue could be observed after two weeks post-implantation in all rhBMP-treated groups. Compared to the effects induced by only BMP-2 or -7 alone, well-organized osteoid trabeculae and new blood vessel invasion were significantly exhibited in response to implantation with both rhBMPs. To a point of interest, BMP-2 (or -4) binds preferentially to BMPRIA/ALK-3 and BMPRIB/ALK-6 whereas BMP-7 (or -6) had higher affinity to ALK-2 [16, 17]. Under these conditions, it was worth noting that significant ectopic bone formation observed with the combination of neovascularization could be due to the synergistic or combined effect of these two potent osteoinductive cytokines.

The mechanism underlying the synergistic or combined effect of these two BMPs remains to be elucidated. Despite the different receptors required for individual BMP signaling, overlapping expression and co-localization during mouse embryogenesis had been reported [18, 19]. Israel et al. [20] established that heterodimeric BMP-2 and -7 was more potent than BMP-2 in both *in vitro* and *in vivo* assays. Intriguingly, it was possible that the more potent *in vivo* osteoinductive event in case of BMP-2 and -7 combination may be accompanied with the activation of downstream signaling cascades via two different subtypes of type I BMP receptors, ALK3/6 and ALK2. In parallel with our speculation, Aoki et al. [21] concluded that transcriptional activity determined by luciferase assay was significantly up-regulated in C2C12 cells co-transfected with ALK-2 and ALK-3 DNAs in synergistic fashion. In addition, *in vitro* alkaline phosphatase activity of C2C12 cells stimulated by two different BMPs isoforms, BMP-4 and -6, was synergistically increased, in comparison to induction with either BMP alone. Their findings might reflect the synergistic effect of BMP-2 and -7 in cooperation determining osteoblastic differentiation in the present study.

All our experimental groups at eight weeks showed that the implants disappeared, consistent with the results of several studies. We hypothesized that type I collagen after treatment with pepsin might lose the structural stability resulting in the short half-life. These findings also were attributed by Lee et al. [22] to the lack of ACS capacity to maintain space and the varying characteristics of ectopic site. Kim et al. [23] had also proposed that collagenase activity might be induced during early development of new bone. Therefore, the limitation of this carrier system was incapability to be stable enough and maintain space for a long period to fully exhibit bone maturation and remodeling in ectopic model. Other carrier systems, i.e. calcium phosphate ceramics and synthetic polymers, were choice of interest for combination with collagen sponge to provide more structural integrity and space maintenance property [24].

In conclusion, the osteogenic induction of rhBMPs was combined with ACS carrier. The combination of these two potent rhBMP-2 and -7 with ACS might be a more promising and effective choice for therapeutic applications.

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The authors have no conflict of interest to report.

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