Various dosages of BMP-2 for management of massive bone defect in Sprague Dawley Rat

Running title: Management of massive bone defect

Abstract

Introduction: The use of BMP-2 plays an important role in the treatment of extensive bone defect. However, data about the optimal dosage of BMP-2 in the massive bone defect cases is rare.

Material and Method: Twenty-five SD rats were randomly divided into a control group ofhydroxyapatite (HA) alone (Group I), HA+BMP-2 1µg/mL (Group II), HA+BMP-2 5 ug/mL (Group III), HA+BMP-2 10 µg/mL (Group IV), and HA+BMP-2 20 ug/mL (Group V). Osteotomies were performed in each group with 10 mm bone defect in the right femur, followed by fixation and filling the defect. The fracture healing was evaluated by histomorphometry, and radiographs using RUST score.

Results: We found there were significant differences in the mean total area of callus between the treatment groups ($p < 0.001$); there were significant differences in the mean area of woven bone between group II, III, IV and V with the control group (respectively $p = 0.009$, $p = 0.016$, $p = 0.009$ and $p = 0.016$), the area of the cartilage between the treatment groups and control group (respectively $p = 0.009$, $p = 0.009$, $p = 0.009$ and $p = 0.028$). A statistically significant difference was found in the average area of fibrosis between group II and control group, group IV and control group (respectively $p = 0.047$ and $p = 0.009$). RUST scores showed significant differences between the control group and group II, III, IV, V (respectively $p = 0.005$, $p = 0.006$, $p = 0.005$ and $p = 0.006$).

Conclusion: The–administration of BMP-2 stimulates the formation of bone bridging in a massive bone defect. The bone bridging filling massive bone defect depends on the dose or concentration of BMP-2.Administration of an optimal dose (10 μg/mL) of BMP-2 demonstrates better result than lower or higher dose for massive bone defect healing in SD rate.

Level of evidence: II

Keywords: BMP-2, optimum dose, massive bone defect, fracture healing

Introduction

Management of massive bone defect has been a challenging problem for orthopedic surgeons [1,2]. Besides the complexity of treatment regiments, massive bone defect also has a significant morbidity on the long run [2]. There are a variety of interventions available to orthopedic surgeons in managing massive bone defect, including autograft, allograft and transplantation with synthetic bone substitutes [3].The gold standard in treatment is an autograft, but graft substitutes and/or delivery of osteoinductive proteins such as the bone morphogenetic protein (BMP) family are also common alternatives with demonstrated efficacy in bone regeneration [1,3].

Several disadvantages have been reported for allograft transplantation, including incomplete or delayed graft incorporation, poor osteoinductivity, the potential for eliciting a deleterious immune response, and the risk of disease transmission [3]. To circumvent these problems of autograft and allograft, various synthetic bone substitutes such as hydroxyapatite (HA), calcium phosphate cements or biodegradable polymers have been developed [4,5]. In addition, these synthetic bone substitutes provide the benefits including availability, sterility and reduced morbidity at the graft site [3].

Special skills and novel techniques along with recent knowledge are necessary to creating an effective healing [2].Although new advances in technology have broadened the alternative treatment strategies, these recent advances have been dreary, complicated and sometimes non-feasible [4,5].

In cases of critically sized (massive) bone defect where the osteoinductive and osteoconductive components have been lost, a good bone regeneration properties could not be achieved without the application of osteogenic and osteoinductive materials [5]. There are three complementary elements in the process of bone healing and these are: osteoconductive matrix, osteoinductive signal and osteogenic cells when combined together with mechanical fixation could enhance a positive osteoinductive and adequate blood flow [6].

Example of an osteoinductive agent that could be rapidly absorbed is bone morphogenic protein-2 (BMP-2) and demineralized bone matrix (DBM)[2,4].The use of BMP-2 in a massive bone defect management plays a critical role in the differentiation, proliferation and inhibition of various cells acting in the microcellular environment and interacts with many regulatory factors [7-9].BMP-2 plays a role in the process of osteogenesis and chondrogenesis and inhibits osteoclastogenesis via theRANKL signaling[10].Several studies have suggested the increase of BMP-2 doses will accelerate the rate of bone healing up to an optimum dose which rate will go into plateau[4-6].This study aims to determine the effect of differences in various BMP-2 doses on the healing of the fracture with massive bone defects.

Materials and methods

This is an experimental study in white Spraque Dawley (SD) rats with post-test control group design. All procedures in this study was approved by the ethical committee in our institution. The SD rats were aged 3-4 months, weighed 250-350 grams, were of male gender, and had no

physical disability. Twenty-five SD rats were randomly divided into five groups. Group I as the control group underwent segmental osteotomy of the femur which resulted in 10 mm bone defect, followed by internal fixation with K-wire and application of HA to fulfill the bone defect. Similar procedures were conducted in group II to group V with additional administration of 1 mL rhBMP-2 in each group with 1 ug/mL, 5 ug/mL,10 ug/mL, and 20ug/mL dosage to HA for group II-V respectively at the bone defect site (Fig. 1).

Surgical procedures

The SD rats were anesthesized using an intraperitoneal injection of ketamine 80 mg/kg body weight and xylazine 10 mg/kg body weight. By anterolateral approach, vastuslateralis and biceps femoris muscles were retracted from femoral bone meanwhile the periosteum was kept intact. Segmental osteotomy including its periosteum of 10 mm long was done at mid-diaphysis of the femur with a manual saw. The osteotomy site was fixed with a retrograde intramedullary 1.4 mm Kirschner (K) wire through the intercondylar femur. Granules of HA were administered at the bone defect area. The fascia and skin were sutured. Prophylactic antibiotic (ampicillin 100 mg/kg/day) and analgesic (paracetamol 50 mg/kg/day) was given for 3 days.

Radiographic examination

Radiographic examination was conducted with E7239X Rotanode Toshiba X-ray machine serial number 2A009, with a maximum exposure of 125 kV and 500 mA. X-ray exposure used in this study was 52 kV and 6.4 mA for 400 ms on ventrodorsal and laterolateral projection. Radiological evaluation was performed using RUST score (Table 1).Each score in the cortex (anterior, posterior, medial, lateral) was added to a total of 12 (fully healed) and 4 (not yet healed).

Histomorphometry

After 6 weeks, the rats were sacrificed and the right femur was resected immediately. By maintaining a K-wire inside, the harvested femur was fixed in 10% neutral buffered formalin for 48 hours. They were decalcified with Plank Rychlo's solution (Wako Pure Chemical Industries Ltd., Osaka, Japan). These samples were embedded in paraffin and cut transversely with a microtome for 5 µm thickness section for six times with an interval of 300nm before –being stained with hematoxylin-eosin.They were examined with a Leica microsystems IC C50 HD microscope with a magnification of 40 x.

The histological images were taken by a digital microscope camera and merged with the help of PTGUI Pro 9.1 software for digital evaluation. Histomorphometry evaluation included the evaluation of the total area of callus, the area of ossification, cartilage andfibrosis (Figure 3). Determination of each area was conducted manually using Image J version 1.4 software.

Statistical analysis

Statistical analysis was performed with SPSS 21 using Kruskal-Wallis or One Way ANOVA analysis. Differences between the means were considered statistically significant when $p < 0.05$.

Results

In the radiographic evaluation of RUST score, Kruskal Wallis test showed a significant difference among the groups with p-value of 0.001 (Table 2). Mann Whitney test revealed a significant difference between group II and I, group III and I, group IV and I, and also group V and I with a p-valueof 0.005; 0.006; 0.005 and 0.006 respectively. Group IV had the highest mean RUST score 11.6 while group I as a control had the lowest RUST score which was 4.4 (Fig. 2).

By using the Image J software, we evaluated the area of total callus, ossification, cartilage and fibrosis (Table 3). Group IV (10 µg/mL) had the largest total area of callus 57.8 mm^2,the area of

ossification 52.5 $mm^2$and area of cartilage 4.2 mm^2 (Fig.3). Meanwhile, group I had the smallest total area of callus, the area of ossification and area of cartilage which were 15.8 mm^2, 6.7 mm^2, and 0.4 mm^2 respectively. ANOVA test showed a significant difference in the total area of callus area with a p-value of 0.001. The Bonferroni posthoc test revealed a significant difference between group II, III, IV ,V and I with a p-valueof 0.033; 0.001; 0.001; and 0.017 respectively. In the area of ossification, Kruskal Wallis test showed a significant difference among all groups with a p-value of 0.001. Mann Whitney test revealed a significant difference between group II and I (control group), group III and I, group IV and I, and also group V and I with a p-valueof 0.009; 0.016; 0.009 and 0.016 respectively. Kruskal-Wallis test also showed a significant difference with p-value of 0.001 in the evaluation of the area of cartilage. Mann Whitney test revealed a significant difference between group II and I (control group), group III and I, group IV and I, and also group V and I with a p-valueof 0.009; 0.009; 0.009 and 0.028 respectively. In contrast to the area of ossification and cartilage, the group I had the highest fibrosis area (8.7 mm2) while the lowest area of fibrosis was found in the group IV (1 mm^2).

Discussion

Fracture healing is a complex physiological process, which consists of three phases: the inflammatory phase, repair and remodeling. It needs cooperation among some factors such as cells, growth factors, differentiation factors, cytokines and the interaction of the extracellular matrix. All of the above are controlled mainly by the expression of members of the TGF-β (transforming growth factor) super family, such as BMPs [10-16].BMP-2 has an important role affecting chondrogenesis, osteogenesis and re-vascularization process. BMP-2 also affects the formation of fibrotic tissue minimally and accelerates the process of maturation and remodeling of callus[10].

BMP has the potency to induce mesenchymal stem cell (MSC) differentiation into osteoblast [17,18], maintaining its maturity and enhancing endochondral ossification which includes recruitment and proliferation of monocyte and MSC, differentiation of MSC into chondrocyte, chondrocyte hypertrophy, cartilage matrix calcification, vascular invasion with osteoblast differentiation and bone formation to finally new bone remodeling and the creation of bone marrow [19].In the process of callus formation, BMP-2 plays a role in the initial process so that the provision of BMP-2 in this study are given in the early phase [20-24].In this study, administration of rhBMP-2 produces extensive callus thereby creating a bridge between proximal and distal osteotomy. Groups treated by rhBMP-2 had larger total callus, ossification, and cartilage, but a smaller area of fibrosis.

Sasso et al. [25] mentioned that BMP has osteoinductive property in massive bone defect healing. An animal study of segmental bone defect resulted in the knowledge that BMP promoted a similar or better result compared to autologous bone graft [26]. Cuomo et al. [26] reported that the addition of rhBMP-2 as an osteoinductive component caused more effective MSC differentiation by giving signal to the cells which produces 100% healing rates. In addition, Kamal et al.[27] reported that application of rhBMP-2 accelerated the healing process, increased bridging callus, and also prevented implant failure.

The dose or concentration of BMP-2 is very important for bone formation. The concentration or dose of BMP-2 required to induce ectopic bone formation depends on the type of carrier material used [28]. Tazaki et al {29] reported using 5 μg of BMP-2, 32 % bone formation is achieved using a 9 mm3 β-tricalcium phosphate scaffold, whereas HA, with the same amount of BMP-2, it only yielded 3 % bone formation in a rat ectopic model.

In this study, various rhBMP-2 dosages influenced the total area of callus, ossification and cartilage. The increasing doses of BMP-2 significantly increase the formation of callus, bone (ossification) and cartilage. However, if it goes beyond the optimal dose (in this study 10 ug/mL) it would eventually decrease in the formation of callus, bone (ossification) and cartilage. In other words, the 20 ug/mL rhBMP-2 (group V) which actually resulted in counter-productive effects or biphasic dose dependant response [30].

Cheng et [1] al reported that1 mg BMP-2 dose samplesqualitatively seemed to have higher local iNOS expression within the bone defect compared with the 10mg dose samples. This indicates that the defects treated with 1 mg BMP-2 exhibited prolonged inflammation locally, which may have contributed to the poor healing observed in these samples. Overall, the degree of ectopic bone was much lower than that observed in a previous study, involving a higher dose of BMP-2. In addition, the low-dose BMP-2 samples demonstrated only small sparse islands of new bone formation surrounded by mostly fibrous tissue with cellular infiltrate [1].

Our evaluation also revealed 10 µg (group IV) more dense and more homogenous callus microstructure compared to group III and V. Thus, group IV showed to stimulate the bone formation with mineral content close to that of the cortical bone. The similarity in the mineral microstructures group III and V suggests a comparable progression in the mineralization. A previous study observed with a calculated optimum dose of 12 µg of rhBMP- 2, above which and below which less stimulation of bone occurs. The reduction in bone content could be attributed to the activation of osteoclasts by the higher doses of rhBMP-2 [30]. Boyce et al.[31]examined two separate doses of rhBMP-2 but did not notice any difference in the cure rate between the two groups. Jones et al. [32] also stated a decrease or plateauing in histological examination of callus

at a higher BMP-2 dosage although it was not significant. Sciadiniet al. [33] also stated that there was a certain individual optimal dosage for BMP-2 in each species.

However, administration of BMP-2 in the treatment groups produced a smaller area of fibrosis than in the control group. It is consistent with our previous study which also showed the fewer area of fibrosis in the group given BMP-2 [27]. We assumed that BMP-2 plays a role in all phases of fracture healing and improves the process of osteogenesis and chondrogenesis.

We foundthat the least area of fibrosis was in group IV compared with group II, III, and V. Group I appears to develop into a critical sized non-union model. This finding is comparable with other studies[30].Theoretically, HA has the same chemical composition with a bone mineral fraction. It may fillabone defect and also stimulate bone growth. HA is a non-resorbable scaffold, and it is a good substrate for adhesion, proliferation, and differentiation of mesenchymal stem cells and osteoblasts. Furthermore, differentiated cells would produce extracellular matrix and integrated with the host tissue[34-38].However, according to some researcher, HA may cause osteolysis if it was exposed to bone marrow and soft tissue. HA debris allegedly caused implant failure by stimulating phagocytosis (by macrophage). After that, there would be an inflammatory process, triggering differentiation of osteoclast precursor into mature osteoclast, and it might cause impairment in bone remodeling and result in osteolysis[34].In this study, all rats from group I had nonunion and failure fixation.

We only evaluated after 6 weeks of treatment and we could not evaluate the progress of dynamic fracture healing, which is the limitation of this study. Hence, a further study at different periods of time is needed. Another limitation is the biomechanic evaluation of fracture healing thatis also needed.

CONCLUSION

Administration of BMP-2 stimulates the formation of bone bridging in a massive bone defect, The bone bridging filling massive bone defect depends on the dose or concentration of BMP-2.Administration of an optimal dose (10 µg/mL) of BMP-2 demonstratesbetter result than lower or higher dose for massive bone defect healing in SD rate.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests ~~regard ing~~regardingthe publication of this paper.

AKNOWLEDGMENTS

We would like to express our deepgratitude to Daewoong Pharmaceutical for the donation of HA and rhBMP-2

References

1. Cheng A, Krishnan L, Tran L, Stevens HY, Xia B, Lee N,et al. The Effects of age and dose on gene expression and segmental bone defect repair after BMP-2 delivery. JBMR1 Plus. 2018 ;2: 1–11.DOI: 10.1002/jbm4.10068
2. Rivera JC, Strohbach CA, Wenke JC, Rathbone CR. Beyond osteogenesis: an in vitro comparison of the potentials of six bone morphogenetic proteins. Front Pharmacol. 2013;4:125.
3. Peng T, Hsieh M, Lin CT, Chen C, Lee MS, Huang Y, et al. Treatment of critically sized femoral defects with recombinant BMP-2 delivered by a modified mPEG-PLGA biodegradable thermosensitive hydrogel. BMC Musculoskeletal Disorders (2016) 17:286 DOI 10.1186/s12891-016-1131-7
4. Ishida K, Haudenschild DR. Interaction between FGF21 and BMP-2 in osteogenesis. BiochemBiophys Res Commun. 2013; 432(4):677-82.

5. Blokhuis TJ, Calori GM, Schmidmaier G. Autograft versus BMPs for the treatment of non-unions:what is the evidence? Injury. 2013; 44 Supp 1:S40-2.
6. Schwabe P, Greiner S, Ganzert R, Eberhart J. Effect of a novel nonviral gene delivery of BMP-2 on bone healing. Scientific World J. 2012; 542-60.
7. Schwarz C, Wulsten D, Ellinghaus A, Lienau J. Mechanical load modulates the stimulatory effect of BMP-2 in a rat non union model. Tissue Eng Part A. 2013;19 (1-2):247-54.
8. Yilgor P, Yilmaz G, Onal MB, Solmaz I, Gundogdu S, Sousa RA, et al. An in vivo study on the effect of scaffold geometry and growth factor release on the healing of bone defect. J Tissue EngRegen Med. 2013;7(9):687-96.
9. Tressler MA, Richards JE, Sofianos D, Comrie FK, Kregor PJ. Orthopedics. 2011; 34 (12):877-84.
10. Doi Y, Miyazaki M, Yoshiiwa T, Hara K, Kataoka M, Tsumura H. Manipulation of the anabolic and catabolic responses with BMP-2 and zoledronic acid in a rat femoral fracture model. Bone 2011; 42(8):746-52.
11. Christopher G. Bone grafting and bone graft substitutes. J Bone Joint Surg. 2002; 84(3):108-17.
12. Brydone A, Meek D, Maclaine S. Bone grafting, orthopaedics biomaterials, and the clinical need for bone engineering. J Eng Med. 2010;224:1329-43.
13. Noldsletten L, Madsen JE. The effect of bone morphogenetic proteins in fracture healing. Scand J Surg. 2006; 95(2):91-4.
14. Whelan DB, Bhandari M, Stephen D, Kreder H, McKee MD, Zdero R, Schemitsch EH. Development of the radiographic union score for tibial fractures for the assessment of tibial fracture healing after intramedullary fixation. J Trauma.2010; 68(3):629-32.

15. Mokbel N, Bou SC, Matni G, Naaman N. Healing patterns of the critical size bony defect in rat following bone graft. J Trauma. 2008;12:73-8.
16. Laurencin CR, El-Amin SF. Xenotransplantation in Orthopaedic Surgery. J Am AcadOrthop Surg. 2008;16:4-8.
17. Wutzl A, Rauner M, Seemann R, Milles W, Krepler P, Pietschmann P, et al. Bone morphogenetic protein 2,5 and 6 in combination stimulate osteoblasts but not osteoclasts in vitro. J Orthop Res. 2010; 28(11):1431-9.
18. Garrison KR, Donell S, Ryder J, Shemit I, Mugford M. Clinical effectiveness and cost-effectiveness of bone morphogenetic proteins in the non-healing of fractures and spinal fusion: a systematic review. Health Technol Assess. 2007;11(30):1-150.
19. Louis CG, Thomas JW, Jeffrey OH, Thomas AE. Application of Histomorphometric Methods to the Study of Bone Repair. J Bone Miner Res 2005; 20:1715–22.
20. Vaccaro R. The role of the osteoconductive scaffold in synthetic bone graft. Orthop Blue J. 2002;25(5):571-8.
21. Mont MA, Ragland Ps, Biggins B, Friedlaender G, Patel T, Cook S, et al. Use of bone morphogenetic proteins for musculoskeletal applications. J Bone Joint Surg Am. 2004;86:41-55.
22. Lieberman JR, Daluiski A, Einhorn TA. The Role of growth factors in the repair of bone. J Bone Joint Surg Am. 2002;84:1032-44.
23. Mills LA, Simpson AH. In vivo models of bone repair. J Bone Joint Surg Br. 2012;94-B:865-74.

24. Carreira AC, Zambuzzi WF, Rossi MC, Filho RA, Sogayar MC, Granjeiro JM. Bone Morphogenetic Proteins: Promising Molecules for Bone Healing, Bioengineering, and Regenerative Medicine.VitamHorm. 2015:99:293-322
25. Sasso RC, LeHuec JC, Shaffrey C. Iliac crest bone graft donor site pain after anterior lumbar interbody fusion: a prospective patient satisfaction outcome assessment. J Spinal Disord Tech.2005;18(Suppl):S77–81.
26. Cuomo AV, Virk M, Petrigliano F, Morgan EF, Lieberman JR. Mesenchymal stem cell concentration and bone repair: potential pitfalls from bench to bedside. J Bone Joint Surg (Am) 2009;91:1073–83.
27. Kamal AF, Dilogo IH, Hutagalung EU, Iskandriati D, Susworo R, Siregar NC, et al. Transplantation of mesenchymal stem cells, recombinant human BMP-2 and their combination in accelerating the union after osteotomy and increasing, the mechanical strength of extracorporeally irradiated femoral autograft in rat models. Med J Islam Repub Iran. 2014;28:129.
28. Mumcuoglu D, Fahmy-Garcia S, Ridwan Y, Nicke J, Farrell E, Kluijtmans SG, et al.Injectable BMP-2 delivery system based on collagen-derived microspheres and alginate induced bone formation in a time- and dose-dependent manner. Europ Cells Materials. 2018; 35: 242-54.
29. Tazaki J, Murata M, Akazawa T, Yamamoto M, Ito K, Arisue M, et al. BMP- 2 release and dose-response studies in hydroxyapatite and beta-tricalcium phosphate. Biomed Mater Eng.2009; 19: 141-6.

30. Angle SR, Sena K, Sumner DR, Virkus WW, Virdi AS. Healing of rat femoral segmental defect with bone morphogenetic protein-2: a dose response study. J Musculoskelet Neuronal Interact. 2012;12:28-37.
31. Boyce AS, Reveal G, Scheid DK, Kaehr DM, Maar D, Watts M, et al. Canine investigation of rhBMP-2, autogenous bone graft, and rhBMP-2 with autogenous bone graft for the healing of a large segmental tibial defect. J Orthop Trauma. 2009; 23(10):685-92.
32. Jones AL, Bucholz RW, Bosse MJ, Mirza SK, Lyon TR, Webb LX, et al. Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphysealtibial fractures with cortical defects: a randomized controlled trial. J Bone Joint Surg Am. 2006;88(7):1431-41.
33. Sciadini MF, Johnson KD. Evaluation of Recombinant Human Bone Morphogenetic Protein-2 As a Bone-Graft Substitute in a Canine Segmental Defect Model. J Orthop Res 2000;18(2):289-302.
34. Nandi S. Orthopaedics application of bone graft & bone substitutes : a review. Indian J Med Res. 2010 Jul;7:15-30.
35. Greenwald A, Boden S, Goldberg V. Bone-graft substitutes: facts, fictions & applications. J Bone Joint Surg Am. 2001;83(2):98-103.
36. Moore W, Stephen E, Gravesbain G. Synthetic bone graft substitutes. ANZ J. Surg. 2001;71:354-61.
37. Leboucher J. Design and characterization of a scaffold for bone tissue engineering. Nat Bio J. 2003;12:1-46.
38. Jahangir A, Nunley R, Mehta S. Bone-graft substitutes in Orthopaedics surgery. Orthop Surg. 1995;90:111-9.

Figures

Figure 1.Surgical procedures. A) K-wire fixation after 10 mm osteotomy; B) HA and BMP-2 administration

Figure 2. RUST score for each groups. Group IV had the highest mean RUST score (11.6) while group I as control had the lowest RUST score which was 4.4

Figure 3. Histomorphometry evaluation included the evaluation of the total area of callus, the area of ossification, cartilage and fibrosis. A). Group I; B Group II; C) Group III;D) group IV and E).Group V.

Fugure 4. Histomorphometry evaluation of total area callus in all group. Group IV had the highest mean total callus area, 57.8 mm^2. While group I (control group) showed the lowest total callus are, 15.8 mm^2.

Tables

Tabel 1. Radiologic Criteria RUST Score

Table 2. The radiographic evaluation RUST score of all groups

Table 3. Histomorphometry of all groups evaluated using Image J