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Use of Native Bovine Bone Morphogenetic Protein Extract in Healing Segmental Tibial Bone Defects in Goats

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Abstract

We tested the new bone-forming activity of injectable native bovine BMP extracted from cadaveric bones obtained from the abattoir after SDS PAGE electrophoresis was used to determine the molecular weights and extract was bio-assayed in the goat thigh muscle pouch model. The bovine BMP extract was further used as an implant in an absorbable collagen sponge (ACS) and hydroxyapatite to heal segmental bone defects in a large animal tibia fracture model. Open tibia fractures were created in 20 adult goats with loss of 1.5 cm segment of the bone. 10 goats were treated with an implant of the study device (0.2 mg of extracted bovine BMP, ACS and hydroxyapatite) while 10 goats were treated with an implant of Buffer, ACS and hydroxyapatite. The devices were implanted as a mould in the segmental defects. The animals were monitored for callus formation which was measured on lateral radiographs and mean callus indexes determined. Radiographs indicated increased callus at 3 weeks in the extracted bovine BMP/ACS/hydroxyapatite treated tibiae. At 6 weeks, the extracted bovine BMP/ACS/hydroxyapatite treated tibiae had superior radiographic healing scores compared with the control group. The extracted bovine BMP/ACS/hydroxyapatite treated tibiae produced significantly larger volume of callus ($p<0.02$) compared to the buffer/ACS/hydroxyapatite treated tibiae. Total callus and new bone volume was significantly increased ($p<0.02$) in the extracted bovine BMP/ACS/hydroxyapatite treated tibiae compared with buffer/ACS/hydroxyapatite groups. Extracted bovine BMP/ACS/hydroxyapatite altered the timing of onset of periosteal/endosteal callus formation in the treated groups compared to untreated controls.

Keywords: Bone morphogenetic protein; Osteo-induction; Hydroxyapatite; Absorbable collagen sponge; Tibia; Segmental bone defects; Fracture; Bone graft; Bone healing

Introduction

Approval from the Ahmadu Bello University Committee on Animal Care and Use (ABUCAUC) was obtained before the commencement of the study.

The use of autogenous bone has always remained the standard in bone grafting. However, the limitations of obtaining autograft bone are obvious: the amount of bone that can be harvested is limited and the procedure causes secondary morbidity at the harvesting site. Recently introduced substitutes for autologous bone grafts include frozen or freeze-dried allografts from cadaveric sources [1]. These materials are less osteo-inductive, though they do retain their osteo-conductive property, permitting the growth of bony trabeculae. Bone allografts are rarely rejected, but there are some risks such as the transmission of infection, including human immunodeficiency virus infection. Occasionally, incomplete healing is seen in spite of proper grafting procedures. Bone healing involves a series of molecular events such as cellular proliferation and differentiation that leads to gradual restoration of bone integrity. The cellular signals that control these molecular events are poorly understood, although recent advances in molecular genetic technologies have begun to unravel this complex biological event. Numerous growth and differentiating factors (cytokines, hormones) and the extracellular matrix are known to be involved in fracture healing [2]. Stimulation of bone regeneration is a challenging idea, which would solve the problem of healing large bone defects. Thus, other methods of obtaining bone graft substitute and stimulating osteo-induction are being researched. The earlier research done by Urist aroused the idea of agents that are able to induce new bone. It showed the ability of demineralized bone matrix (DBM) to induce bone ectopically, when implanted in rabbits and rats intramuscularly [3]. It was later shown that low-molecular weight proteins extracted from DBM had osteogenic activity [4]. They were called bone morphogenetic proteins (BMPs). Bone morphogenetic proteins (BMP) are cytokines of the transforming growth factor beta family. The BMP plays a major role in skeletal development and the maintenance of homeostasis during bone remodeling. The objectives of this study were to: (i) extract and characterize BMP from bovine bone obtained from cadaveric sources, (ii) confirm osteo-inductive capacity of native BMP extracts from bovine bone, (iii) to evaluate the rate of bone healing using different implants with and without BMP, (iv) test absorbable collagen sponge and hydroxyapatite implants as suitable carrier materials for BMP and (v) evaluate the effect of extracted native bovine BMP in healing segmental long bone defects in goats. The importance of this work lies in the carefully controlled demonstration that new bone formation can be induced independent of the surrounding bone tissue and that low-molecular weight proteins extracted from demineralized bone matrix; the bone morphogenetic proteins (BMPs) has osteogenic activity. Segmental long bone defects have been used as models for bone reconstruction to evaluate different transplant materials as well as the efficacy of BMP. This model is valid in studying osteo-inductive agents when the defect is too large to heal spontaneously [5]. The tibia bone defect model has been used to test bone substitute by many researchers [6,7]. DBM combined with BMP either extracted from bone tissues or produced by gene therapy and

engineering has been shown to be effective in the reconstruction and repair of bone defects [8-14]. In this study the effect of biocompatible carriers ACS and hydroxyapatite implants impregnated with bovine BMP was tested to demonstrate that native bovine BMP extract could be ostegenic and serve as graft substitute in the repair of large segmental bone defects in a large animal model. It has been reported that local delivery of BMP has disadvantages which includes short half-life, need for repeated application and large dose requirement resulting in high cost [11,15] In this study however high BMP cost was reduced by substitution of urea for guanidine hydrochloride (GuHcl) at the initial stage of extraction [16,17].

Materials and Methods

Extraction purification and characterization of bovine BMP

Ten kilogram of fresh bovine long bones was obtained from cattle slaughtered at the Nsukka abattoir. The epiphyseal ends were cut off with a bone saw, the diaphysis were mechanically scraped clean of soft tissues and extensively washed in cold water. The washed bones were frozen and then reduced to particulate sizes of about 1 mm^2, defatted in chloroform/methanol (1:1) and washed again in cold water. The bone particles were demineralized in 0.6M HCl for 72 hrs, the demineralized bone matrix was extensively washed in NaN_3 solution and deionized water and extracted in 8M urea pH 7.4 (Carbamide, May and Baker Dagenhamp, Germany) 1M $NaCl_2$ and 50mN Tris [Tris(hydroxymethyl) aminomethane] (Merck Specialities Ltd, USA) at 4°C for 48 hrs with a magnetic stirrer. The supernatant was filtered using a cheese cloth, allowed to stand overnight in the cold and dialysed against 23 volumes of deionized water over night at 4°C while stirring. The extracted solution was passed through a Millipore filter (pore size 0.6 m, Millipore incorporated MI, USA). The filtered solution was then dialysed against deionized water and the water-insoluble precipitate was re-dissolved in 4M GUHCL. Gelatin peptides were removed by dialysis against 0.52 M citrate buffer and the precipitate was centrifuged and lyophilized. The water insoluble bovine BMP was then collected [17]. SDS PAGE technique was used to test for the molecular weight of the proteins extracted; presence of series of low molecular weight proteins in the extracted material indicated the presence of proteins similar to BMPs.

Bio-assay of extracted bovine BMP

Bioassay was done by injecting 2 mg of native bovine BMP extract into the right thigh muscles of goats. While the left corresponding thigh muscles of the same goats were injected with physiological saline alone which served as controls. New bone formation was evaluated radiographically 14 days and 21 days after the injections respectively.

Surgery

Twenty adult male Kano brown goats used for the clinical study were randomized into two groups of 10 each. Blood samples were collected from the goats and iSTAT EC8+cartridges were used to analyse them. ACS and hydroxyapatite were used as the carrier device for the bovine BMP. Goats in group 1 were treated with ACS, hydroxyapatite and extracted bovine BMP and goats in group 2 were treated with ACS, hydroxyapatite and phosphate buffered saline (PBS). For surgery, all animals were pre-medicated and general anesthesia was induced using ketamine (5 mg/kg) intravenously and maintained with xylazine (0.05 mg/kg) intramuscularly. Cephazolin sodium (35 mg/kg) intravenous injection; was administered prior to surgery for prophylaxis. The left hind limbs were shaved of hair and aseptically prepared for surgery and the proposed site for surgery was locally infiltrated with lidocaine 2% in a ring block. Animals were placed in a posterior oblique position. On the lateral surface of the tibia, a 5 cm linear cutaneous incision was made 12 cm distal from the stifle joint to access the tibia. The gastronemius and soleus on the postero-lateral aspect of the limb extending inferiorly to the lateral aspect of the malleolus were displaced caudally to isolate the tibia. A manual retractors was used to pull the posterior muscle mass medially to allow the exposed tibia to be stabilised using a bone forceps. The tibialis posterior was then dissected in part from its origin on the posterior aspect of the interosseous membrane. The posterior tibia artery, vein and nerve were not exposed but retracted medially in the muscle mass. An osteotome and a ronguers were used to transect the bone to create a mid-shaft fracture. The proximal and the distal segments of the fractured left tibia were carefully proned to excise some chips and create a gap of about 1.5 cm. The prepared BMP implants were used as fillers to bridge the gap. The retracted muscles and tendons were rearranged and cutaneous closure was in 3 layers using polyglactin 910 2/0 in simple continuous patterns for the deep fascia and the subcutis while nylon 2/0 was used to close the skin in horizontal mattress suture patterns. All the goats were placed on cephazolin sodium (35 mg/kg i/v) and dexamethasone (0.5 mg/kg i/m) for 5 days post-surgery. Following successful surgery and implantation, a plaster of paris cast and a compression bandage was placed over the affected limbs for fixation and partial stabilization. There was no attempt to fix the fractures with any internal fixation device. Radiographs were taken immediately after surgery and repeated every two weeks post-surgery for twenty weeks to assess rate of callus formation, cortical bridging and restoration. Maximum callus diameter (MCD) in centimeters were measured on lateral radiographs and recorded for each animal (Figure 1) and callus index (CI) was calculated by dividing the callus diameter by the Bone diameter (BD). By using a ratio in this way, any variation in magnification or rotation was minimized. The mean of the callus index for each group was then recorded against time in weeks.

Statistical analysis

Data generated were analyzed using student t-test with Statistical Package for Social Sciences (SPSS) version 16.0 for windows to determine significant differences between the means of the two groups (Table 1). Significance was accepted at 5% probability level. Data were further presented in charts (Figure 2).

Week	GROUP 1	GROUP 2
2	2.48 ± 0.06a	1.48 ± 0.03b
4	2.50 ± 0.06a	1.66 ± 0.07b
6	2.40 ± 0.22	2.00 ± 0.05
8	1.74 ± 0.04a	2.30 ± 0.05b
10	1.48 ± 0.07a	2.08 ± 0.05b
12	1.26 ± 0.08a	2.04 ± 0.04b
14	1.16 ± 0.04a	1.82 ± 0.02b
16	1.14 ± 0.05a	1.72 ± 0.05b
18	1.08 ± 0.02a	1.42 ± 0.04b

20	1.06 ± 0.02	1.16 ± 0.05

Table 1: Mean callus index of goats in group 1 and group 2. Values with different superscripts in the same horizontal row are significantly different from each other at $p<0.05$.

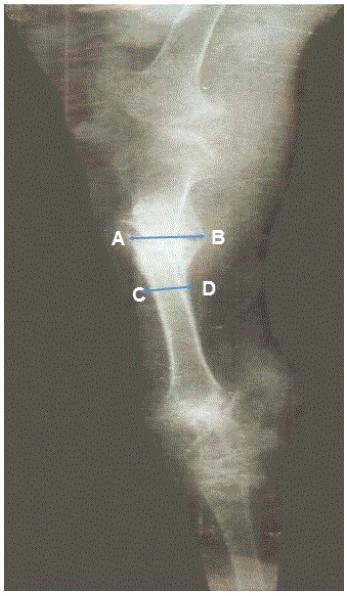

Figure 1: Measurement and determination of callus index on lateral radiographs.

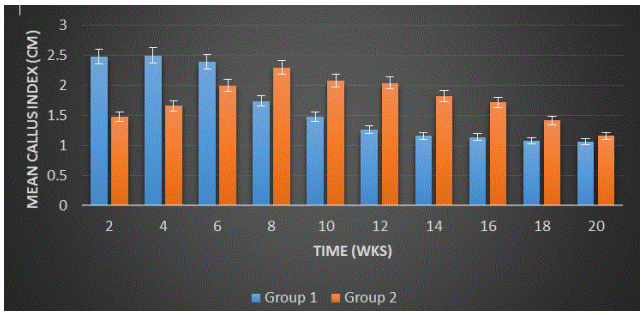

Figure 2: Chart of mean callus Index (cm) against (wks).

Results

10 gm of the water insoluble protein bovine BMP extract was collected after the alkaline urea and GuHcl dissociative extraction of demineralized bone matrix. Purity of proteins was therefore determined by 15% SDS PAGE (3 µg non-reduced). Electrophoretic patterns on SDS PAGE gel followed by western blotting from extracted bovine BMP with Dalton Marks VII-L standard mixture to act as marker in the corresponding band denoted proteins of approximately 13.5-25 kDa molecular mass markers. Blots corresponded to the position and approximate sizes in kDa of molecular mass markers (Figure 3). None of the injections without extracted bovine BMP were able to induce new bone visible in radiographs, whereas 2 mg injections with crude extracted bovine BMP induced osteogenesis effectively. A radiopaque density at the injection sites in the radiographs of treated group was evident 14 days post-surgery (Figure 4).

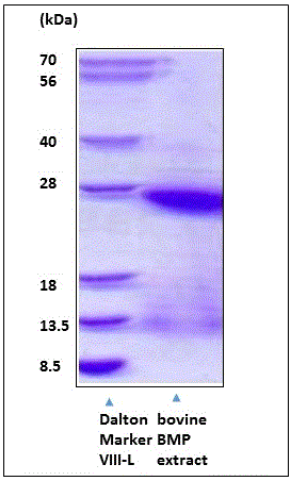

Figure 3: SDS PAGE result showing extracted bovine BMP on right column with molecular weight range 13.5 -25 kDa.

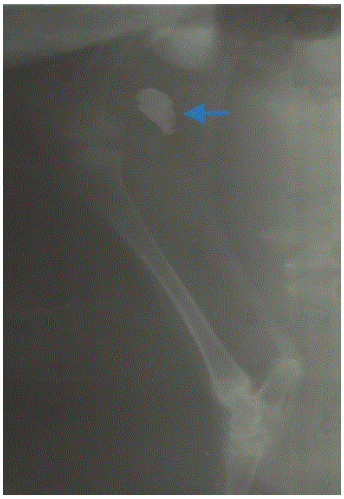

Figure 4: Radiograph of goats at day 14 post injection of bovine BMP extract showing osteo-induction.

Surgeries were successful and well tolerated by the goats. Weight bearing began within four days post-surgery. There was no infection.

There was significant differences ($p<0.05$) in the mean callus index (MCI) of both groups at week 2, 4 and 8-18 post-surgery. There were no significant differences ($p>0.05$) in the MCI of all the groups at week 6 and 20 post-surgery. At week 2, the MCI of goats in group 1 was significantly higher ($p<0.02$) when compared to group 2. At week 8, the MCI of goats in group 1 was significantly ($p<0.05$) lower than those of goats in group 2. There was significantly higher volume ($p<0.05$) of new bones formed in the bovine BMP treated group when compared to untreated group (Table 1).

Discussion

The study of BMPs began in the 1960s, with the observation that demineralized bone matrix had the capacity to induce endochondral bone formation in subcutaneous and intramuscular pockets in rodents [18,19]. The research group subsequently isolated a low-molecular weight glycoprotein from bone and demonstrated that it promoted bone formation when ectopically located Urist called these substance Bone Morphogenetic Proteins [20,21].

A major stumbling block to purification was the insolubility of demineralized bone matrix. To overcome this hurdle, Reddi [17] used dissociative extractants, such as 4M guanidine HCl, 8M urea, or 1% SDS. The soluble extract alone or the insoluble residues alone were incapable of new bone induction. This work suggested that the optimal osteogenic activity requires a synergy between soluble extract and the insoluble collagenous substratum. In spite of the method used, purification of BMPs is an extremely laborious process and the yields are usually low. Preparations must be initiated with a minimum of 10 kg of washed fresh cortical bone free of bone marrow. Since their molecular weight ranges from 15 to 30 kDa, partial purification of BMPs yields 1 mg of the pool of BMPs per kg of fresh bone. Isolation of a particular native BMP yields even smaller amounts of the order of µg/kg tissue. The small amounts of BMPs resulting from such a laborious purification process have stimulated the application of molecular biology techniques for the cloning and expression of these proteins [22]. In this study considerable cost of extraction of BMP was reduced by substituting 8M Urea for 4M GuHCl at the early stage of extraction and dialysis was done to remove the urea before further extraction in 4M GuHCl native protein yield was comparable to when GuHCl alone was used and the osteoinductive capacity of the extracted proteins were retained. Result of this study showed that about 0.1% solubilized protein and 0.01% of the initial 10 kg bovine bone used for the extraction was purified bovine BMP extract. It should be noted that solubilized protein must at some stage be folded into the native conformation; hence, the most effective solubilizing conditions might not necessarily be the best, especially if they cause irreversible denaturation. Irreversible denaturation appears to result from chemical modification of the protein and is induced by such factors as high temperature, extremes of pH, and tight binding of denaturants such as SDS.

BMPs have been isolated from the bones of a variety of mammals: mouse, rats, bovine, monkey and man [23-26] and the extracted BMP were found to induce bone formation in not only the same species but also in other species. In this study BMP was extracted from bovine bone. The extracted BMP induced new bone formation in the thigh muscle pouches of goats. While the structure of the bovine BMP extracted in this study was unknown, there might be a common structure between dentin derived BMP and bovine bone derived BMP in this study as the bovine BMP extract was capable of inducing new bone growth in the same period reported for dentine derived BMP after implantation 5-14 days [27,28]. There have been many reports on the molecular weight of BMP, suggesting values of 17-18 kDa [29], 22 kDa [30] from bovine bone, 25 kDa [28,31] from rabbit dentin. The molecular weight of the dentin and bovine bone derived BMP was somewhat larger than BMP derived from other sources and similar to bovine BMP 13.5-25 kDa extracted and used in this study.

Segmental long bone defects have been used as models for bone reconstruction to evaluate different transplant materials as well as the efficacy of BMPs. This model is valid in studying osteoconductive agents when the defect (large enough) does not heal spontaneously [32]. Segmental bone defects and non-unions resulting from trauma, resection or pathology represent significant clinical challenges world-wide, for the orthopaedic, reconstructive and maxillofacial surgeons [33]. To date, bone grafting techniques that use materials such as autografts, allografts or metallic implants face considerable limitations for bone repair due to lack of available bone tissue, disease transmission, cell-mediated immune responses, and significant costs. These difficulties have resulted in the search for alternative bone graft substitutes to be used in the repair of non-union fractures, spinal fusions and bone tumour resections.

In this study, tibia segmental bone defects were created and treated effectively with native bovine BMP extract. The rate of bone healing was significantly faster and there were significantly higher volume of callus formed in the bovine BMP treated group when compared to the untreated group. Previous animal studies with bone defects treated with bone substitute materials or BMP include dog radius [34,35], dog femur [36], sheep tibia [37], rabbit radius [38] and dog ulna [39].

Many different carrier materials have been used in a variety of animal models, in which bone morphogenetic proteins have been tested [40] but the optimal carrier material for BMPs still remains to be found. The optimal type of carrier material used will probably depend on the clinical indication to which the morphogenetic protein will be applied [41]. ACS/hydroxyapatite molds used as the carrier material in this study provided support for the fractures and when impregnated with the bovine BMP extract resulted in significantly higher rates of union and repair of the large segmental bone defects.

This study further demonstrated that bovine BMP implanted using ACS and hydroxyapatite enhanced normal fracture healing mechanisms in this segmental tibial defect model. The increased amount of callus formation found in the study suggests that an osteo inductive potential exists in bovine BMP which tend to heal fractures more rapidly. Absorbable Collagen Sponge and hydroxyapatite impregnated with native bovine BMP extracts implanted in the segmental defects in this study enhanced bridging of the defects by increasing osteoinduction, osteoconduction and promoting rapid and exuberant callus formation which has also been earlier reported [42].

In evaluating the results, various methods of analysis were used, the principal method being radiography. Several radiographic features are easily observed during indirect or secondary fracture healing with the production of an external callus, these being the formation and growth of a calcified callus and the bridging of the fracture callus. Measurement of external callus on radiographs was first described by Spencer [43] and thereafter it was used to measure callus index and calculates callus volume from the radiographs [44]. Spencer's method was refined in this study using serial radiographs to produce a quantifiable measurement which we refer to as the "Mean Callus Index. (MCI)" Callus index is defined as the ratio of the maximum callus diameter to bone diameter at the same level as the callus. As callus grows the callus index will increase, indicating that healing is progressing, and since remodeling of the fracture callus does not commence until sufficient stability of the fracture has been achieved, the time taken to reach a "Maximum Callus Index" becomes a useful measurement of the time to clinical union.

The results of this study showed that the implant of extracted bovine BMP induces early regeneration of large segmental tibia diaphyseal bone defect in goats. The implanted defects showed an exuberant regenerative process with union occurring within two months and there was significantly ($p<0.05$) larger volume of new bones formed in

the bovine BMP induced regeneration. The release of growth factors and osteo-progenitor cell populations by biocompatible scaffolds have been shown to enhance the regenerative capacity of bone [45,46].

The bovine BMP, ACS and hydroxyapatite implants resulted in increased callus formation. Ordinarily, the cortical bone of ruminant species being plexiform type bone are always slow in remodelling into secondary osteonal bone. In this study, bone fracture and periosteal stripping markedly increased the bone turnover associated with secondary remodeling within the cortical bone. The increased secondary remodeling at 8 weeks implies an advanced state of healing and a potentially more rapid return to mechanical integrity. The increased callus formation associated with bovine BMP, ACS and hydroxyapatite treatment, therefore, appears to be more of increased osteo-progenitor cell recruitment than stimulation of individual cellular activity.

Conclusion

From the study it can be concluded that Bovine BMP can be extracted successfully from fresh bovine bone obtained from the abattoir, the molecular weights of bovine bone derived BMPs ranges between 13.5-25 kDa; protein extracts from fresh bovine bones collected from abattoir can be used to stimulate new bone growth (osteo-induction), bone fracture healing can be significantly enhanced by the use of bovine BMP extracts and absorbable collagen sponge and hydroxyapatite are suitable carriers of BMP for fracture healing in goats.

References

1. Laurencin C, Khan Y, El-Amin SF (2006) Bone graft substitutes. Expert Rev Med Devices 3: 49-57.
2. Nandi SK, Roy S, Mukherjee P, Kundu B, De DK, et al. (2010) Orthopaedic applications of bone graft & graft substitutes: a review. Indian J Med Res 132: 15-30.
3. Urist MR (1965) Bone: formation by autoinduction. Science 150: 893-899.
4. Urist MR, Mikulski A, Lietze A (1979) Solubilized and insolubilized bone morphogenetic protein. Proc Natl Acad Sci USA 76: 1828-1832.
5. Einhorn TA (1999) Clinically applied models of bone regeneration in tissue engineering research. Clin Orthop Relat Res : S59-67.
6. Reichert JC, Epari DR, Wullschleger ME, Saifzadeh S, Steck R, et al. (2010) Establishment of a preclinical ovine model for tibial segmental bone defect repair by applying bone tissue engineering strategies. Tissue Eng Part B Rev 16: 93-104.
7. Lippens E, Vertenten G, Girones J, Declercq H, Saunders J, et al. (2010) Evaluation of bone regeneration with an injectable, in situ polymerizable Pluronic F127 hydrogel derivative combined with autologous mesenchymal stem cells in a goat tibia defect model. Tissue Eng Part A 16: 617-627.
8. Gao TJ, Lindholm TS, Kommonen B, Ragni P, Paronzini A, et al. (1997) The use of a coral composite implant containing bone morphogenetic protein to repair a segmental tibial defect in sheep. Int Orthop 21: 194-200.
9. Xu XL, Wang KW, Yang ZH (2001) A biomechanical study of bBMP-collagen-coral composite bone substitute in the repair of bone defects of femoral heads China J Orthop Trauma 14: 17-19.
10. Kirker-Head CA, Gerhart TN, Armstrong R, Schelling SH, Carmel LA (1998) Healing bone using recombinant human bone morphogenetic protein 2 and copolymer. Clin Orthop Relat Res, pp: 205-217.
11. Wozney JM, Rosen V (1998) Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. Clin Orthop Relat Res, pp: 26-37.
12. Friedlaender GE, Perry CR, Cole JD, Cook SD, Cierny G, et al. (2001) Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. J Bone Joint Surg Am 83-83A Suppl 1: S151-158.
13. Johnson EE, Urist MR (1998) One-stage lengthening of femoral nonunion augmented with human bone morphogenetic protein. Clin Orthop Relat Res : 105-116.
14. Schilephake H (2002) Bone growth factors in maxillofacial skeletal reconstruction. Int J Oral Maxillofac Surg 31: 469-484.
15. Dai KR, Xu XL, Tang TT, Zhu ZA, Yu CF, et al. (2005) Repairing of goat tibial bone defects with BMP-2 gene-modified tissue-engineered bone. Calcif Tissue Int 77: 55-61.
16. Urist MR, Nogami H, Mikulski A (1976) A bone morphogenetic polypeptide. Calcif Tissue Res 21 Suppl: 81-87.
17. Sampath TK, Reddi AH (1981) Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. Proc Natl Acad Sci U S A 78: 7599-7603.
18. Reddi AH (1997) Bone morphogenetic proteins: an unconventional approach to isolation of first mammalian morphogens. Cytokine Growth Factor Rev 8: 11-20.
19. Wang EA, Rosen V, Cordes P, Hewick RM, Kriz MJ, et al. (1988) Purification and characterization of other distinct bone-inducing factors. Proc Natl Acad Sci U S A 85: 9484-9488.
20. Gao TJ, Lindholm TS, Kommonen B, Ragni A, Paronzini A, et al. (1996) Enhanced healing of segmental tibial defects in sheep by a composite bone substitute composed of tricalcium phosphate cylinder, bone morphogenetic protein, and type IV collagen. J Biomed Mater Res 32: 505-512.
21. Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, et al. (1990) Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci U S A 87: 9843-9847.
22. Sampath TK, Rashka KE, Doctor JS, Tucker RF, Hoffmann FM (1993) Drosophila transforming growth factor beta superfamily proteins induce endochondral bone formation in mammals. Proc Natl Acad Sci USA 90: 6004-6008.
23. Urist MR, Lietze A, Mizutani H, Takagi K, Triffitt JT, et al. (1982) A bovine low molecular weight bone morphogenetic protein (BMP) fraction. Clin Orthop Relat Res : 219-232.
24. Bauer FC, Urist MR (1981) Human osteosarcoma-derived soluble bone morphogenetic protein. Clin Orthop Relat Res : 291-295.
25. Urist MR, Lietze A, Mizutani H, Takagi K, Triffitt JT, et al. (1982) A bovine low molecular weight bone morphogenetic protein (BMP) fraction. Clin Orthop Relat Res : 219-232.
26. Luyten FP, Cunningham NS, Ma S, Muthukumaran N, Hammonds RG, et al. (1989) Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. J Biol Chem 264: 13377-13380.
27. Bessho K, Kusumoto K, Fujimura K, Konishi Y, Ogawa Y, et al. (1999) Comparison of recombinant and purified human bone morphogenetic protein. Br J Oral Maxillofac Surg 37: 2-5.
28. Uwagie Ero EA, Kene ROC, Chilaka FC, Kadima KB, Awasum CA, et al. (2014) New bone formation induced by bovine bone morphogenetic protein extract. Asian Acad Res J Multidisciplinary 17: 472-480.
29. Langer R, Vacanti JP (1993) Tissue engineering. Science 260: 920-926.
30. Johnson KD, August A, Sciadini MF, Smith C (1996) Evaluation of ground cortical autograft as a bone graft material in a new canine bilateral segmental long bone defect model. J Orthop Trauma 10: 28-36.
31. Johnson KD, Frierson KE, Keller TS, Cook C, Scheinberg R, et al. (1996) Porous ceramics as bone graft substitutes in long bone defects: a biomechanical, histological, and radiographic analysis. J Orthop Res 14: 351-369.
32. Heckman JD, Ehler W, Brooks BP, Aufdemorte TB, Lohmann CH, et al. (1999) Bone morphogenetic protein but not transforming growth factor-beta enhances bone formation in canine diaphyseal nonunions implanted with a biodegradable composite polymer. J Bone Joint Surg Am 81: 1717-1729.

33. Sciadini MF, Johnson KD (2000) Evaluation of recombinant human bone morphogenetic protein-2 as a bone-graft substitute in a canine segmental defect model. J Orthop Res 18: 289-302.
34. Enneking WF, Burchardt H, Puhl JJ, Piotrowski G (1975) Physical and biological aspects of repair in dog cortical-bone transplants. J Bone Joint Surg Am 57: 237-252.
35. Johnson EE, Urist MR, Schmalzried TP, Chotivichit A, Huang HK, et al. (1989) Autogeneic cancellous bone grafts in extensive segmental ulnar defects in dogs. Effects of xenogeneic bovine bone morphogenetic protein without and with interposition of soft tissues and interruption of blood supply. Clin Orthop Relat Res : 254-265.
36. Cook SD, Baffes GC, Wolfe MW, Sampath TK, Rueger DC (1994) Recombinant human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model. Clin Orthop Relat Res : 302-312.
37. Marcacci M, Kon E, Zaffagnini S, Giardino R, Rocca M, et al. (1999) Reconstruction of extensive long-bone defects in sheep using porous hydroxyapatite sponges. Calcif Tissue Int 64: 83-90.
38. Zellin G, Linde A (1997) Treatment of segmental defects in long bones using osteopromotive membranes and recombinant human bone morphogenetic protein-2. An experimental study in rabbits. Scand J Plast Reconstr Surg Hand Surg 31: 97-104.
39. Teixeira JO, Urist MR (1998) Bone morphogenetic protein induced repair of compartmentalized segmental diaphyseal defects. Arch Orthop Trauma Surg 117: 27-34.
40. Winn SR, Uludag H, Hollinger JO (1999) Carrier systems for bone morphogenetic proteins. Clin Orthop Relat Res: S95-106.
41. Yang XB, Whitaker MJ, Sebald W, Clarke N, Howdle SM, et al. (2004) Human osteoprogenitor bone formation using encapsulated bone morphogenetic protein 2 in porous polymer scaffolds. Tissue Eng 10: 1037-1045.
42. Lind M, Eriksen EF, Bunger C (1996) Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and -6 stimulates chemotactic migration of human osteoblast, human marrow osteoblast, and U2-OS cells. Bone 18: 53-57.
43. Spencer RF (1987) The effect of head injury on fracture healing. A quantitative assessment. J Bone Joint Surg Br 69: 525-528.
44. Oni OO, Dunning J, Mobbs RJ, Gregg PJ (1991) Clinical factors and the size of the external callus in tibial shaft fractures. Clin Orthop Relat Res : 278-283.
45. Kaigler D, Wang Z, Horger K, Mooney DJ, Krebsbach PH (2006) VEGF scaffolds enhance angiogenesis and bone regeneration in irradiated osseous defects. J Bone Miner Res 21: 735-744.
46. Huang YC, Kaigler D, Rice KG, Krebsbach PH, Mooney DJ (2005) Combined angiogenic and osteogenic factor delivery enhances bone marrow stromal cell-driven bone regeneration. J Bone Miner Res 20: 848-857.