Bone marrow-derived osteoblasts seeded into porous beta-tricalcium phosphate to repair segmental defect in canine's mandibula

Köpek mandibulasındaki defekti tamir etmek üzere kemik iliğinden elde edilen osteoblastların poroz beta-trikalsiyum fosfat içine tohumlanması

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BACKGROUND

Bone regeneration is often needed for many aesthetic and reconstructive procedures. Tissue engineering provided a promising approach to supplement existing treatment strategies. In this study, we aimed to evaluate the effect of reconstructing mandibular defect by using bioceramics seeded with bone marrow derived osteoblasts.

METHODS

Canine's autologous marrow stromal cells were Culture-expanded and induced to osteoblastic phenotype, then were seeded into prepared porous beta-tricalcium phosphate, after being incubated *in vitro*. The cell/ scaffold complexes were implanted into the prepared defect in canines' mandibula and fixed by internal rigid fixation. In control groups, beta-tricalcium phosphate alone and autologous iliums were implanted into the prepared defects. Twelve weeks after implantation, the specimens were examined macroscopically and histologically.

RESULTS

In experimental group and autologous iliums group, new bone grafts were successfully developed at 12 weeks after implantation and repaired the continuity of the mandibula. Histologically, newly formed bone could be observed on the surface and in the pores of beta-tricalcium phosphate in the cell/scaffold group, whereas incomplete bone repair was found in pure beta-tricalcium phosphate group.

CONCLUSION

The harvested bone marrow derived osteoblasts possess the ability to form new bone tissue when seeded onto porous beta-tricalcium phosphate, which shows the potential of using this method to repair large segmental mandibular defect clinically.

Key Words: Beta-tricalcium phosphate; bone defect; mandibula; tissue engineering.

AMAÇ

Kemik rejenerasyonuna birçok estetik ve rekonstrüktif prosedürde sıklıkla gereksinim duyulmaktadır. Doku mühendisliği, mevcut tedavi stratejilerini desteklemeye yönelik umut verici bir yaklaşım sağlamıştır. Bu çalışmada, kemik iliğinden elde edilen osteoblastlar ile tohumlanan biyoseramikler kullanılarak gerçekleştirilen mandibüler defekt rekonstrüksiyonunun etkisini değerlendirmeyi amaçladık.

GEREÇ VE YÖNTEM

Köpekte otolog kemik iliği stromal hücreleri, kültürle genişletilip osteoblastik fenotipe indüklendi. Daha sonra hazırlanan poroz beta-trikalsiyum fosfat içine tohumlandı, *in vitro* olarak inkübe edildikten sonra hücre/iskelet kompleksleri canine'nin mandibulasındaki defektin içine implante edilerek internal rijit fikasyon yöntemi ile sabitlendi. Kontrol gruplarında, hazırlanan defektlere tek başına beta-trikalsiyum fosfat ve otolog iliumlar implante edildi. İmplantasyondan on iki hafta sonra, örnekler makroskopik ve histolojik olarak incelendi.

BULGULAR

Deneysel ve otolog ilum gruplarında, implantasyondan on iki hafta sonra başarılı bir şekilde yeni kemik greftleri gelişti ve defekti tamir ederek mandibulanın devamlılığını sağladı. Histolojik olarak, hücre/iskelet grubunun yüzey kısımlarında ve beta-trikalsiyum fosfat porlarında yeni oluşmuş kemik gözlenebilirken saf beta-trikalsiyum fosfat grubunda tam oluşmayan kemik tamiri olduğu bulundu.

SONUÇ

Elde edilen kemik iliği kaynaklı osteoblastlar, poroz beta-trikalsiyum fosfat üzerine tohumlandıklarında yeni kemik dokusu oluşturma yeteneğine sahiptirler. Bu özellik, klinik olarak geniş segmental mandibuler defektlerin tamirinde kullanılabilecek olası bir yönteme işaret etmektedir.

Anahtar Sözcükler: Beta-trikalsiyum fosfat; kemik defekti; mandibula; doku mühendisliği.

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Despite the progresses that had been made in bone regeneration, repair of segmental defects in mandibula remains one of the challenging problems in oral and maxillofacial surgery. Autologous bone grafting is widely accepted as the gold standard for the treatment of bone defects and nonunions,[1] however, serious drawbacks, such as a prolonged operation time and donor site morbidity in about 10-30% of the cases,[2] stimulate people to find better approach to repair bony defects. Recently, distraction osteogenesis has been reported to successfully repair limited defect in mandibula,[3] but these techniques often involve multistage surgical procedures, inhibit early mandibular function, and require several revision procedures to maintain acceptable alignment and achieve osseous healing.

Advances in tissue engineering during the past decades have enabled this technique to hold promise for the eventual application of guided osseous tissue regeneration in the mandible. Many biomaterials, such as polyester, bioceramics and hydrogels have been tested to be scaffold in bone tissue engineering. Among these biomaterials, although scaffolds made of polyesters own better mechanical properties, they may provoke inflammation due to the hydrophobic property and the acidity of their hydrolysis products.[4,5] Synthetic porous bioceramics such as hydroxyapatite (HA) and beta-tricalcium phosphate (beta-TCP) represent primary candidate bone substitutes. They display excellent osteoconductive properties, can be fabricated into custom-designed shape and size, and are free from risks of rejection or infection. In previous studies, hydroxyapatite was reported to be hardly resorbed,[6,7] whereas beta-TCP was reported to degrade *in vivo* and was completely replaced with remodeled host bone.[8,9] In order to adjust the degrading rate of the beta-TCP, the use of biphasic calcium phosphate ceramics with different ratios of HA to beta-TCP have also been investigated.[10] However, the HA mixed into the beta-TCP will existed in the new born bone tissue for a long time and the almost disappearance of those biomaterials and their simulated replacement by mature bone have rarely been reported. Based above consideration, we selected porous beta-TCP as scaffold and combined it with marrow-derived osteoblasts for bone regeneration.

In previous experiments, beta-TCP has been proved as a biocompatible scaffold and bone can be regenerated *in vitro* and *in vivo* by tissue engineering techniques on it.[11-13] Orii H, et al.[14] demonstrated beta-TCP graft combined with bone marrow-derived osteoblasts achieved massive bone formation in Macaque lumber posterolateral spine fusion models. In many other studies, ectopic sites, cancellous bone and calvarial defects, or small animal models were used,[15,16] however, the defect sizes in practical conditions are always larger than that in previous experiments, so we should investigate the behavior of porous calcium phosphate ceramics in repairing large segmental mandibular defects.

In this study, the combination of beta-TCP and marrow-derived osteoblasts were evaluated in large animal segmental mandibular defect model. We prepared 3 cm segmental defect in mandibula and reconstructed it with engineered autologous constructs, in canine model, using porous beta-TCP ceramics, then the reparative effects of three different treatments were compared. Good results in this model would indicate a highly effective bone graft substitute, which could be useful in many situations of traumatic bone loss encountered in clinical practice.

MATERIALS AND METHODS

Scaffold preparation

Sintered porous beta-TCP ceramics (porosity of 58% and pore size of 100-250 μm in diameter) was provided by Sichuan University (Chengdu, China). In order to obtain porous beta-TCP ceramics high-purity beta-TCP micropowder was mixed with pore-forming material and high temperature binder which chiefly consisted of CaP and P_2O_5 then foamed through the rosin foaming method and sintered at 850°C for 2 h. Implants of 30 mm in length, 8 mm in thickness and 20 mm in height were made and sterilized by high pressure steam. Part of the scaffold was processed for scanning electronic microscope (Fig. 1).

Harvesting and inducing marrow stromal cells in canines

Marrow stromal cells in canines were harvested and induced as described previously with some modification.[17] The animals were anesthesized before an 11-gauge needle was used to penetrate the

cortex of the canine iliums, 20 ml of bone marrow was aspirated into a syringe containing 5.000 units of heparin. The marrow was washed once with Dulbecco phosphate buffered saline (PBS), then it was resuspended and cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco), containing 10% fetal bovine serum (Gibco), ascorbic acid 5 mg/L (Sigma), L-glutamine 0.272 g/L (Sigma). When the cells covered about 70-80% of the bottom of the dishes, induction medium containing ascorbic acid 50 mg/L, dexamethasone 10 nmol/L (Sigma), β-glycerophosphate sodium 10 mmol/L (Sigma), recombinant human bone morphogenetic protein 2100 μg/L (rhBMP-2, Department of biological chemistry, FMMU) was applied to improve the osteoblastic phenotype of the marrow stromal cells. Before they reached the confluent monolayer, the cells were digested by using trypsin 0.25% (Sigma) digestion and harvested by centrifugation. The cell density was adjusted to $2x10^8$/mL with medium before cell seeding.

Cell seeding and *in vitro* incubation

The beta-TCP scaffolds were prewetted by culture medium and $8x10^8$ cells in about 3 mL suspension were carefully seeded onto each scaffold. The cell-scaffold complexes were put into dishes and moved into incubator for 2 hours so that most cells adhered to the scaffolds. Then, 2 mL of medium was carefully added around the complexes. Twelve hours later, an additional 10 mL medium was added and the complexes were incubated *in vitro* for 3 days (Fig. 3b). Prior to implantation, a fragment in one complex was obtained and processed for the investigation of cell-scaffold interaction. Cell morphology and cellular mitosis on the scaffolds were visualized by using scanning electronic microscope (SEM) and the confocal laser scanning microscope (CLSM) (Fig. 2).

In vivo implantation and specimen harvesting

Six male beagle canines (age, 10 months; weight, 11-13 kg) was used for this experiment according to the experimental protocol approved by the animal experiment committee of Fourth military medical university, China. Surgery was performed under aseptic conditions and general anesthesia induced with intramuscular injection of xylazine (2 mg/kg) and ketamine (20 mg/kg), in addition, a solution of 1% lidocaine was locally administrated. Initially, canines' teeth were extracted, after the healing period of 1 month; the animals' mandibular defects were prepared and underwent implantation procedures. The right mandibular body were exposed through a submandibular incision, full thickness bone defect, 3x2 cm, were created with a reciprocating saw (Fig. 3a). The animals were divided randomly into 3 groups: 2 control group and an experimental group. Plain scaffolds were implanted into the control I and autologous iliums were implanted into the control II; the implants were further fixed by 5 cm titanium plate and titanium mesh. The wounds were sutured with 3-0 silk threads. At twelve weeks postoperatively, the animals were sacrificed and the mandibles were harvested for the further analysis as follows.

Clinical evaluation and Radiologic examination

After the mandibles were harvested, they were examined clinically and photographed, the lateral view radiographs of the specimen were obtained (set at 76KV, 100mA, and exposure time of 3.2 second). Computed tomography (CT) data on harvested mandibula at 12 weeks was collected with computerized tomography (CT; 65-80 kV; 20s) and images were reconstructed using 3-dimensional image reconstruction software (Marconi, Mx8000).

Histological examination

After specimens had been fixed for 24 h, the specimens were demineralised and embedded in paraffin, and sectioned. The sections were stained with haematoxylin and eosin and Masson's trichrome before examination.

RESULTS

Scaffold evaluation

The pore size of the prepared scaffold ranged from 100 to 250 μm, with a mean porosity of about 58%. Fig. 2 shows the three-dimensional structure of the beta-TCP used in the study. Cells adhered and spreaded well on beta-TCP scaffold, fibrous extracellular matrices was observed around cells in scaffold (Fig. 2a). CLSM showed good vitability of cells seeded into the scaffolds and cells were observed around and deep into the pores.

Clinical examination

All the animals survived the intervention and concluded the study. No sign of inflammation or

adverse tissue reaction was observed around implants. Gross examination showed that the submandibular wound was well healed. From the gross specimens resected from dog models in experimental group (Cell + Scaffold) and positive control group, observations and palpation showed that newly formed bone had repaired the defect and restore the continuity of the mandibula (Fig. 4a1,

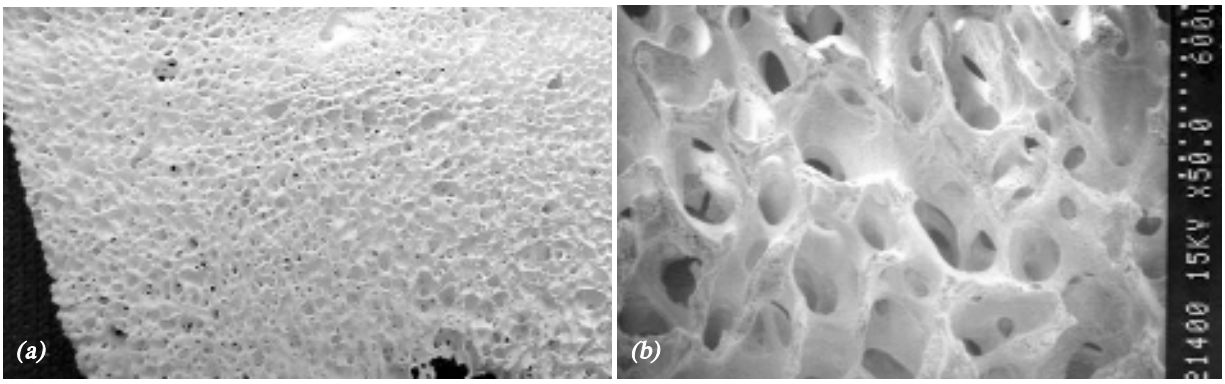

Fig. 1. **(a)** Beta-TCP used in the study. **(b)** Three-dimensional and interconnective pore structure of beta-TCP were observed by using scanning electron microscopy (bar scale: 600 µm).

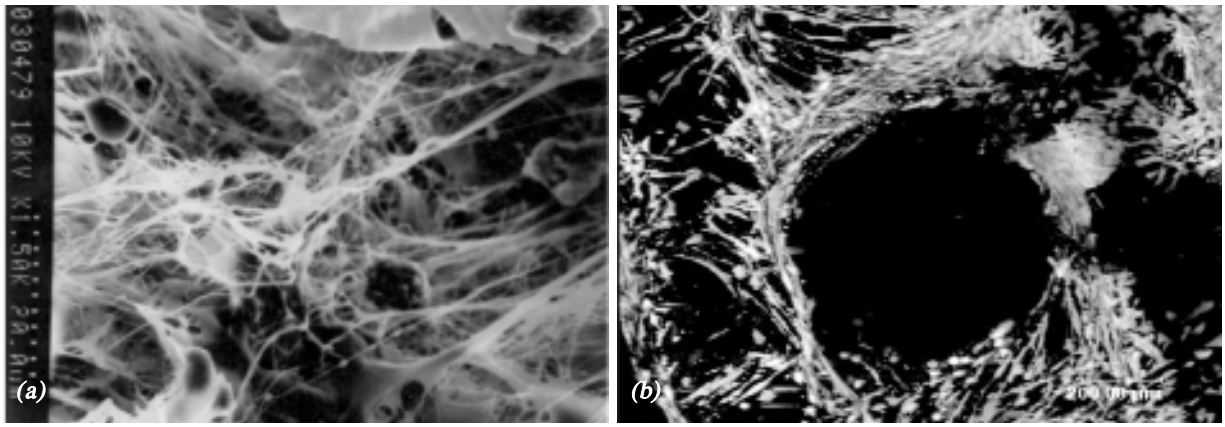

Fig. 2. **(a)** Scanning electron microscopic (SEM) observation of complexes prior to implantation. Cells covering the outer surface of the scaffold. Extracellular matrix formation is observed (scanning electron microscopy, bar scale: 20 µm). **(b)** CLSM images of cells in pores: Depth projection micrograph of initial attachment of cells on pore bottom surface (arrow indicate), bar scale: 200 µm.

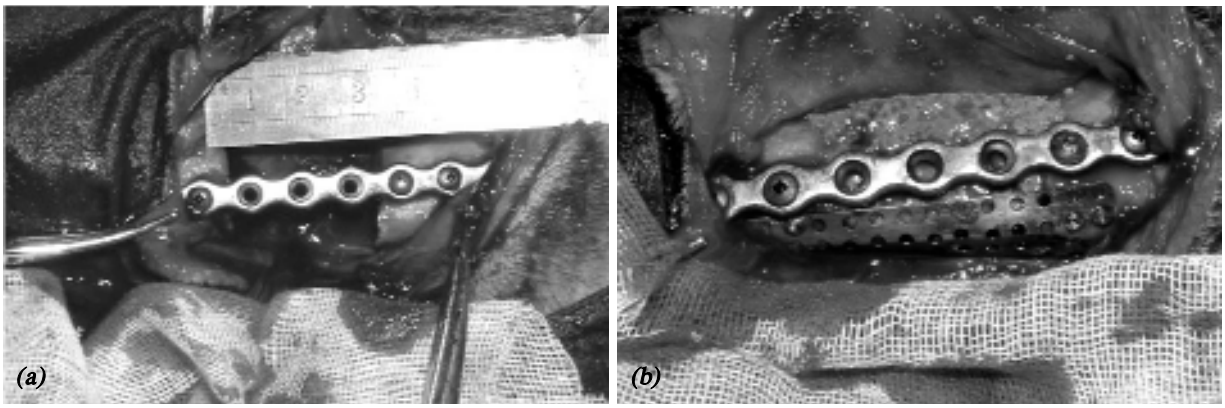

Fig. 3. Photographs of the surgical procedure. **(a)** The 3 cm bony defect was fabricated. **(b)** Cell/beta-TCP was successfully implanted into the defect and fixed by titanium plate and titanium mesh.

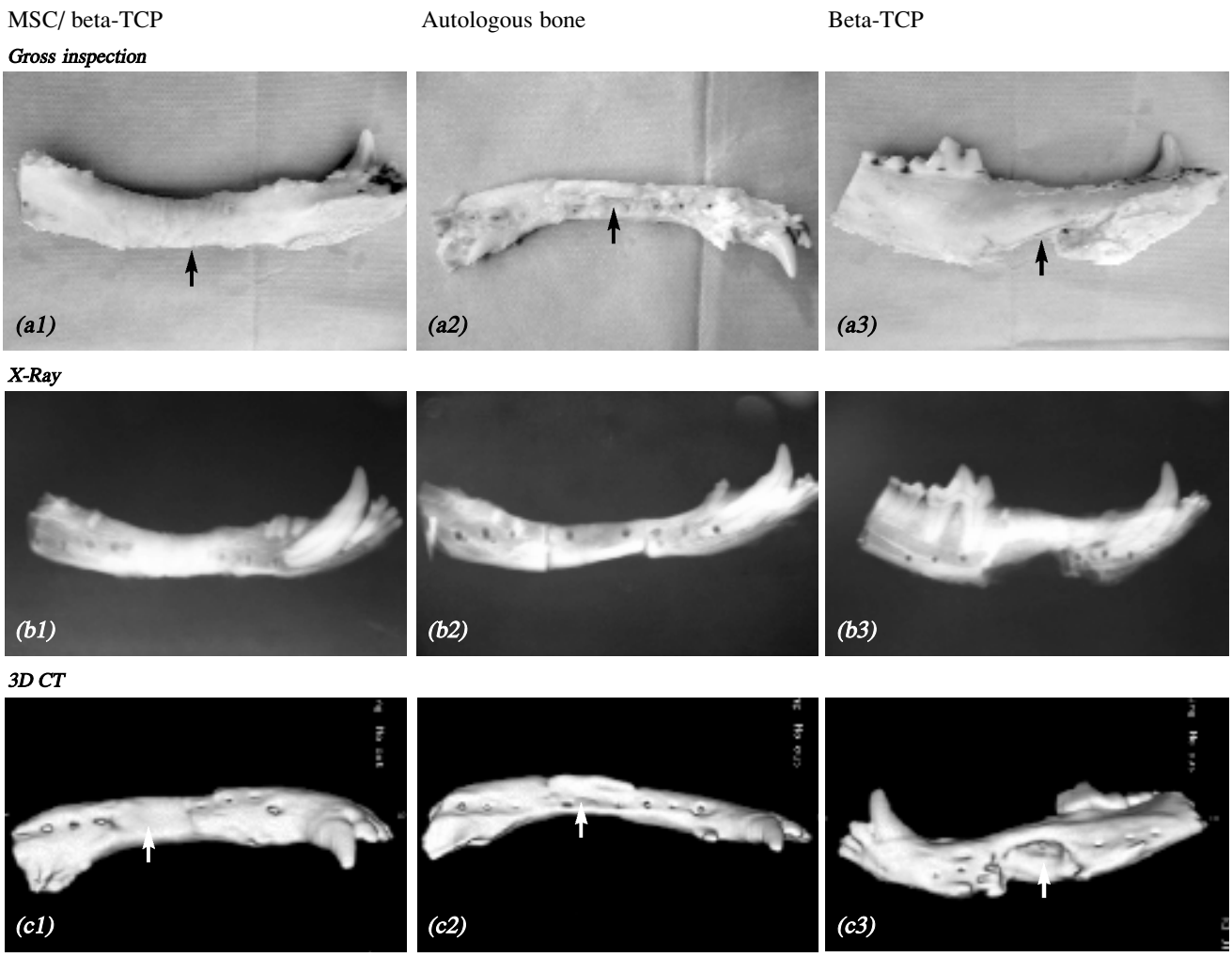

Fig. 4. Gross inspection, X-ray and 3D-CT images of the harvested mandibles at the 12th week after surgery. The repaired defect with MSC/ beta-TCP complex is shown at left **(a1, b1, c1)**. Images **(a2, b2, c2)** are of the mandible in the control group (autologous bone group). At right **(a3, b3, c3)** are the images of mandible in the beta-TCP without cells group. Arrows indicated the repairing site in specimens.

Fig. 4a2). Tissue filling each defect in the mandibular body was hard and non-compressible. In group (Cell + Scaffold), tissue engineered bone was observed to completely bridge the defect and was indistinguishable at the margins from native bone. In contrast, only partial repair can be seen in group pure scaffold, only a thin bone bridge was observed on the top of the defect, no tissue filling was observed in other part of the defect site (Fig. 4a3).

Radiological examination

The defects reconstructed with cell-scaffold construct appeared similar as compared with the native bone, radiopacity was found in defect site, which also showed good chemical bond between implants and bone (Fig. 4b1), 3D-CT supported the X-ray image, showed a smooth surface on the reparative tissue (Fig. 4c1). Autologous bone transplant group showed a good healing in bone defect site, X-ray image showed autologous bone have been replaced by new bone, however, radiolucent still can be seen in the interface between new bone and native bone (Fig. 4b2), 3-D CT showed a good morphologic reparation with autologous bone (Fig. 4c2). The defects reconstructed with unseeded scaffold appeared radiolucent in most part of the defect (Fig. 4b3); 3-D CT showed defect still existed in the implanted site (Fig. 4c3).

Histological analysis

In defects filled with cells/scaffold constructs, an endochondral ossification pattern was observed. Histological analysis with H.E and Masson's trichrome staining revealed abundant bone that was observed in the healed defects at 12 weeks. New bone was mature with histological appearance of trabecular bone on the surface. Crosslinked osteoid tissue was formed in the pores of the scaffold. Small diameter blood vessels had penetrated into the bone graft. The development of the bone trabecular and medullary cavity filled with bone marrow were also observed in newly formed bone (Fig. 5a and 6a). Fig 5b and Fig 6b revealed the substantial new bone formation occurred at the defect site in autologous bone group. New woven bone was observed in bone graft and Masson's trichrome verified the calcification within transplant. In contrast, the group of scaffold without cells showed large part of scaffold degraded with only scattered bone tissues enclosed (Fig. 5c). Osteoid synthesis was poor compared with the corresponding cell-scaffold transplant; limited cellular infiltrations were observed (Fig. 6c)

DISCUSSION

As a promising technique in regeneration medicine, tissue engineering has been used to recreate many types of tissues. Restoration of bone and repair of bone defects are its main aspects and are promising for general clinical application. Its advantages include less donor site morbidity and control of shape, which can overcome the drawbacks of autologous bone graft.[18,19] In this study, we attempted to restore large segmental defect (3 cm) in mandibula by using the techniques of tissue engineering. According to the principles of the tissue engineering, we aspirate bone marrow from ilium of animals, after expansion and induced to osteoblastic phenotype *in vitro*, the bone marrow derived osteoblasts were seeded into the prepared beta-TCP scaffold and the cell/scaffold complex was implanted into the defect of mandibula in canine model. Twelve weeks later, cell/scaffold construct was replaced by newly formed bone and successfully restored the continuity of the mandibula.

An important advantage of tissue engineering is the availability of bone tissue without donor site

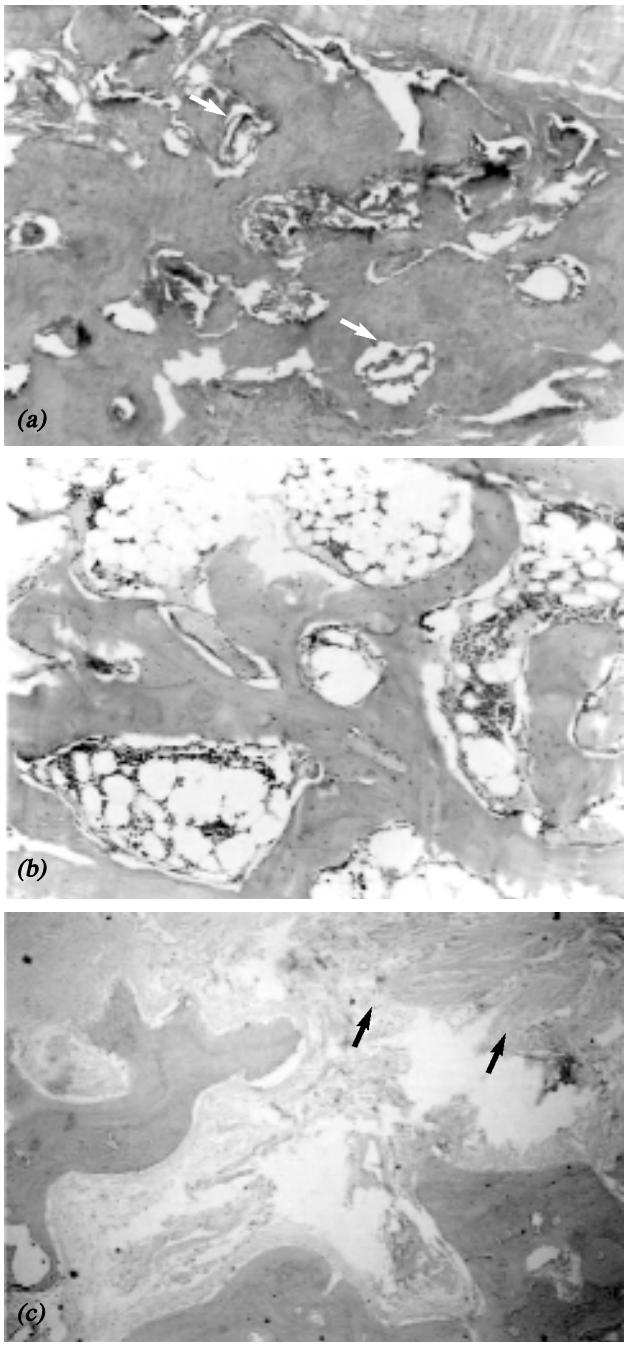

Fig. 5. The histological appearance of segmental mandibular defects at 12 weeks postoperatively. **(a)** The bone defect site has been fully closed in the MSC/β-TCP treated defect small vessels were observed to penetrated into the formed bone tissues (arrow). H-Ex100. **(b)** Bone defect site treated with autologous bone. An extensive network of newly formed bone trabeculae is seen adjacent to the original cortical bone (H-Ex100). **(c)** Bone trabecula are seen at the periphery of the bone defect site of a control beta-TCP treated defect, while a gap containing connective tissue is still observed in the defect site (arrow) H-E x100.

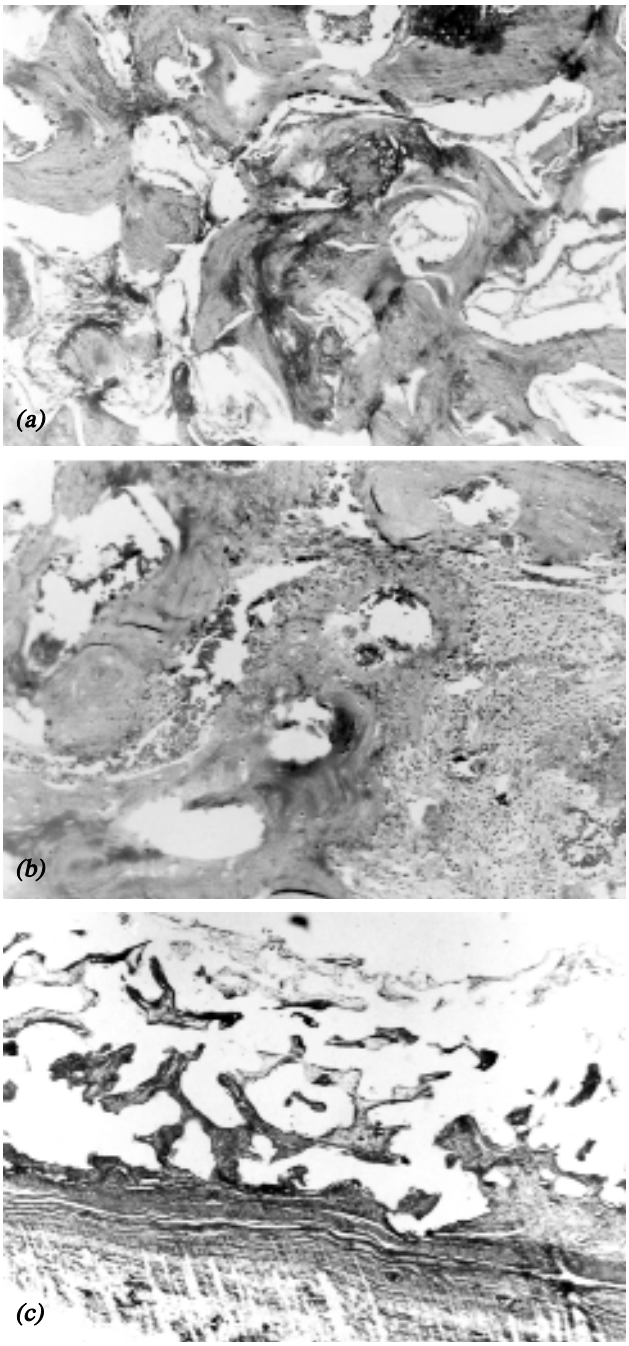

Fig. 6. **(a)** Masson's trichrome showed massive formation of mature bone-like tissues in the defect of MSC/β-TCP group (x100), which is also showed in **(b)** group (Autologous bone group, (x100)). **(c)** In beta-TCP without cells group, Masson's trichrome showed less new bone formation at the surface of the specimen, The rest of the defect is filled with fibrous tissue (x40).

morbidity. Mesenchymal stem cells are multipotential cells which can be induced to differentiate into bone, cartilage, fat and connective tissue, Mesenchymal stem cells can be aspirated from bone marrow and expanded *in vitro* to a large number,[20] thus provided an ideal source for autologous bone tissue engineering. In previous study, we have observed bone formation using culture-expanded marrow derived osteogenic cells was through endochondral bone formation.[21] As compared with two other kinds of osteogenic cells, osteoblast and periosteal cells, bone marrow derived osteoblast could be easily obtained by needle aspiration, which avoids traumatic harvesting operations.

As critical element in bone tissue engineering, scaffold plays an important role in determining the effect of osteogenesis. In traumatic bone defects, a bone graft substitute should contain a material with some structural integrity and a space-occupying effect in order to maintain a proper reduction and continuity between the bone fragments during the operation and to enable an adequate osteosynthesis to be performed.[22,23] Beta-TCP has been considered as a very promising osteoconductive, ceramic bone substitute, its properties are much like the inorganic phase of bone, which constitutes 60-70% of human bone.[24,25] The macro-porosity and crosslinked pores of the material facilitate bone ingrowth and vessels' infiltration. In animal studies tricalcium phosphates have shown favourable biocompatibility, osteoconduction and resorption properties. It appeared that the beta-TCP gradually resorbs and in the end is completely replaced by remodelled bone.[26,27] It could be also argued that the brittleness of beta-TCP, as used in this study, would make this material unsuitable for this purpose. However, in bone defect of mandibula, a space-occupying effect and little structural support is sufficient, because the major part of the stability is provided by the osteosynthesis and the involved implants will be kept unloaded until healing has been achieved. In our experiment, the implants were fixed by titanium plates and meshes. So during the osteogenesis process, the scaffolds were in fact free of stress. Based above consideration, we believe that it is an ideal material in the use of bone tissue engineering, as shown in the present study.

Although some papers reported that bone defect can be repaired by filling beta-TCP alone, in these studies, the size of the defects made in animal model was limited, and no result in segmental defect was reported. In our experiment, beta-TCP

/cells constructs were capable of forming a sufficient amount of bone to repair segmental defect, whereas beta-TCP alone did not repair. In other studies with segmental bone defects, an improvement of healing with the combination of hydroxyapatite and bone marrow compared to hydroxyapatite alone was found as well.[28,29] This is in agreement with the general assumption that bioactive ceramics itself has no osteoinductive activity.[30]

Angiogenesis is essential for the delivery of oxygen and nutrients required for bone formation. Osteoblast-like cells need a supply of oxygen and nutrients from surrounding blood vessels or tissue fluid *in vivo*, the cells inside the porous materials can not obtain a supply of oxygen and nutrients during the culture *in vivo*, this fact remains a difficult problem in tissue engineering when repair large tissue defect. Appropriate porous structure can facilitate the ingrowth of blood vessels,[31] in addition, vascular endothelial growth factor is highly expressed by osteoblastic cells,[32] which means addition of osteoblastic cells also facilitate the growth of vessels into the scaffold. In this study, to achieve uniform bone formation and better vascularization, the pore size and interconnected pore structure was controlled during the processing procedures of scaffold, in addition, construct's early implantation (3 days' *in vitro* incubation) was performed. In cell/scaffold group, many small diameter vessels were observed in the abundant bone tissue which were formed at the outer part of the implants and restored the continuity of the mandibula. However, complete replacement of the central part which was filled with scaffold/biomaterial by growing bone tissue requires longer term investigations.

Several problems remain to be solved before any clinical applications can be undertaken. First of all, it is important to use auto-serum to culture seeded cells, instead of fetal bovine serum which is thought to lead to immune rejection. Another problem is that even if beta-TCP is a good scaffold for this method, could we produce a scaffold with specialized shape? Recently, using computer aimed design and manufacture (CAD/CAM); the graft could be made into the shape exactly contouring the lost bone, such as the mandibular ramus, and other bones with complicated three-dimensional structures in the oral and maxillofacial region.[33]

In conclusion, healing of segmental bone defects, treated with porous beta-TCP can be improved considerably by the addition of osteogenic autologous mesenchymal cells. In 12 weeks, these composite biosynthetic grafts yielded results comparable to those of autologous bone grafting. Therefore, beta-TCP combined with bone marrow is a valuable alternative to autograft in the treatment of traumatic bone defects and atrophic non-unions, by which the disadvantages of the harvest of autologous bone can be prevented.

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