Effects of Local Delivery of Vascular Endothelial Growth Factor on Biological Performance of the Composite Biomaterial Used to Accelerate Bridging of Critical-Sized Mandibular Bone Defect in Rabbit Model

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***Abstract*—This study was aimed to produce a clinically applicable model of composite bone graft capable of treating CSBDs in the mandible with a single surgical procedure. Twelve male white New Zealand rabbits were divided into two groups: *control*, n=4, consisting of A (untreated) and B (BCP/FS); *experimental*, n=8, named C (BCP/FS) and D (BCP/FS+VEGF). A circular CSBD (8-mm Ø) was created on both sides of the mandible. Four rabbits were prepared for BCP/FS on one side of the mandible, leaving CSBD on the other side of the mandible untreated. Another set of eight rabbits with CSBDs created on both sides of the mandible was treated with BCP/FS on one side and BCP/FS loaded with 500ng/ml VEGF on the other side. Two rabbits each from the experimental group and one rabbit each from the control group were sacrificed at 14, 30, 45 and 60 days post operatively. All collected samples from CSBDs-site were subjected to micro computed tomography (μCT) and quantitative and semi-quantitative histological examinations. The results showed that the residual composite was significantly lower at 8 weeks in D (BCP/FS loaded with 500ng/ml VEGF) with more new bone formation compared with other groups. Data collected confirmed the applicability of this novel composite matrix for application in bone regeneration in CSBDs.**

***Index Terms*—critical sized bone defects, biphasic calcium phosphate, fibrin sealant, vascular endothelial growth factor**

I. Introduction

Bone tissue engineering is an interdisciplinary field developed in an attempt to resolve formidable challenges in the repairing process of hard and soft tissues [1]. A critical sized bone defects (CSBDs) will not regenerate spontaneously during a reasonable period of time and needs bone grafts [2]. Autografts and allografts are the most widely used techniques to repair bone defects. However, these grafts have the disadvantages of an inadequate supply, infectivity or unfavorable immune response and create the possibility of surgical complications at the site (donor-site morbidity) [3]. One alternative to autograft is allograft, or tissue taken from a cadaver. However, there is real risk of disease transmission from donor to recipient with allografts [4]. Bone xenografts are now considered to be unsuitable as bone graft due to a potential risk of contamination with viral and bacterial infections, immunogenicity, and finally rejection [5]. Composite biomaterials containing calcium phosphate and polymers (natural or synthetic) have been developed to address these limitations [6]. In general, bone possesses a high intrinsic repair and remodeling capacity, and the vast majority of bone defects can heal spontaneously. However, CSBDs have limited intrinsic regeneration potential, in part because of the failure of certain healing processes such as blood supply and a deficiency in factors regulating these processes. Angiogenesis, is the formation of new blood vessels from preexisting ones, promoted after bone injury, to deliver nutrients, remove waste products, and provide cells and biological mediators [7]. Vascular endothelial growth factor (VEGF) which is considered mitogenic - specific for endothelial cells plays an important role in the development of a functional vascular supply during the early stages of bone repair. That VEGF has a role in bone tissue engineering has long been known to control the behavior of the cells within the construct, and several approaches have been applied to this end [8]. Biphasic calcium phosphate (BCP) granules have become a strong choice recently in the reconstruction of CSBDs [9]. The most attractive feature of BCP is its ability to form direct bonding with the host bone, resulting in a strong interface [9]. However, the granules may be difficult to handle with their low mechanical stability and possibility of migration from the implanted site due to brittleness [10].

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Fibrin sealant (FS) is a natural polymer and the combination with BCP granules, as a composite, represents a new approach to improve the properties of the granules by reinforcement and reduction of their overall brittleness with good seal [11]. A smart scaffold loaded with a powerful growth factor is needed for effective bone healing. VEGF acts as a strong promoter agent in neovascularization which is required for effective coupling of angiogenesis and osteogenesis in bone substitutes. Systemic administration of VEGF or local administration in high dose usually has negative effects in healing process [12]. Systemic application of exogenous VEGF was unstable and had a clearance half-life of less than 1 hour following injection *in vivo* [13]. Thus, slow releasing VEGF sustained for 2-8 weeks would be the choice.

II. Materials and Methods

The experimental surgical protocol for this study was analyzed and approved by the Animal Ethics Committee USM (AECUSM) Universiti Sains Malaysia USM/2012/1-July-2012/1-July-2014), No. of Animal Ethics (81) (419). For adaptation to the animal house, rabbits were kept under veterinary and operator supervision for a minimum of 1 week before surgery. The animals were housed separately in metal cages in an experimental animal room (22 ℃, 55% humidity, 12/12-hour light/dark cycle) and fed a standard laboratory diet and water. FS was purchased from Baxter Healthcare Corporation, Westlake Village, CA-91362 USA, US License No. 140. VEGF was purchased from (GIBCO, USA). Macro-microporous biphasic calcium phosphate (MBCP) ceramic granules were synthesized in Ceramic Laboratory, School of Materials and Mineral Resources, Engineering Campus, Universiti Sains Malaysia (USM). The preparation of FS and VEGF was in accordance with the manufacturer's recommendations. Twelve male white New Zealand rabbits (Oryctolagus cuniculus), aged between 5 and 6 months, with a mean weight 2.63kg±0.35kg were divided into two main groups: (*control*, untreated, named **A** vs BCP/FS named **B**, n=4, and *experimental*, BCP/FS named **C** vs BCP/FS+VEGF named **D**, n=8). In Fig. 1, Rabbits were sedated before surgery with ketamine xylazine (ketamine 35mg/kg and xylazine 5mg/kg) by I.M injection [14]. Anaesthesia was induced and maintained by administration of 1%-3% sevaflourane mixed with oxygen via a face mask. Rabbits were put in the lateral position during surgery, and the heart rate, respiratory rate, pulse oximetry, and temperature of the animals were monitored. Before the operation, the area was shaved and prepared with Betadine, and a subcutaneous injection of 0.5% lidocaine with 1:200,000 epinephrine was administered in the line of incision (incision line block) to control bleeding and provide additional anesthesia. One circular CSBD (8-mm Ø) was created bilaterally in the mandibular body area (the premolar/molar region) removing only the lateral bony cortex, trabecular bone, and tooth roots (partial thickness). Four rabbits were prepared for BCP/FS on one side of the mandible while leaving the CSBD on the other side of the mandible (untreated). Another set of eight rabbits were prepared with BCP/FS on one side of the mandible and BCP/FS loaded with 500ng/ml VEGF on the other side of the mandible. The periosteal flap was repositioned and the wound closed in layers using 4-0 coated Vicryl suture. Postoperatively, each animal was observed closely by a veterinary technician and operator until it regained consciousness, at which time, it was transferred to the individual cages. Subcutaneous infusion of 5% glucose water was given and oral orange juice for the initial feeding. Thereafter, the animals were fed normally. In each animal, the wound site was debrided daily and cleaned with normal saline and povidon iodine for 7 days. Neomycin local antibiotic cream was applied daily for the first week to the incision site. Intramuscular injection of Baytril (Enrofloxacin), 10mg/kg/day (Bayer, Shawnee Mission, KS, USA) once a day for 7 days followed. Tramadol hydrochloric 2mg/kg once daily was given for pain relief for 7 days. The weight of the rabbits was recorded daily and any change in the dietary habits or activity was monitored closely. The animals were euthanized, one rabbit from control and 2 rabbits from experiment Fig. 1 at 2, 4, 6 and 8 weeks respectively after surgery with an intravenous injection of overdose sodium pentobarbitone (100ml/kg) and the tissues from the defect areas were collected. The samples were fixed in 4% paraformaldehyde for 12h at room temperature. All samples were scanned for bone formation within the defect site using a Scanco system (µCT 80, Scanco Medical Switzerland). After the µCT scanning, all tissue samples were decalcified in 10% formic acid and processed for paraffin sections. All sections were stained with haematoxylin and eosin (H&E) for histological and histomorphometric analysis (Carl Zeiss image analysis system MMI 0684).

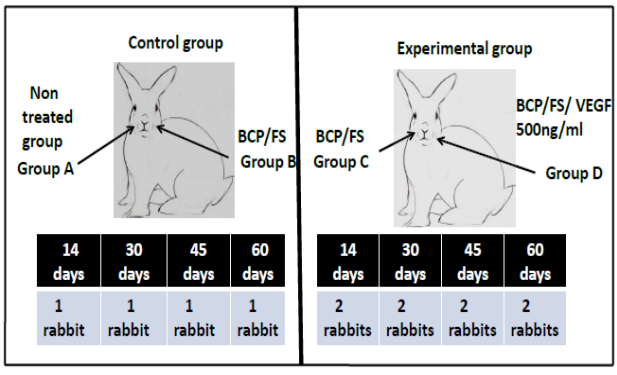

Figure 1. Schematic of CSBDs performed on the mandibular body area of the rabbit on both sides and euthanization of the rabbits according to the post operative time points.

A B C

Figure 2. A=FS, B=CSBD before implantation of composite bone graft, C=CSBD after implantation with composite bone graft

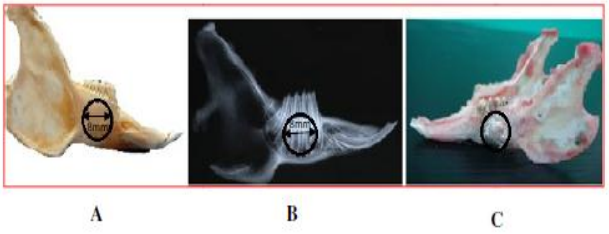

Figure 3. Design of the mandibular alveolar bone defect. A: Photograph of the lateral aspect of a harvested intact rabbit mandible. The location of the cylindrical defect is shown by the black circle. B: Radiograph showing the defect within the alveolar bone, which by definition is the bone that surrounds the roots of the teeth. C: Rabbit mandible dissected from the cranium and the soft tissues attached around the area of the implant.

III. Results

Statistical analyses were performed using PASW® Statistics 20 (SPSS, Chicago IL). A good clinical follow-up was conducted and there was no complication. The quantitative results of serial postoperative examination represented by a new bone formation and composite resorption were studied for statistical purposes using non parametric tests Kruskal-Wallis test and Mann-Whitney to determine significance of difference among groups and between groups of treated and non- treated cases, and the values of $P<0.05$ were considered significant.

A. µCT Evaluation

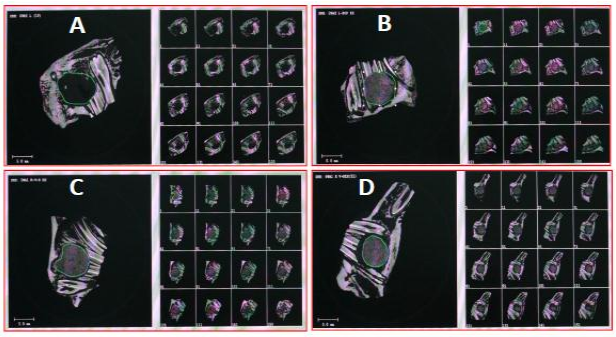

Figure 4. µCT results at week 2 showed a radiolucent zone (halo) surrounding the graft material with less size in group D compared to others groups.

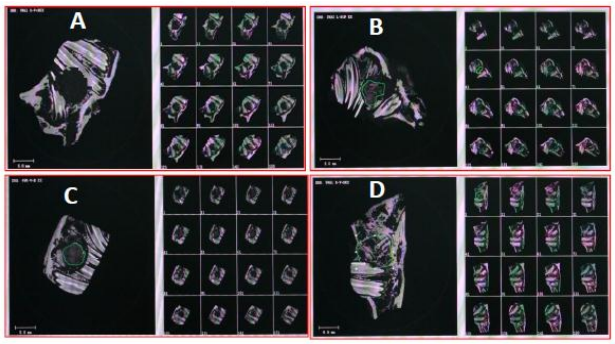

Figure 5. µCT results at week 8 showed a radiolucent zone (halo) surrounding the graft material with less size in group D compared to others groups.

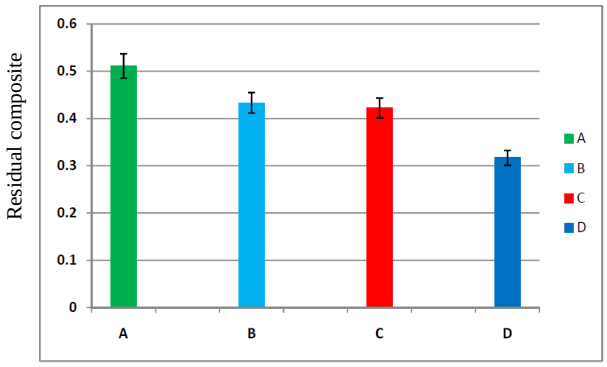

Figure 6. Mean difference of remaining residual composite at 2 weeks post surgery (radio-lucent) in group B, C and D in relation to group A which was measured by µCT less radiolucency mean more new bone formation which appears clear and significantly difference in group D compare to the others groups.

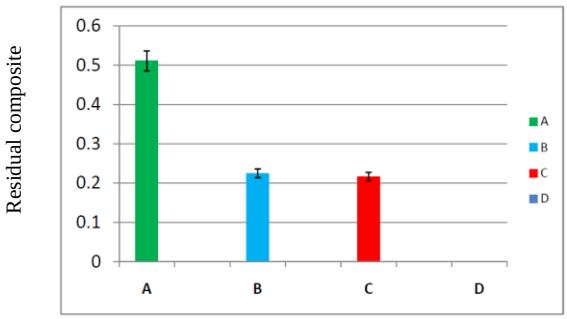

Figure 7. Mean difference of remaining residual composite at 8 weeks post surgery (radio-lucent) in group B and C with complete resorption of composite in group D (young bone formation) in relation to group A which was measured using µCT.

The percentage of residual composite was significantly lower at 2, 4, 6 and 8 weeks in BCP/FS loaded with 500ng/ml VEGF (group D) than in the other groups A, B and C and the difference was significant ($p<0.001$), while the difference between group A and other groups was also significant ($p<0.002$). There was no difference between group B and C ($p<0.873$), while the difference between group B and D was significant ($p<0.002$) and the difference between group C and D appear significant ($p<0.002$). µCT pictures of 2 and 8 weeks post surgery (treated and non treated groups) showed radio-lucent area which was gradually reducing in size and was replaced by opacities encroaching from the periphery to the centre of the bone defect with time, according to the rate of new bone formation after surgery. In Fig. 4 and Fig. 5, Group B- (BCP/FS) showed more opacities than group A. The bone regeneration was marked by increasing radio-opacity. A clear boundary with the host bone and different radio-opacities from the periphery toward the centre of the defect was observed. The amount of newly formed bone in the total area in group D was significantly greater than that in group B and C. Mean difference of residual composite at 2, 4, 6 and 8 weeks in group D was less than the mean difference in group B and C in Fig. 6 and Fig. 7.

B. Histological and Histomorphometrical Findings

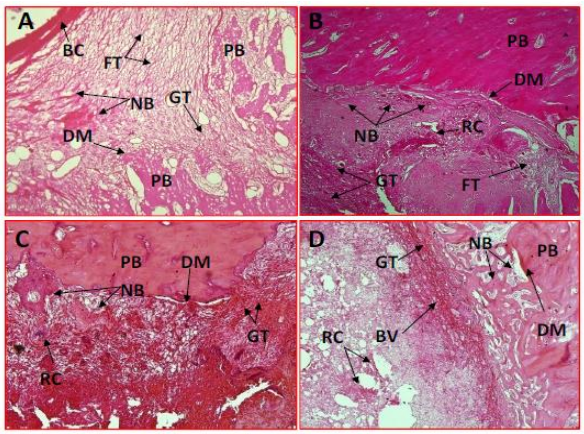

Figure 8. Histological presentation at 2 weeks post surgery of CSBDs healing in group A, B, C and D (H&E 40x). Group A (surgical defect only) shows no or very minimum new bone formed, with more fibrous tissue and thick layer blood clot. Group B shows more residual composite (RC) and minimal granulation tissue (GT) and new bone formation (NB). Group C shows more (RC) and minimum (GT and NB). Group D shows scattered extensive new blood vessels (BV) from defect margin (DM), more (GT), with minimal (RC), marked (NB) in thick layers and pre-existing bone (PB) appear clearly.

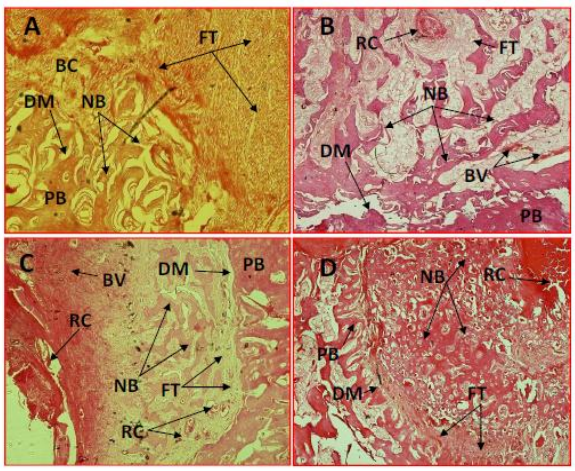

Figure 9. Histological presentation at 8 weeks post surgery of CSBDs healing in group A, B, C and D (H&E 40x). Group A (surgical defect only) shows very minimum new bone formed, with extensive fibrous tissue fill the defect. Group B still shows residual composite (RC) and minimal granulation tissue (GT) and more new bone formation (NB). Group C still shows (RC) and minimum (GT and NB). Group D shows extensive new bone formation (marked) thick layers fill the defect from defect margin (DM) toward the defect centre, with minimal (RC), and pre-existing bone (PB) appear clearly.

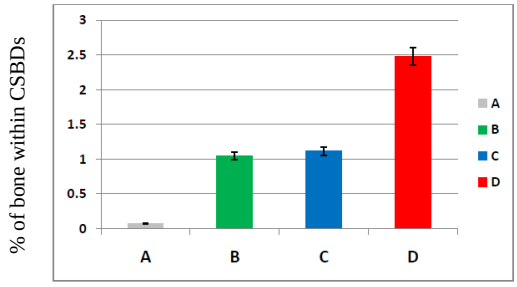

Figure 10. Mean difference of new bone formation at 2 weeks post surgery in group A, B, C and D. group D shows highly significant difference compare to other treatment groups and surgical defect only group (A).

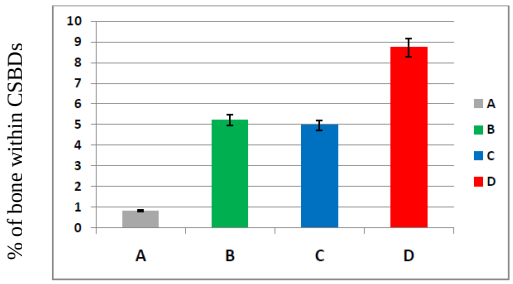

Figure 11. Mean difference of new bone formation at 8 weeks post surgery in group A, B, C and D. group D shows highly significant difference compare to other treatment groups and surgical defect only group (A).

Light-microscopic examination of the sections revealed no difference between the groups B and C at all post operative time points. At 2 weeks there was a mild inflammatory reaction with sporadic giant cells and minimal fibrous tissue. In Fig. 8, very little amount of newly formed bone from the marginal defect line and blood vessels scattered at the periphery of defect with slight resorption of the composite graft particles could be observed compared to group A which showed extensive fibrous tissue formation with mild focal inflammatory reaction at some area at the defect margin. Newly formed bone was hardly visible at the borders of the defect and the defect was filled with blood clot. In Fig. 8, for group D implanted with BCP/FS composite loaded with 500ng/ml VEGF showed extensive scattered blood vessels and granulation tissue from the margins of the defect toward the center with more new bone formation, while fibrous tissue was very scanty. In Fig. 8, at 4, 6 and 8 weeks, group A showed minimum difference when compared with results of week 2, and the defect was filled with fibrous connective tissue. Slightly increased new bone formation was observed compared with that at 2 weeks of healing at some areas close to the defect margin. In Fig. 9, group B and C showed more new bone formation starting from the margin of the defect and spreading toward the center of defect along with scanty granulation and increased fibrous tissue. Residual composite particles were still present at week 8. In Fig. 9, high degree of direct contact between newly formed bone and composite graft was also seen. Group D showed marked new bone formation at 4, 6 and 8 weeks post surgery. More new bone formation was observed compared with that in the 2 week sections. The circular defect was filled with newly formed bone tissue grown from the defect margins resulting in relatively large bony islands. Bone healing in all specimens was incomplete. Even though some portion of composite graft was surrounded by new bone originating from the defect margins, most of grafts were still surrounded by minimum fibrous connective tissue. In Fig. 9, histomorphometric analysis of new bone formation showed significant difference between the groups ($p<0.05$). At each time point 2, 4, 6 and 8 weeks the differences between groups was different ($p<0.001$), while group B and C showed no difference ($p<0.964$). The statistical difference between group B and C comparing with group A was significant ($p<0.001$). Mean

difference of new bone formation at 2, 4, 6 and 8 weeks in group D was higher than the mean difference in groups A, B and C and it was considered statistically highly significant in Fig. 10 and Fig. 11.

IV. Discussion

Repairing CSBDs in the craniofacial skeleton is the major challenge in maxillofacial surgery, because traditional grafting techniques require abundant time and effort, with an associated risk of morbidity [15]. Combining BCP and FS produces a mouldable material that is easy to both handle and use in surgical conditions. The mouldable composite is capable of cementing the granules at the implantation site. Quantitative bone histomorphometry is the gold standard to evaluate cellular events of bone modelling activity with quantitative study of microscopic features that substantially reduces subjectivity and observer variation [16]. Bone colonization occurred consistently during the 6-week implantation period [17]. The association of BCP granules with a biocompatible polymer provided an injectable bone substitute whose injectability could be adapted. The particulate form of the BCP/FS composite provides a suspension of the ceramic granules into the polymer solution much more efficiently than the macroporous architecture of many massive bone substitutes [18]. The ideal bone substitute material should be osteoinductive, osteoconductive and should be subject to complete resorption during the process of new bone formation [11]. A well balanced equilibrium between biomaterial degradation and new bone formation is desirable [11]. The described scaffold requirements may vary depending on type, size and anatomic location of defects [11]. In the present study, significant resorption of composite bone graft particles loaded with the VEGF was observed only in this group (D). On the other hand, the resorption of BCP/FS composite was little and slow in the groups B and C. This suggests that there is a relationship between the degree of composite resorption and the physiological function of VEGF which shows clearly more abundant blood circulation in the bone defect of group D. For successful CSBDs wound healing, VEGF must be maintained for a period of time more than 6 weeks, because test of biomaterial used in bone defect healing needs 6 to 8 weeks post operatively [19]. Our finding showed that the highest amount of new bone formation was obtained in group D compared to other groups with a 17:83 HA to β-TCP ratio of BCP at 6 and 8 weeks, suggesting the potential of using faster resorbable ceramics in bone tissue engineering and the osteogenic potential of calcium phosphates is based on their osteoconductive properties [20]. The total porosity of BCP granules used in this study was approximately 79%. It has been found that high porosity and large pores enhance bone ingrowth because, they allow migration and proliferation of osteoblasts and mesenchymal stem cells as well as vascularisation [21]. Delivery of nutrients materials and the removal of anabolites by the vascular network are considered critical for the viability of biological tissues [22]. For this reason, nearly every cell in the human body lies at a distance no further than 100-200 μm from a blood vessel, which correlates with the mathematically-calculated limit at which oxygen tension reaches a critical level for cell survival [22]. In bone reconstruction process, Graft implantation results in inflammation, which represents the first phase of tissue repair. This favours vascular response, but angiogenesis is generally limited to less than 1mm from the interface implant-host tissue [23]. The neoformed vessels of the implant must anastomose to the systemic circulation. In the absence of a vascular supply, the transport of nutrients occurs mainly by diffusion, which is only efficient for distances from 100 to 200 micron [21]. The insufficient vascularization compromises the supply of oxygen and nutrients to the newly formed tissue and does not remove the waste products of cells and the local accumulation of toxic substances may trigger an inflammatory reaction [21]. Therefore, the scaffold must not only support the growth of the cells that will replace the specific tissue in vivo, but it must also support endothelial cell adhesion and proliferation, and develop an effectively functioning vasculature to supply the cells with oxygen and nutrients with a good outcome [21]. Delivery of growth factors is a critical aspect in tissue engineering strategies [21]. A successful delivery system candidate should provide vascular and cellular invasion to favor osteoinduction by the growth factor [20]. In this study, a rabbit model of a critical-sized mandibular bone defect was established and then bone healing was observed post-surgery. The CSBDs concept depends not only on healing delay, defect size, location and animal species, and periosteum preservation but also on the quality and stability of the osteosynthesis [20]. The osteopromotive effect of BCP/FS composite scaffold loaded with 500ng/ml VEGF was evaluated by implanting the scaffold into the bone defect area. The μCT were used to monitor the newly formed bone in defect sites and histology was used to quantify the amount of the newly formed bone at the defect site and observe the biological response of scaffold. In the present study, all new bone formation leans to colonize the defect from the periphery to the center of the defect. In the case of large bone defects such as in our model, osteoconductive properties are unable to attain bone colonization, and the peripheral osteogenic cells are not likely to be recruited in the center of such a large defect, leading to insufficient ceramic resorption and bone substitution which is clearly seen in group B and C using BCP/FS composite only. The findings confirm Jégoux's work [20]. Statistically, no significant difference in the quantity of bone formation appears between control group B and the experimental group C meaning that the VEGF effect is localized.

V. Conclusions

The present study shows that the use of BCP/FS composite bone graft loaded with VEGF is ideal for local controlled release of VEGF resulting in accelerated bone formation and healing process of mandibular CSBDs and this may contribute to the scope of modern surgery.

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REFERENCES

[1] T. Kaully, K. Kaufman-Francis, A. Lesman, and S. Levenberg, "Vascularization—The conduit to viable engineered tissues," *Tissue Eng Part B Rev.*, vol. 15, no. 2, pp. 159-69, 2009.
[2] C. N. S. Chang, H. Chuang, Y. R Chen, L. C. Yang, *et al.*, "Cranial repair using BMP-2 gene engineered bone marrow stromal cells," *J. Surg. Res*., vol. 119, no. 1, pp. 85-91, 2004.
[3] J. Y. Wang, L. Wu, Y. Gao, J. S. Zhang, and C. C. Zhang, "Experimental study on the osseointegration of foam TiC/Ti composites," *Biomed. Material*, vol. 8, no. 4, 2013.
[4] M. Mehta, K. Schmidt-Bleek, G. N. Duda, and D. J. Mooney, "Biomaterial delivery of morphogens to mimic the natural healing cascade in bone," *Adv. Drug. Deli. Rev*., vol. 64, no. 12, pp. 1257-1276, 2012.
[5] Y. Seiichi, H. Ken, I. Mika, L. Terry Y., H. Shigeru, D. Jisen, and M. Amr M., "The potential of tissue engineering and regeneration for craniofacial bone," *Dentistry*, vol. 2, no. 136, 2012.
[6] C. Fricain, S. Schlaubitz, L. Visage, C. Arnault, *et al*., "A nano-hydroxyapatite-Pullulan/dextran polysaccharide composite macroporous material for bone tissue engineering," *Biomat*., vol. 34, no. 12, pp. 2947-2959, 2013.
[7] X. Wu, T. Hou, F. Luo, J. Xing, *et al.*, "Vascular endothelial growth factor and physiological compressive loading synergistically promote bone formation of tissue-engineered bone," *Tissue Eng Part A*., 2013.
[8] Y. P. Huri, G. Huri, U. Yasar, Y. Ucar, N., N. H. Dikmen, and V. Hasirci, "A biomimetic growth factor delivery strategy for enhanced regeneration of iliac crest defects," *Biomed. Materials*, vol. 8, no. 4, 2013.
[9] J. L. Ricci, E. A. Clark, A. Murriky, and J. E. Smay, "Three-dimensional printing of bone repair and replacement materials: Impact on craniofacial surgery," *J. Craniofac. Surg.*, vol. 23, no. 1, pp. 304-308, 2012.
[10] S. Tadier, N. van Garderen, A. de Gasparo, N. Doebelin, G. Baroud, and M. Bohner, "Synthesis of spherical calcium phosphate particles for dental and orthopedic applications," *Biomater*., vol. 3, no. 2, 2013.
[11] S. Reppenhagen, C. Reichert, L. J. Rackwitz, Rudert, *et al.*, "Biphasic bone substitute and fibrin sealant for treatment of benign bone tumours and tumour-like lesions," *Int. Orthop*., vol. 36, no. 1, pp. 139-148, 2012.
[12] Y. Bai, G. Yin, Z. Huang, X. Liao, *et al.*, "Localized delivery of growth factors for angiogenesis and bone formation in tissue engineering," *Int. Immunopharmacol.*, vol. 16, no. 2, pp. 214-223, 2013.
[13] C. Wu, Y. Zhang, Y. Zhou, W. Fan, and Y. Xiao, "A comparative study of mesoporous glass/silk and non-mesoporous glass/silk scaffolds: Physiochemistry and in vivo osteogenesis," *Acta Biomaterialia*, vol. 7, no. 5, pp. 2229-2236, 2012.
[14] G. Daculsi, A. Uzel, P. Weiss, E. Goyenvalle, and E. Aguado, "Developments in injectable multiphasic biomaterials. The performance of microporous biphasic calcium phosphate granules and hydrogels," *J Mater. Sci. Mater. Med.*, vol. 21, no. 3, pp. 855-861, 2010.
[15] M. X. Sun, W. Y. Tan, K. T. Wang, Z. Q. Dong, H. H. Peng, and F. C. Wei, "Effects of allogenous periosteal-derived cells transfected with adenovirus-mediated BMP-2 on repairing defects of the mandible in rabbits," *J. Oral Maxillofac. Surg*., vol. 71, no. 10, pp. 1789-1799, October 2013.
[16] K. S. Joiner, "Enhancing histologic assessment of experimental disease using histomorphometrics," *Poult Fish Wildl Sci*, 2013.
[17] O. Gauthier, R. Müller, D. von Stechow, B. Lamy, *et al*., "In vivo bone regeneration with injectable calcium phosphate biomaterial: A three-dimensional micro-computed tomographic, biomechanical and SEM study," *Biomaterials*, vol. 26, no. 27, pp. 5444-5453, 2005.
[18] P. Bartolo, J. P. Kruth, J. Silva, G. Levy, *et al.*, "Biomedical production of implants by additive electro-chemical and physical processes," *CIRP Annals-Manufacturing Technology*, vol. 61, no. 2, pp. 635-655, 2012.
[19] J. Y. Sohn, J. C. Park, Y. J. Um, U. W. Jung, *et al*., "Spontaneous healing capacity of rabbit cranial defects of various sizes," *J. Periodont. & Impl. Sci.*, vol. 40, no. 4, pp. 180-187, 2010.
[20] F. Jégoux, E. Goyenvalle, R. Cognet, O. Malard, *et al.*, "Mandibular segmental defect regenerated with macroporous biphasic calcium phosphate, collagen membrane, and bone marrow graft in dogs," *Archives of Otolaryngology—Head & Neck Surg*., vol. 136, no. 10, pp. 971, 2010.
[21] S. Kachgal, K. A. Mace, and N. J. Boudreau, "The dual roles of homeobox genes in vascularization and wound healing," *Cell Adhesion & Migration*, vol. 6, no. 6, pp. 457-470, 2012.
[22] M. M. Porter, S. Lee, N. Tanadchangsaeng, M. J. Jaremko, J. Yu, M. Meyers, and J. McKittrick, "Porous hydroxyapatite-polyhydroxybutyrate composites fabricated by a novel method via centrifugation," *Int. Mechanics of Biological Systems and Materials*, Springer, vol. 5, pp. 63-71, 2013.
[23] E. Cenni, F. Perut, and N. Baldini, "In vitro models for the evaluation of angiogenic potential in bone engineering," *Acta Pharmacologica Sinica*, vol. 32, no. 1, pp. 21-30, 2010.

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