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Microencapsulated rBMMSCs/calcium phosphate cement for bone formation in vivo

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Abstract. As an injectable scaffold material for bone tissue engineering, calcium phosphate cement (CPC) has good biocompatibility, self-setting, and osteoconduction properties. Alginate-microencapsulated seed cells can pick up the degradation speed and bioactivity of CPC. The aim of this study was to explore the osteogenic ability of a composite of microencapsulated rabbit bone marrow mesenchymal stem cells (rBMMSCs) with β-tricalcium phosphate/calcium phosphate cement (β-TCP/CPC) in vivo. Cavity defects were created in both femoral condylar regions of New Zealand White rabbits. β-TCP/CPC (control group) and alginate microencapsulated rBMMSCs/β-TCP/CPC composite (composite group) were implanted separately into the bone defects of both femurs. Bone substitute degradation and new bone formation were evaluated by CBCT, and the defects were examined histologically 8, 16, and 24 weeks after implantation. In addition, fluorescent carbocyanine CM-Dil was used to track the rBMMSCs in vivo after implantation. The results showed that far more new bone and bone marrow grew into the bone defects in the composite group. Few CM-Dil labeled positive cells were observed postoperatively. However more native cells were detected in the graft areas of the composite group than those of the control group. The study indicates that a composite of microencapsulated seed cells/β-TCP/CPC might be considered as a promising injectable material for the generation of new bone tissue.

Keywords: Calcium phosphate cement, microencapsulation, injectable, bone marrow mesenchymal stem cell

1. Introduction

In clinical practice, there is a significant need for treatment of bone nonunions and bone defects caused by numerous reasons such as trauma, tumor resection and congenital malformations. Bone tissue engineering is of major interest for bone reconstruction to repair bone defects, in which scaffold, seed cells, and growth factors are three classical elements. In order to implant engineered bone, an open operation is often required. Compared with the surgical implantation method, the injection technique has obvious advantages, such as easier administration, minor invasiveness, reduced scar formation and lower risk. β-tricalcium phosphate/calcium phosphate cement (β-TCP/CPC) is one type of injectable composite bone cement material produced by the Institute of Nuclear and New Energy Technology of Tsinghua University. As an injectable scaffold material, β-TCP/CPC has good biocompatibility, self-setting and osteoconduction properties. However the process of solidification of

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β-TCP/CPC paste has a toxic effect on cells and the degradation speed and bioactivity of β-TCP/CPC need to be increased [1]. Other studies have confirmed that calcium alginate microcapsules may protect the seeded cells during the setting reaction of CPC paste [2, 3]. However, so far there has been little *in vivo* study to demonstrate the osteogenic capacity of such a composite of alginate microencapsulated seed cells/CPC.

Bone marrow mesenchymal stem cells (BMMSCs) are one of the best-characterized postnatal stem cell populations which are considered suitable for application in cell-based clinical therapies. With strong regeneration potential and immunosuppressive properties, they are important for allografts [4, 5]. BMMSCs cultured in scaffolds can induce new bone formation *in vivo* and lead to improved healing of critical-size defects [6].

In this study, β-TCP/CPC was used as the scaffold material. First, rabbit bone marrow mesenchymal stem cells (rBMMSCs) encapsulated within calcium alginate micro-beads were seeded into this scaffold. Then, the bone reconstructive ability of the alginate-microencapsulated rBMMSCs/β-TCP/CPC composite *in vivo* was analyzed in an experimental defect of the femoral condyle in New Zealand White rabbits.

2. Materials and methods

2.1. Cell culture and preparation of alginate-microencapsulated seed cells

The rBMMSCs were obtained from Cyagen Biosciences Inc. and cultured in rabbit mesenchymal stem cell growth medium (Cyagen Biosciences Inc.). The cells were incubated at 37°C in an atmosphere of 5% CO2 in air and 100% humidity and passaged every 3-4 days. Medium was changed every 3 days. Passage 5 rBMMSCs were used for the experiments and labeled by CM-Dil (Invitrogen) to enable later identification in the animal study. The cells were trypsinized, washed, and resuspended in 1.5% (w/v) sodium alginate solution. The cell concentration was maintained at 1.0 × 106 cells/mL alginate. The cell-alginate suspension was released in droplets from a 5 mL syringe into CaCl2 solution (1.11% w/v) in a high-voltage electrostatic field and allowed to gel for 15 min to form alginate microencapsulated rBMMSC beads with a diameter of about 200 μm. Before implantation into the rabbit models, the alginate-microencapsulated rBMMSCs were first cultured in rabbit mesenchymal stem cell growth medium for 7 days and then in rabbit mesenchymal stem cell osteogenic differentiation medium (Cyagen Biosciences Inc.) for 3 days. By the end of the culture period, all of the rBMMSCs appeared to be positively labeled with CM-Dil.

2.2. Implantation of bone substitute material into the rabbit model

Eighteen New Zealand White rabbits (4 weeks old, with an average weight of 2.5 kg) were used in this study. All protocols were approved by the Animal Welfare Committee of Peking University Health Science Center.

After anesthesia, the rabbits were placed in a supine position and the condyles of both femurs were exposed. A cylindrical bone defect was created in each femur, perpendicular to the surface of the bone from the medial to the lateral aspect, using a dental drill cooled with normal saline. The final dimension of the bone defects were 4 mm in diameter and 5 mm deep. Alginate microencapsulated rBMMSCs and β-TCP/CPC slurry were mixed in a volumetric proportion of 1:1 to form the composite material. β-TCP/CPC (control group) and alginate microencapsulated rBMMSCs/β-TCP/CPC

composite (composite group) were injected into the bone defects of the bilateral femurs, one on either side. The bone substitute material solidified in approximately 15 min. Finally the wounds were sutured in two layers. Thirty-six femoral defects (n = 6 per group) were created in eighteen rabbits (Fig. 1). And 8, 16, and 24 weeks after surgery, animals were sacrificed by air embolism after anesthesia.

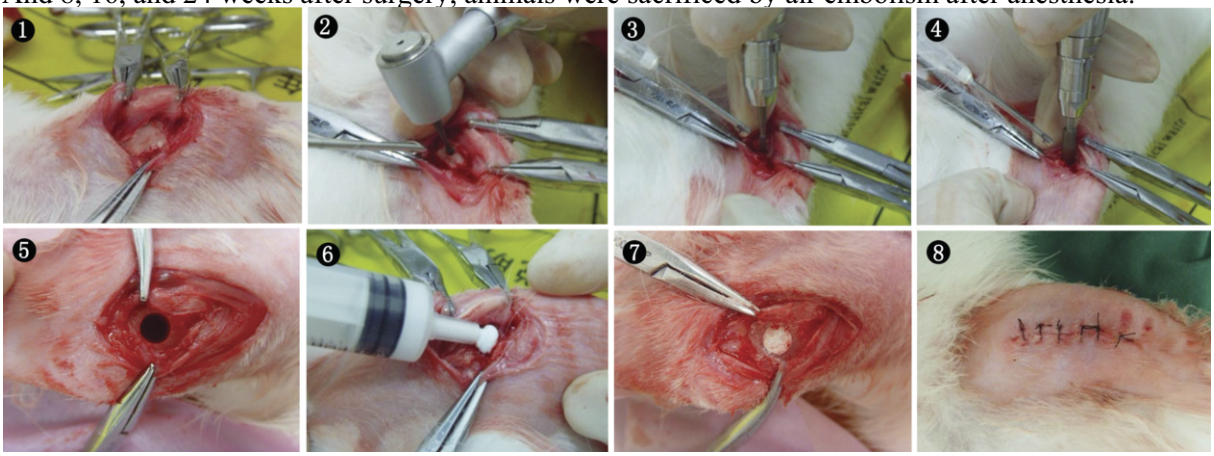

Fig. 1. The surgical implantation process of bone substitute material into a rabbit model.

2.3. Cone beam computed tomography, cell tracing in vivo, and histological examination

Three days after surgery, all animals were anesthetized and cone beam computed tomography (CBCT) was performed to observe the filling of the bone defects. And 8, 16, and 24 weeks after surgery, 6 animals were anesthetized for CBCT again to observe any degradation of the bone substitute material.

Cell tracing was performed 8 weeks after implantation. Animals were euthanized and tissue samples from bone defect areas were harvested. Half of each sample was used for cell tracing. The samples were fixed in formalin for 24 h, decalcified in Decalcifying Fluid (Zhongshan, Beijing, China) for approximately 48 h at room temperature, then washed in running water for 24 h. Samples were then frozen and 5 μm cryosections were prepared and counterstained with DAPI (Zhongshan, Beijing, China) for 5 min. The results were observed using a Confocal Laser Scanning Microscope (TCS SP5, Leica, Germany). All procedures were performed in the dark.

The other half of each 8 week sample, as well as tissue samples harvested 16 and 24 weeks after implantation, was used for histological examination. The samples were fixed, decalcified, and washed the same as above, and then dehydrated in a graded alcohol series and embedded in paraffin. Five μm sections of each sample were prepared and stained with hematoxylin and eosin (H&E) and Masson's trichrome. The stained sections were photographed digitally under a microscope (BX51, Olympus, Japan).

3. Results

3.1. CBCT scanning post-surgery

The CBCT images of both femurs of rabbits at 3 days post-surgery demonstrated that the bone substitute material filled the bone defects well on both the composite side and the control side (Fig. 2).

No material run out of the defects or flowed into the bone marrow cavity.

At 8, 16, and 24 weeks post-surgery, the CBCT images show that the bone material gradually degraded. Comparing the area and density of the remaining bone substitute material, the authors observed that the bone material on the composite side was absorbed more rapidly than that of the control side (Fig. 3).

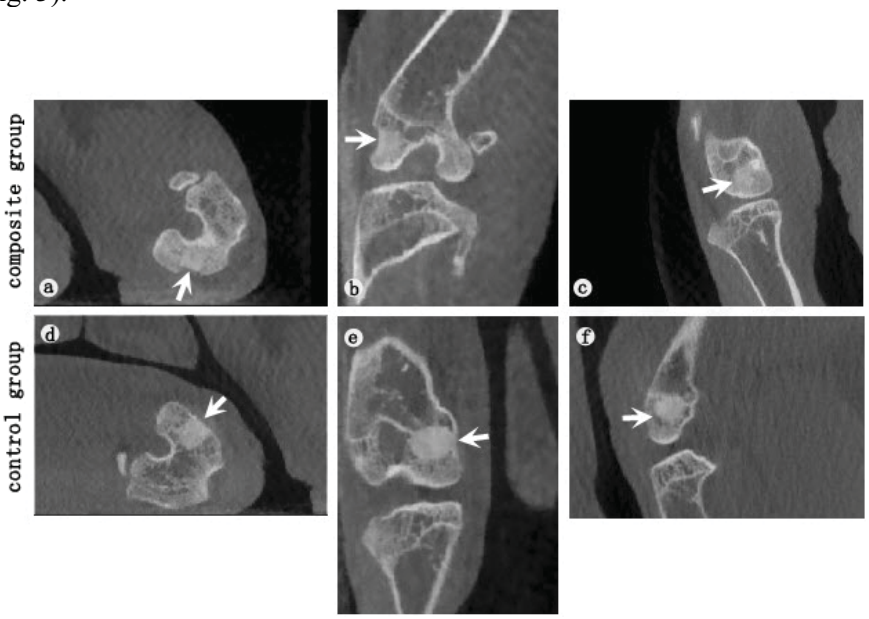

Fig. 2. CBCT images at 3 days post-surgery. White arrows show the bone material. The bone substitute material filled in the bone defects well on both the composite side (a–c) and the control side (d–f).

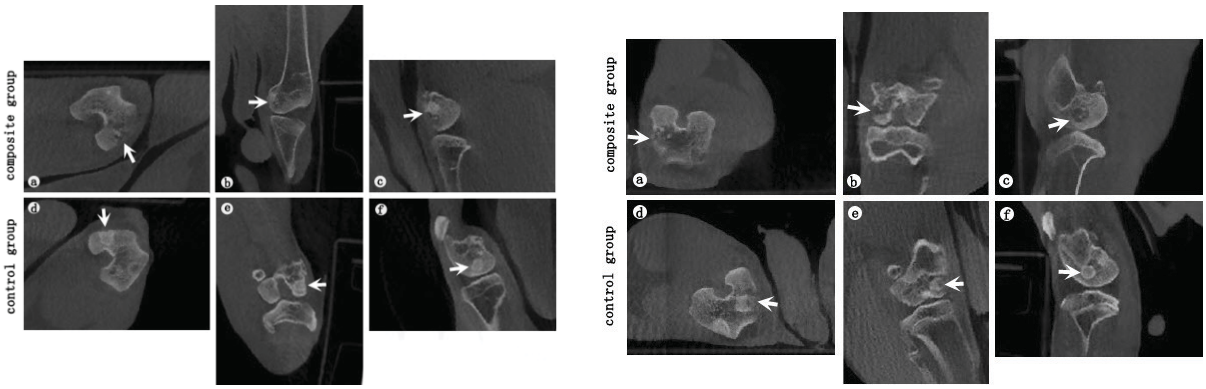

Fig. 3. CBCT images at 8 weeks (left) and 16 weeks (right) post-surgery. White arrows show the bone material. The bone material on the composite side (a–c) was absorbed more rapidly than that on the control side (d–f).

3.2. Cell tracing in vivo

Eight weeks after implantation, the graft area was harvested for cell tracing analysis. Under a confocal laser scanning microscope, CM-Dil labeled cells should appear stained red. Simultaneously all live cells of the graft area would be stained blue by DAPI. The cells that were double stained red

and blue could thus be identified as the seeded rBMMSCs, while native cells would be stained only blue. The results showed that few CM-Dil labeled seeded cells were present in the graft area of the composite group, while counterstaining with DAPI showed that native cells had grown into the interior of the graft area. In contrast with the control group, the native cells only aggregated at the border region of the substitute material (Fig. 4).

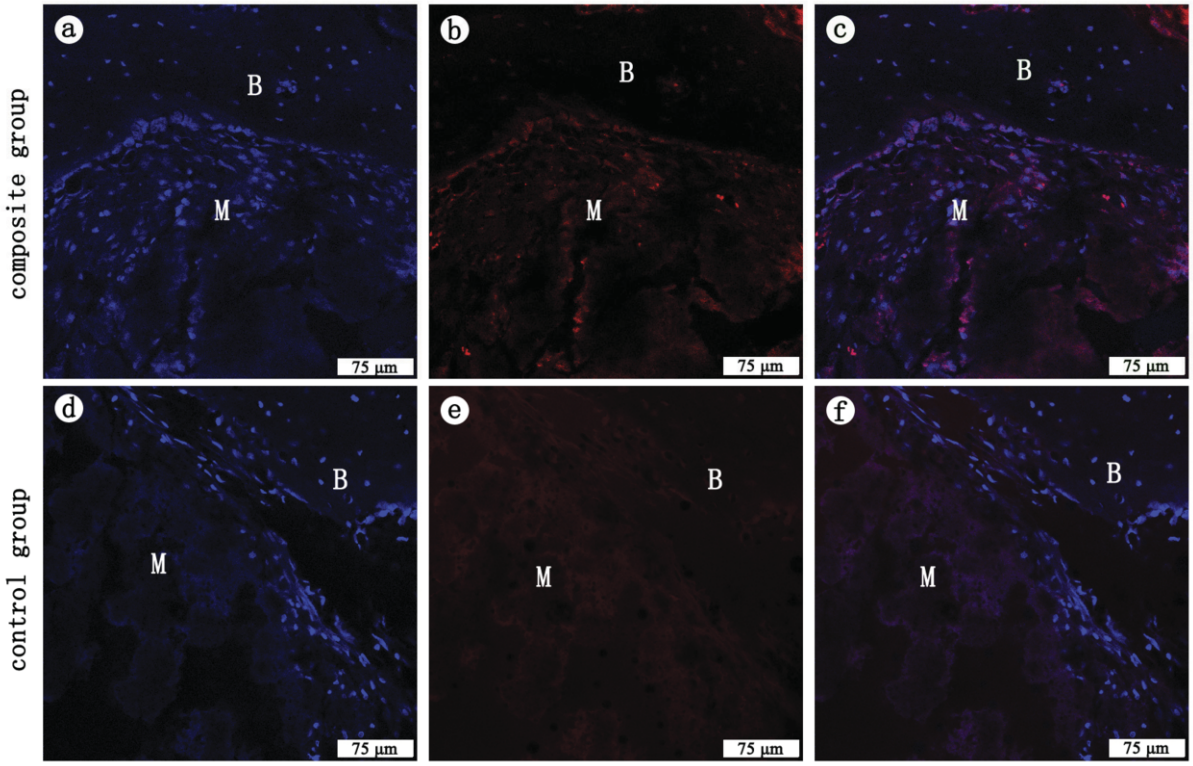

Fig. 4. Cell tracing *in vivo* at 8 weeks after transplantation. (a) and (d) are counterstaining results with DAPI. (b) and (e) are staining results of rBMMSCs with CM-Dil. (c) and (f) are overlaps of the first two images. More native cells were observed to have grown into the substitute material in the composite group (a–c) than in the control group (d–f). B: host bone. M: substitute material.

3.3. Histological examination

The reconstruction processes of bone in the defects were evaluated by histological analysis. Representative histological images are shown in Fig. 5 (H&E staining) and Fig. 6 (Masson's trichrome staining). There were distinct differences in scaffold degradation and new bone formation between areas implanted with β-TCP/CPC and those implanted with the alginate microencapsulated rBMMSCs/β-TCP/CPC composite.

Eight weeks after implantation, in the composite group, the scaffold was divided into numerous small pieces. New bone and bone marrow tissue could be seen growing into the bone defects. Osteoblasts were arranged in rows and were present at the surface layer of the new bone. A large number of multinucleated giant cells and foam cells were detected within the β-TCP/CPC material. In images of Masson's trichrome staining, numerous vessels and red blood cells were detected, but little bone collagen. In the control group, the scaffold material was still relatively integrated and of high

density. Osteoblasts and bone collagen were observed attached to the edge of the scaffold. Few vessels or red blood cells were detected within the β-TCP/CPC material, while bone collagen, a major extracellular matrix constituent, was present in the newly formed tissues at the border area of the scaffold as detected by Masson's trichrome staining. No obvious inflammatory reaction was observed in either the composite or the control group.

Sixteen weeks after implantation, in the composite group, mature fragments of bone trabeculae and bone lacunae had formed in the new bone. The scaffold had been greatly degraded except for some residual scaffold materials scattered in the graft area. In the control group, there were still large pieces of β-TCP/CPC material present in the graft area. The new bone grew inward from the edge of the scaffold to the interior. Some vessel-like tissue and bone collagen were detected in the spaces between the scaffold fragments.

Twenty-four weeks after implantation, in the composite group, the scaffold was almost completely absorbed, with only a small amount of residue remaining. Woven new bone and abundant bone marrow tissue was formed in the graft area. In contrast with the control group, there were still many remaining fragments of β-TCP/CPC material within the bone defects, and bone collagens were observed at the frontier of the new bone.

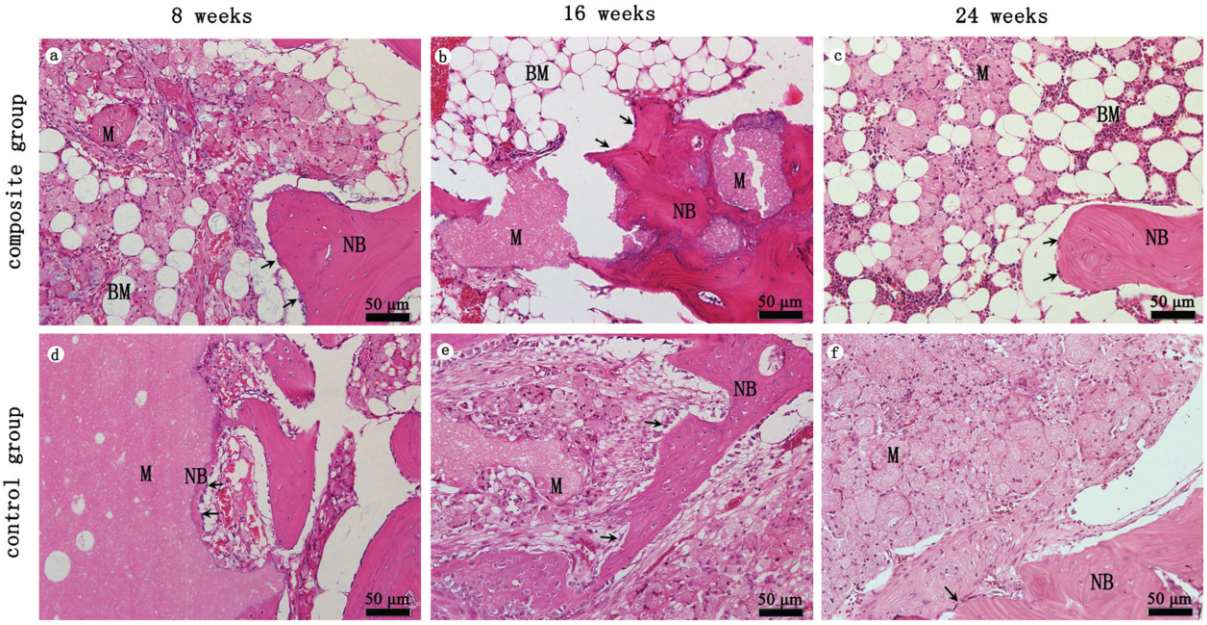

Fig. 5. H&E staining images of the graft areas after implantation (×200). New bone formation and scaffold material degradation are shown in these images. Black arrows show the osteoblasts. NB: new bone. BM: bone marrow. M: scaffold material.

4. Discussion

In this study, it was found that a composite scaffold of microencapsulated rBMMSCs/β-TCP/CPC had good bioactivity and biodegradation properties. Compared with pure β-TCP/CPC material, the composite material degraded fast, and was highly osteogenic. The two components, alginate micro-

capsules and rBMMSCs, might each contribute to promoting the degradation of the β-TCP/CPC scaffold material and new bone formation.

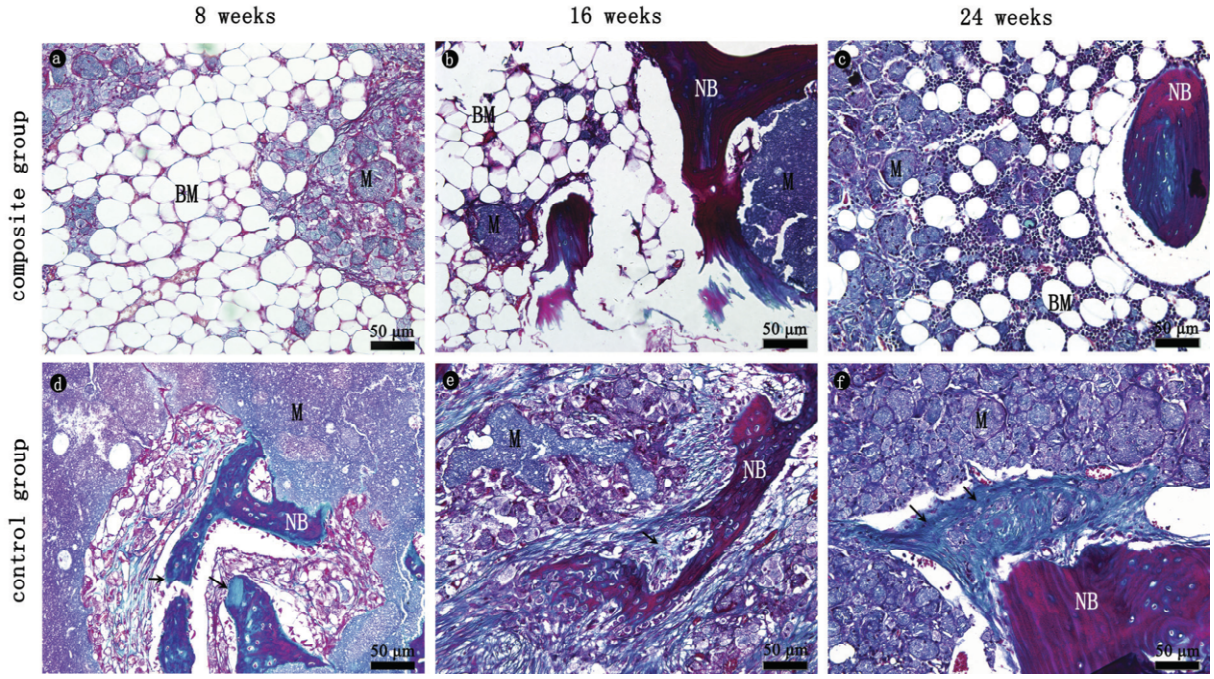

Fig. 6. Masson's trichrome staining images of the graft areas after implantation. Black arrows show the bone collagen which was stained green. Red blood cells and vessels were stained red in these images. NB: new bone. BM: bone marrow. M: substitute material.

Some *in vitro* studies previously indicated that alginate hydrogel beads might form alternative cell carriers for bone regeneration [7]. In previous studies by our research group, it was confirmed that alginate microcapsules could spontaneously dissolve after being mixed with β-TCP/CPC paste, releasing seed cells into the β-TCP/CPC scaffold. The calcium alginate hydrogel beads provided good protection for the seed cells during the setting reaction of the CPC paste [2]. After the alginate microcapsules had dissolved, pores remained in the scaffold material. In several previous studies, it had been proven that the pore size and percentage of the scaffold material had a great influence on the development of new bone in scaffold material used in bone defect areas [8-10]. In addition they might also play an important role in the osteogenic differentiation of seed cells *in vivo* [8]. In our previous studies, the diameter of the microcapsules was measured, and it was found that they were approximately 200 μm, and the percentage of pores produced by degradation of the microcapsules was 40%-50%. These results indicated that the addition of alginate microcapsules made the scaffold material more porous. Pores could provide space to allow for migration and proliferation of mesenchymal cells and native osteoblasts within the scaffold, as well as vascularization. As a result, in the composite group, the authors detected more native cells, more osteoid tissue and increased ingrowth of vessels, along with faster scaffold resorption.

In the present study, scaffold degradation and new bone formation in areas grafted with β-TCP/CPC with alginate microencapsulated rBMMSCs composites were only compared with β-TCP/CPC, since only one bone defect could be made at the condyle of each femur in each rabbit. However, in our

previous studies β-TCP/CPC paste with alginate microcapsules (CPC-A) and β-TCP/CPC with alginate microencapsulated seed cell (CPC-A-cell) composites, as well as β-TCP/CPC paste were implanted subcutaneously into the backs of nude mice at separate sites. In these experiments, histological examination showed that the CPC-A-cell group exhibited better bone-forming ability than the CPC-A group, suggesting that the cells seeded into CPC secrete growth factors to enhance bone formation or recruit native cells into the defective area to generate new osseous tissues. The CPC-A composite seemed to have an accelerated degradation rate compared with grafts of CPC alone, because the introduction of microcapsules into CPC concomitantly created pores in situ, which were suitable for cell migration and tissue ingrowth (These results have been accepted for publication in the journals "Rare Metal Materials and Engineering" and "Materials Science and Engineering C" in 2013).

Eight weeks after implantation, few seeded rBMMSCs could be detected by cell tracing *in vivo*. By contrast, in other studies, seeded cells could be detected 6 or 10 weeks after implantation [11, 12]. By comparing these studies, the authors found that in studies in which the labeled seeded cells were detected, a scaffold with the seeded cells attached to the surface, or no scaffold was used. In our study, the microencapsulated seeded cells were embedded within the scaffold material. Bone calcium salt deposition would hamper fluorescence detection. It should be inferred that the seeded cells only function as osteoblasts during the early stage after implantation. The most important function of rBMMSCs might be secreting some growth factors to recruit native cells into the area of the defect to generate new osseous tissues, in the same way as previously demonstrated in an *in vitro* study [13]. This possibility should be explored in further studies.

The process of new bone formation is affected by many factors. Generally, the reconstruction of bone involves the interaction of osteoblasts with osteoclasts. The process consists of a catabolic part, with resorption of the dead bone graft by osteoclasts, and an anabolic part with osteoblasts forming a new trabecular structure. On one hand, anabolic substances such as bone morphogenetic proteins (BMPs) stimulate bone formation and bone graft remodeling. On the other hand, BMPs also boost catabolism and might therefore cause premature resorption, both of the graft and of the newly-formed bone. Bisphosphonates inactivate osteoclasts and can be used to control resorption. The combination of BMPs and bisphosphonate could thus accelerate bone remodeling and increase the amount of remaining woven bone after the completion of remodeling [14]. In our study, abundant bone marrow tissue and a relatively smaller amount of woven new bone formed in the graft area. One possible reason might be an imbalance in the relative proportions of catabolism and anabolism. In order to induce more new trabecular bone formation, additional growth factors might be needed.

5. Conclusion

A composite of microencapsulated rBMMSCs/β-TCP/CPC has good bioactivity and biodegradation properties. The composite material degrades quickly, and has strong osteogenic capability. It could therefore be considered as a new promising material to generate injectable bone tissue. However, additional growth factors might be needed to induce formation of more new trabecular structure.

6. Acknowledgements

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