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Fabrication of pre-determined shape of bone segment with collagen-hydroxyapatite scaffold and autogenous platelet-rich plasma

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Abstract *Background* Reconstruction of large segment of bony defects is frequently needed in hand surgery. Autogenous bone grafting is considered the standard in management of these bony defects but has limited source of graft material. Collagen and hydroxyapatite have been used as bone-filling materials and are known to serve as the osteoconductive scaffold for bone regeneration. On the other hand, platelet-rich plasma is a kind of natural source of growth factors, and has been used successfully in bone regeneration and improving wound healing. This study was designed to evaluate the effectiveness of using biohybrids of platelet-rich plasma and collagen-hydroxyapatite beads for fabricating of protrusive bone in a rabbit animal model. *Methods* Biomaterial beads comprised of particulate hydroxyapatite dispersed in fibrous collagen (type I) matrices were prepared and filled in the ringed polytetrafluoroethylene (PTFE) artificial vascular graft (3 cm long, 1 cm in diameter). New Zealand White rabbits were each implanted with two cylindrical PTFE artificial vascular graft over both iliac crests ($n = 16$). A 2 × 0.5 cm opening was created at the side of each PTFE chamber to allow the content of chamber in contact with the bone marrow of the ileum. The chambers were empty (groups A and D), filled with type I collagen/hydroxyapatite beads (groups B and C). In groups C and D, autologous platelet rich plasma (PRP) was given by transcutaneous injection method into the chambers every week. After 12 weeks, the animals were sacrificed and the chambers were harvested for radiological and histological analysis. *Results* In plain radiographs, the group C chambers had significantly higher bone tissue engineered (average calcified density 0.95, average calcified area 61.83%) compared with other groups ($P < 0.001$). In histological examination, there was a creeping substitution of the implant by the in-growth of fibrovascular tissue in group C. Abundant fibrovascular networks positioned interstitially between these biomaterial beads in all parts of chamber. Degradation of these beads and newly formed capillaries and osteoids around the degraded matrixes are shown. The continually calcification in the matrixes or degraded matrixes is evidenced by the strong green fluorescence observed under the confocal microscope. In group B, looser architecture without evidence of tissue in-growth was shown. In the scaffold absent groups (A and D), there was only fibrous tissue shown within the chamber. *Conclusions* In this study, we have demonstrated a feasible approach to fabricate an osseous tissue that meets clinical need. Using the type I collagen/hydroxyapatite gel beads matrixes and intermittent injection of autologous platelet-rich-plasma, specific 3D osseous tissues with fibrovascular network structure from pre-exist bony margin were successfully created in an in vivo animal model.

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1 Introduction

In the phalangeal and metacarpal bone defects, there are often three-dimensional complex shapes. These bony defects still remain significant challenges for surgeons since it often requires large volumes of donor tissue and complex staged surgical procedures to achieve the desired functional and aesthetic goals. Presently, the strategies to address these bone defects include the use of autogeneous grafts and flaps, allograft or xenograft bone, non-degradable bone cement, metals and ceramics. The use of autogenous bone grafts and bone flap is considered the standard in management of bony defects but has limited source of graft material, especially in children. The allograft and xenograft are good substitutes of autogenous bone graft, however, there are potentials of transferring pathogens and difficulties of shaping into the desired form. Most of synthetic implants are not biodegradable and may result in stress shielding in the surrounding bone or fatigue failure of the implant. Therefore, the search for improved materials and surgical strategies for repairing skeletal defects continues [1–3].

The approaches of tissue engineering have the potential to evolve the present clinical strategies. They attempt to create tissue replacements by providing a scaffold with the proper size and predetermined shape for tissue development and permitting cells from the surrounding tissue to migrate into the matrixes [4–6]. It can provide patient-specific three dimensional shapes while minimizing donor tissue requirements. The scaffolds are often three-dimensional porous polymeric matrices, which provides a space with the proper size and shape for tissue regeneration [5–10]. The surface of the polymers would also ideally promote cell attachment, as many cells are anchorage dependent for survival. In large craniofacial defects, more concerns in truly clinical need, are to engineer structural tissues with supporting vascular network. Under these circumstances, the reconstructions with tissue engineering techniques require different strategies that establish an extensive, patent vascular network within the engineered bone [9, 11].

Collagen and hydroxyapatite, are the two major components of bone tissue and they have been used as the bone substitute materials in orthopedic, oral-maxillo-facial and plastic surgery. The collagen fibers can serve as the scaffold for cell growth and tissue repair, and the hydroxyapatite (HA) is biocompatible and osteoconductive for bone regeneration [3, 9–11]. While most of the collagen matrices are prepared in a slab form, spherical gel beads of collagen and hydroxyapatite are more versatile in biomedical applications [12].

Previous studies have shown that many growth factors are vital modulators during healing process of bone defects [13]. Platelet-rich plasma (PRP) is an concentration of platelets in plasma, which contains many growth factors, such as PDGF, TGF-β, vascular endothelial growth factor, etc. It was reported a significant enhancement of bone and soft tissue healing when PRP is used in bone surgery, cosmetic surgery, and in oral and maxillofacial surgery [14–18]. In periodontal surgery, the combination of PRP and bovine porous bone mineral (BPBM) had been used as a therapy for human intrabony defects [18]. Bovine porous bone mineral (BPBM) is a xenograft prepared by protein extraction of bovine bone, which results in a structure similar to human cancellous bone and has the ability to enhance bone formation.

The guided bone regeneration principle is based on the basis of the hypothesis by placing an inert membrane barrier over the defect in close contact with the bone tissue, which acts as a mechanical barrier preventing the ingrowth of connective tissue into the defect, a secluded space can be created between the bone and the membrane formed (Dahlin et al. and Nyman et al.) [19]. On cellular and molecular mechanism, by reducing the ingrowth of fibroblasts and elevating local concentration of growth and differentiation factors, the osteogenesis is able to occur without interference from other tissue types [19–23].

In this paper, we report the approach to prepare gel beads containing particulate hydroxyapatites dispersed in the reconstituted atelocollagen matrix. Using these degradable biomaterials as scaffold, the platelet-rich plasma (PRP) and the technique of guided bone regeneration, we propose an animal model of in vivo engineering of a predetermined bone fabrication to form the vascularized structural bone tissue.

2 Materials and methods

2.1 Preparation of hydroxyapatite/collagen beads

Collagen was prepared from bull skin according to the procedure described previously [24]. Hydroxyapatite was purchased from Merck (Darmstadt, Germany) and further characterized in the laboratory of National Yang-Ming University, Taipei, Taiwan. Before use, the acidic collagen solution was dialyzed against 0.02 M phosphate buffered saline (pH 7.2) and centrifuged at 48,000 rpm for 3 h. The upper two thirds of the collagen solution was collected from the centrifuge tube and used for reconstitution. The solution was mixed with hydroxyapatite powders with a weight ratio of 35:65 (collagen to hydroxyapatite) at 4°C. Five milliliter of the cold mix was added drop by drop to 100 ml of olive oil stirring at various speeds at 37°C. Collagen was reconstituted in the droplets and further aged for 1 h in the oil bath. At the end of the incubation, 100 ml

of PBS was added to make a suspension of gel beads. The hydroxyapatite/collagen gel beads were collected from the aqueous phase, transferred to a 2.5% glutaraldehyde solution, and incubated at 37°C for three more hours. The cross-linked gel beads were washed repeatedly with 0.02 M PBS. The sizes of the hydroxyapatite/collagen gel beads were controlled between 0.1 and 1 mm by adjusting the stirring speed [12].

2.2 Preparation of platelet-rich plasma

After anesthesia, 5 ml blood were drawn from rabbit's central ear artery by a 10 ml injection syringe. The blood was mixed with 1 ml citrate phosphate dextrose (CPD), agitated for 15 s, then transferred into a centrifugal chamber and centrifuged. The centrifugation process consisted of separation spin and concentration spin. In separation spin, the blood was centrifuged at 1,500 rpm for 20 min to separate the red blood cells from the rest of the whole blood (white blood cells, platelets, and plasma). Platelets and white blood cells were on the upper layer of the preparation, and the red blood cell fraction was in the lower layer. All supernatant and the upper 1–2 mm red blood cells were pipetted into another centrifugal tube and then centrifuged at 3,000 rpm for another 30 min for concentration spin. The concentration spin resulted in the separation and compaction of the platelets, white blood cells, and a small number of residual red blood cells from the plasma. After discarding about three-quarter of supernatant, the remainder, about 0.18 ml, was PRP. All the procedures were manipulated under strict sterile circumstances [14, 15]. Immediately before the application, PRP was mixed with an equal volume of a sterile saline solution and then was injected into the chamber by a No 23 needle. This inactivated PRP was used, instead of activated PRP, because of the feasible application by transcutaneous injection.

Platelet counts of whole blood and PRP of each patient were measured with a standard hemocytometer. Levels of TGF-β1 and PDGF-AB in PRP were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (R &D System, Inc., USA), according to the manufacturer's instruction.

2.3 Animal study

The animal study was conducted only after the protocol had been approved by the Faculty of Mackay Memorial Hospital commission for the animal experimentation. All 16 rabbits underwent the following operative procedure. First, the hydroxyapatite/collagen gel beads were collected from the wells and then filled into the ringed PTFE vascular graft (1 cm × 3 cm) and sealed with 5–0 prolene suture. At the same time, the experimental New Zealand rabbit was prepared. An induction mixture of ketamine (ketamine hydrochloride, 50 mg/kg), Rompun (xylazine, 5 mg/kg) was administered intramuscularly. After adequate anesthesia, the back of rabbit was shaved and prepared with antiseptic solution (10% povidone iodine) and then sterilely draped with the rabbit in the prone position. The iliac crest was exposed through a 4 cm longitudinal oblique incision. The skin and muscle were retracted and the entire iliac crest was exposed. The periosteum was stripped from the bone using a periosteal elevator. The outer cortex of the iliac crest was removed by a bone cut to expose the bone marrow. A longitudinal window (2 cm × 0.5 cm) was made along the long axis of ringed PTFE vascular graft to expose the content of the vascular graft. Then the ringed PTFE vascular graft was then applied to the iliac crest with the opening window in contact with the bone marrow. 4–0 Nylon stitches were used to fix the vascular graft in rigid position (Fig. 1). The wound was then closed in layers, and the rabbits were returned to a padded crib until spontaneous and purposeful movement was noted. The ringed PTFE vascular grafts were left unfilled (groups A and D) or filled with the hydroxyapatite/collagen gel beads (groups B and C). In groups C and D, further PRP solution (0.6 cm^3 per week) was administered into the engineering chamber through direct injection by a No 23 needle. Besides, for the purpose of bone labeling, high dose tetracycline was given intramuscularly each month.

At 12 weeks post-operatively, the animals were euthanized and the implants were removed. The iliac crests with the ringed PTFE vascular graft were fixed in 10% buffered formalin and then cut cross-sectioning every 2 mm along the longitudinal axis of the ringed PTFE vascular graft. All

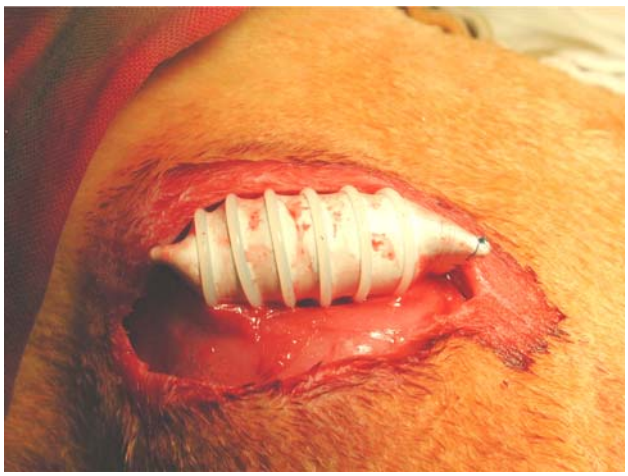

Fig. 1 The 3 cm × 1 cm ringed PTFE vascular graft was implanted on the iliac crest of New Zealand rabbit. Post-operative view

specimen produced were prepared for further radiological, fluoroscopic and histological examinations.

2.4 Radiologic analysis

Rabbits underwent standardized serial radiography of the iliac crest immediately after surgery and at 4, 8, 12 and at 16 weeks. All radiographs were size standardized. The cross-sectioned specimens also received radiographic analysis and the radiographic images were digitized using a scanner. All the images of cross-sectioned specimens were analyzed using Fujifilm image software (Science Lab 2003 for windows, Process Version 2.2). The radio-opaque region of mineralization within the chamber was outlined and calculated. The mean density of the outlined mineralization region within the chamber were also calculated and compared with the mean density of the connecting iliac bone.

2.5 Imaging of tetracycline fluorescence

The cross-sectioned specimens were photographed under ultraviolet (UV) light with a Nikon camera. A custom-built box contained the UV irradiation and limited light from external sources. The UV source consisted of four lamps (365 nm wavelength) positioned inside the custom-built box. A UV filter and a broadband interference filter was fitted to the lens to narrow the band of transmitted light to the 500–600-nm range; the emitted fluorescence of tetracycline was in the 530-nm range.

2.6 Histological analysis

After radiographic analysis, the implants were then decalcified in hydrochloride, processed, embedded in paraffin, and sectioned (6 μm) using standard protocols. Sections were stained using a standard hematoxylin and eosin protocol.

3 Results

3.1 Platelet counts and growth factor levels

Platelet counts of the whole blood from patients yielded a mean value of $(326.1 \pm 20.2) \times 10^3/\mu l$, ranging $(275–370) \times 10^3/\mu l$. The mean platelet count of PRP was $(1{,}100 \pm 332) \times 10^3/\mu l$, ranging $(925–1{,}250) \times 10^3/\mu l$. These values confirmed the sufficient concentration of platelets during the PRP preparing process. The platelet counts of PRP in all samples were more than threefold of baseline whole blood platelet counts [mean: $(337.3 \pm 52.0)\%$; range: (310–430)%]. In unactivated PRP, TGF-β1 level was (95.2 ± 37.0) ng/ml (ranged from 52.2 to 129.6 ng/ml), and PDGF-AB level was (53.2 ± 24.5) ng/ml (ranged from 26.5 to 78.6 ng/ml).

3.2 Radiological and fluoroscopic analysis

All animals tolerated the surgery well and recovered spontaneously. In the time period of experiment, all the rabbits tolerated the presence of the ringed PTFE chambers without difficulties, and no visible inflammatory reaction, infection or extrusion was observed. Before harvesting of the specimens, the grafts could clearly be seen subcutaneously on the dorsal site. The specimens were harvested with a segment of iliac bone. On gross specimen inspection, the structure of the PTFE chambers were all intact except the scaffold absent group (groups A and D), in which collapse was shown. There was not any tissue ingrowth into the PTFE chamber wall which was proved later under histological examination. After cross-sectioning, the chambers showed intact and fully filled except the scaffold absent groups (groups A and D) where empty space was shown within the chamber. Grossly, area of calcification can be seen on the cross-sectional surfaces of groups B and C, and is largest in the group C (Fig. 2). Under fluorescence, there was strong tetracycline labeling of these calcified area which is indicative of new mineralized matrix elaboration (Fig. 3).

In the radiographic analysis of the cross-sectioned specimens, the area of radio-opaque region of mineralization within the chamber was highest in group C $(61.83 \pm 9.35\%)$. There was not radio-opaque region shown in scaffold absent groups (groups A and D). The average calcified density (mean density of the outlined mineralization region within the chamber/mean density of iliac bone) was highest in group B (1.39 ± 0.15) (Table 1).

3.3 Histological analysis

After 12 weeks' implantation, the inflammatory response and foreign body reaction was mild in group C, as testified by the paucity of inflammatory cells (leukocytes, lymphocytes, plasma cells and eosinophils). New tissue had grown into the matrix of the chambers of group C (Fig. 4b and c) compared to cell-free matrixes (group B) where severe foreign body reaction was shown and most of the ingrowth tissues were dominated by macrophages (Fig. 4a). In group C, there was a creeping substitution of the implant by the in-growth of granulation and macrophage tissues. In the middle and upper parts of the chamber, there were also abundant fibrovascular networks positioned interstitially between these biomaterial beads. Degradation of these beads and newly formed capillaries and osteoids around the degraded matrixes are shown simultaneously in these

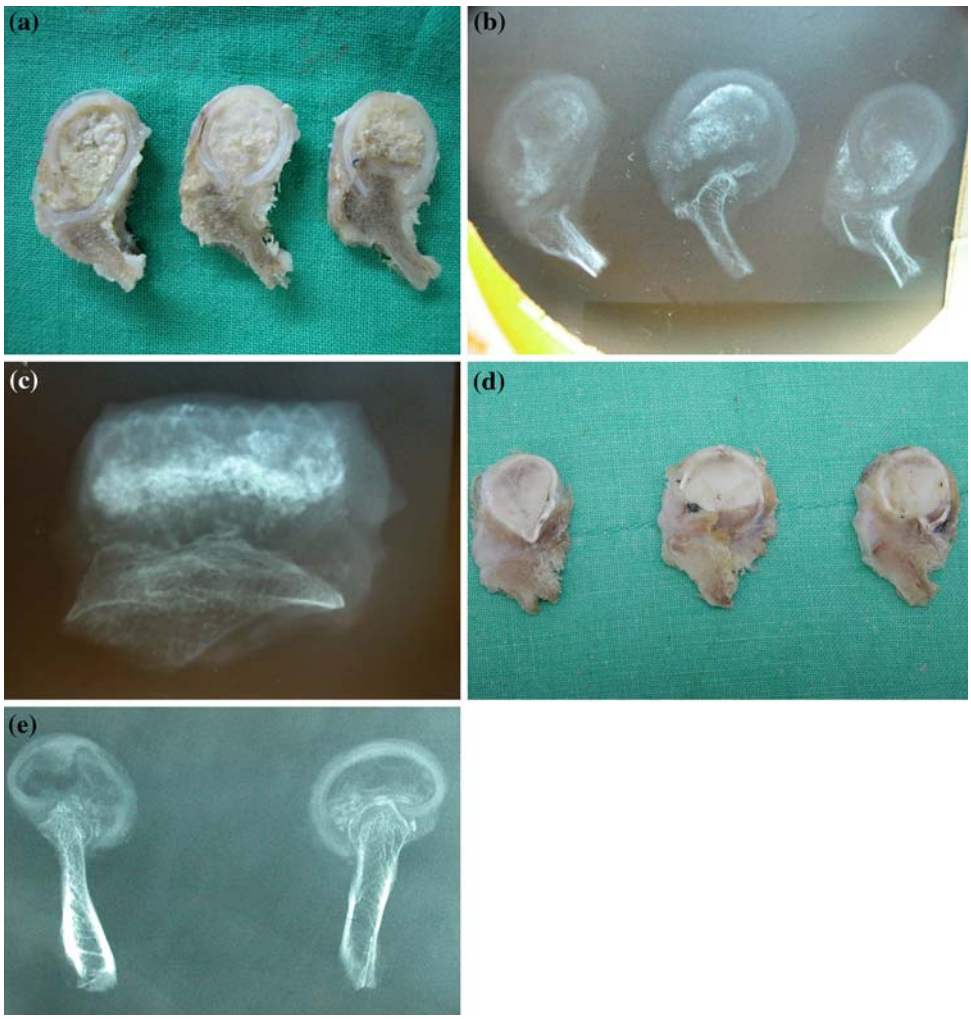

Fig. 2 Cross-sectional specimens and their radiographs. (**a**–**c**) group C, (**d** and **e**) group B. The calcification can be clearly seen in the radiographs

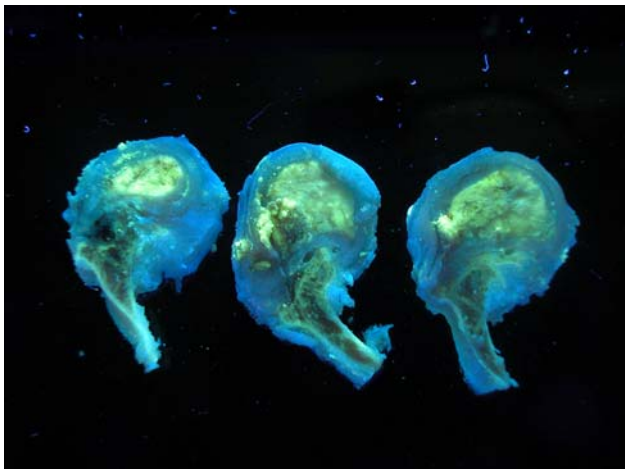

Fig. 3 The cross-sectional specimens of group D. Under fluorescence, there was strong tetracycline labeling of the calcified area which is indicative of new mineralized matrix elaboration

histological analyses. Surrounding osteoprogenitors and osteoblasts invaded the matrix forming new osteoids adjacent to the in-growth of macrophages. The continually calcification in the matrixes or degraded matrixes is evidenced by the strong green fluorescence observed under the confocal microscope (Fig. 3).

Table 1 The average calcified density and average calcified area

Group	Average calcifies area (%)	Average calcifies density
A	0	0
B	25.17 ± 8.21	1.39 ± 0.15
C	61.83 ± 9.35	0.95 ± 0.11
D	0	0

In group B, appearance of foreign body reaction was shown and abundant inflammatory cells can be seen. There was not new tissue formation noted. In the group A, there was only fibrous tissue without evidence of osteogenesis process. In group D, fibrovascular tissue with minimal osteogenesis can be seen at the margin of iliac bone (Fig. 4d and e).

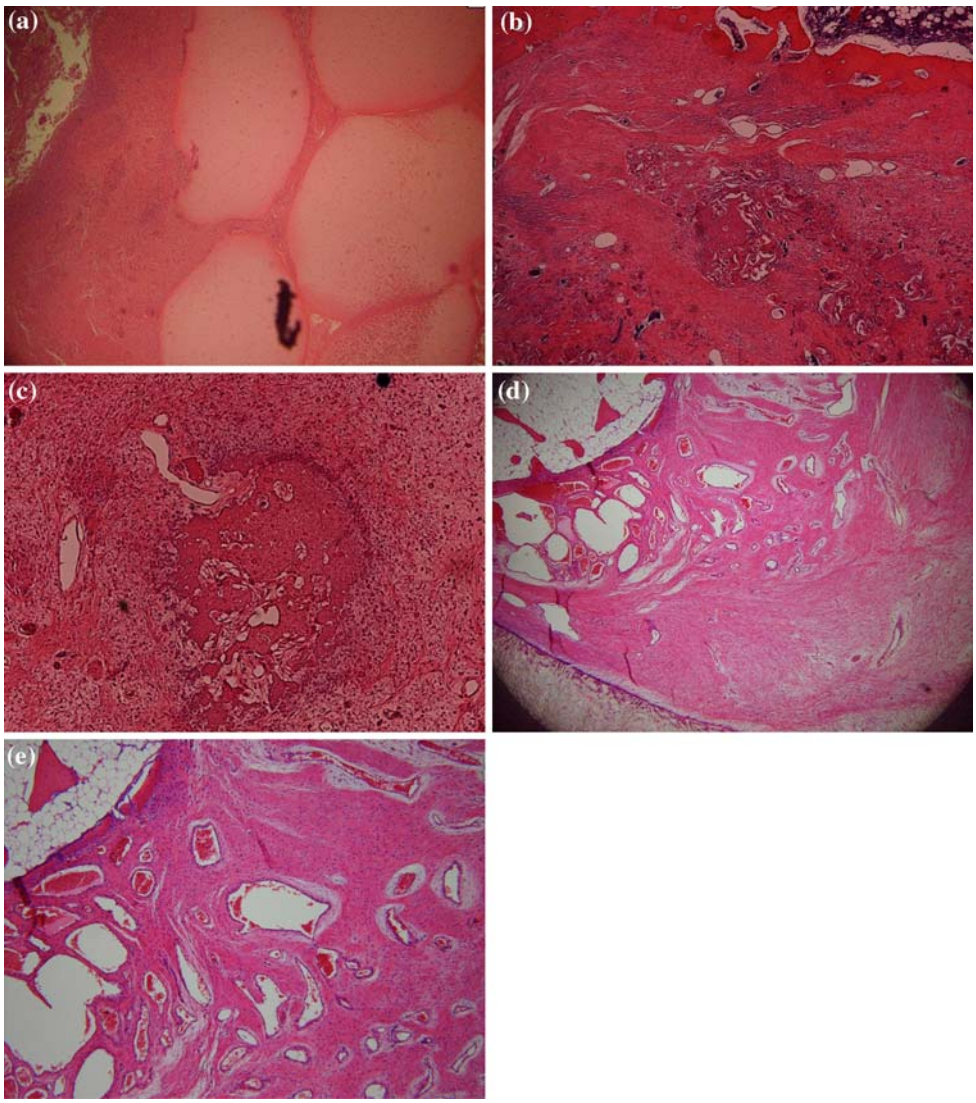

Fig. 4 Histologic sections after 12 weeks' implantation. (**a**) In group B, only foreign body reaction was shown. (**b** and **c**) In group C, calcification and osteoid can be observed in the middle and even the upper portion of the chamber. The matrices were invaded and divided by the fibrovascular tissue. (**d** and **e**) In group D, there was only fibrovascular tissue invasion with minimal osteogenesis which can be seen at the margin of iliac bone

4 Discussion

Reconstruction of three-dimensional phalangeal and metacarpal bone defects after trauma or tumor resection often requires large volumes of donor tissue and complex surgical procedures. Clinically, by which to achieve the desired functional and aesthetic outcomes, these procedures include bone grafting or even free bone flap and are often multiple staged [25, 26]. The techniques of tissue engineering have the potential to revolutionize the traditional reconstructive approaches. They attempt to create tissue replacements by providing a scaffold with the proper size and shape for tissue development and permitting cells from the surrounding tissue to migrate into the matrices [3, 11, 27]. One of the advantages of tissue engineering is to restore tissue in a predetermined shape. Although the previous reports on engineered replacements have already demonstrated the promising results of tissue-engineering approaches, practical clinical need is to restore defects with large volumes of bone in most circumstances. These larger volumes also require methods that establish an extensive, patent vascular network within the engineered bone which is similar to the structure of bone.

Ideally, scaffold for tissue engineering should be non-toxic, resorbable and provide a high surface area to volume ratio for cell–matrix interaction, sufficient space for extracellular matrix regeneration, and minimal diffusion constraints during culture [5–10]. Many different biodegradable scaffolds have been used to study osteogenesis in vivo, such as type I collagen matrix gels, gelatin, hyaluronic acid-based polymers, polylactide/polyglycolide sponges and hydroxyapatite/tricalcium phosphate ceramic [2, 4–6]. The highly porous matrices can provide a high surface area for cell–polymer interactions, sufficient space for extracellular matrix (ECM) regeneration and minimal diffusion constraints during in vitro culture. It have been

commented on the advantage of a construct having a large surface area to volume ratio to provide a conduit for uniform cell delivery to give high cell density with the optimal pore size depending on the intended application of the matrix [4, 6]. The diffusion constrains to the far deep structure of the matrix is another difficulty to overcome, especially when used in vivo. In addition, the difficulty of shaping also limited their application to the craniofacial, especially the orbito-maxillary, bone defects. Type I collagen is a suitable carrier material for various biomedical applications, but unfortunately lacks of mechanical strength. The collagen-based matrix can be reinforced by incorporating hydroxyapatite particulates and in addition shaped by a synthetic membrane [9]. This approach has been applied to the tissue scaffold of guided bone regeneration of periodontology [19]. There are many methods to fabricate the composite of collagen and hydroxyapatite and some of them incorporate a network of microchannels in the interior of the scaffold to increase the mass transport of oxygen and nutrients, such as solid freeform fabrication (SFF) method [28–30]. These problems can also be minimized by using microspheres/beads system. In this study, we use the hydroxyapatite/type I collage gel beads for tissue-engineered bone replacement by providing the scaffold for osteoblasts to attach, migrate, proliferate and differentiate. This injectable microsphere/bead system has high surface area to volume ratio, which depends on the size of the microsphere/bead, and it has been proved to support the growth of osteoblast cells [12].

PRP is a concentration of autologous platelets, which poses no risk for transmissible disease in human being. It is also a source of different protein growth factors and the growth factors of platelet are in their normal ratios, which result in most effective interworks that distinguish PRP from recombinant growth factors. These recombinant growth factors, such as recombinant human PDGF or the recombinant human BMPs, are single growth factors often in super-physiologic doses [14–18]. The protein growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor, and epidermal growth factor β are polypeptides released from the platelets, which have been proved to improve wound healing and bone regeneration. In the previous studies, many growth factors have been demonstrated to stimulate bone formation and bone healing. Marx et al. reported that PRP accelerated bone repair due to high concentration of PDGF, TGF-β and other growth factors [14]. The mechanism of the initiation of bone regeneration may start with the release of PDGF and TGF-β from the degranulation of platelets. PDGF can stimulate mitogenesis of marrow stem cells and transfer endosteal osteoblasts into the graft. It also starts angiogenesis of capillary budding into the graft by inducing endothelial cell mitosis. TGF-β activates fibroblasts and preosteoblasts to mitoses and increase their number, as well as promoting their differentiation towards mature functioning osteoblasts. Continued TGF-β secretion also influences the osteoblasts to lay down bone matrix and the fibroblast to lay down collagen matrix to support capillary in growth. Human platelet concentrations also had been used as a supplement of basic medium and it was found that platelet-supplemented medium stimulates proliferation and maintains the differentiated function of human osteoblast-like cells [14–18]. In their reports, the platelets may play an important role in healing of fracture and also may be useful as a cheap autologous source of multiple growth factors to enhance osteoblast proliferation in vivo and in vitro. Autologous platelet-rich plasma gel had also been used with apparent clinical success in the craniofacial, oral-maxillary and orthopedic surgery. Recently, it has undergone a significant increase in use as adhesive with cancellous bone particles in oral and maxillofacial surgery bone grafting procedures. Several studies reported a positive effect in bone regeneration and soft tissue healing when PRP was applied with autogenous bone grafts, with freeze-dried bone allografts and composites of autogenous bone grafts in sinus lifts, with skin graft healing enhancement [15, 16]. Although PRP can be produced easily and rapidly, however, some questions yet remain: how and when growth factors are secreted by platelets, what is their viability, what is the correlation between their concentration and the number of platelets, and so on. Further investigations are necessary to clarify these points.

The techniques of guided bone regeneration utilizes a mechanical barrier in an intra-bony defect with the aim of creating a secluded space to received only cells with an osteogenic potential so osteogenesis may occur unimpeded within the space [19]. In addition to supply long term mechanical need, this method has been used to enhance bone regeneration within intra-bony defect, to form improved osseous tissue around the implant in bone, to prevent fibrous encapsulation, and to produce additional bone in the area. In this study, combined with the concepts of tissue engineering, we use type I collagen/hydroxyapatite gel beads and scaffold and compounded with PRP to fabricate a protrusive bone segment in vivo. The results of this study showed that successful in vivo engineering of large bone segment from pre-exist bony margin is possible. It can promote the in-growth of surrounding tissues from the bony margin, with established extensive patent vascular network within the engineered bone segment. The in-growth tissues were composed mainly of macrophages and osteoblasts and the matrixes had begun to degrade. Surrounding osteoprogenitors and osteoblasts invaded the matrix forming new osteoids. The results from this study also indicate that continually calcification in the matrixes or degraded matrixes as evidenced by the strong

green fluorescence observed under the confocal microscope. However, further study of the engineered bone, esp. biomechanical analysis, is indicated.

5 Conclusion

There is little doubt that scientists and clinicians alike will continue to challenge the benefits and merits of novel regenerative medical procedures for some time to come. Appropriate combinations of bioengineering (biomaterials, growth factors, and stem cells) are essential for stable long-term function and integration of therapeutic implants. Using the type I collagen/hydroxyapatite gel beads matrixes and intermittent injection of the autologous platelet-rich-plasma, specific 3D osseous tissues with fibrovascular network structure from pre-exist bony margin were successfully created in an in vivo animal model.

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