Effect of expanded bone marrow-derived osteoprogenitor cells seeded into polycaprolactone/tricalcium phosphate scaffolds in new bone regeneration of rabbit mandibular defects

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Abstract
The purpose of this study was to assess and evaluate new bone formation in rabbit marginal mandibular defects using expanded bone marrow-derived osteoprogenitor cells seeded in three-dimensional scaffolds of polycaprolactone/tricalcium phosphate (PCL/TCP). Bone marrow was harvested from the rabbit ilium and rabbit bone marrow-derived osteoprogenitor cells were isolated and expanded in standard culture medium and osteogenic medium supplement. The cells were then seeded into the PCL/TCP scaffolds and the cell/scaffold constructions were implanted into prepared defects in rabbit mandibles. PCL/TCP scaffold alone and autogenous bone graft from the mandible were also implanted into the other prepared defects. The specimens were evaluated at 4 and 8 weeks after the implantation using clinical, radiographic, and histological techniques. The results of the experimental group demonstrated more newly formed bone on the surface and in the pores of the PCL/TCP scaffolds. In addition, the osteoblasts, osteocytes, and new bone trabeculae were identified throughout the defects that were implanted with the cell/scaffold constructions. The PCL/TCP alone group was filled mostly with fibrous cells particularly in the middle region with less bone formation. These results would suggest that the derived osteotoprogenitor cells have the potential to form bone tissue when seeded onto PCL/TCP scaffolds.

1 Introduction
Bone defect secondary to a tumor, trauma, or deformity often presents a significant problem for oral and maxillofacial surgeons and orthopedics. Although autogenous bone is usually considered as the gold standard for the treatment of bone defects and non-unions, its limitations include inadequate bone volume, deformity, and discomfort [1]. However, serious drawbacks, such as prolonged operation time and donor site morbidity in about 10–30% of cases, impel people to find better approaches to repair bone defects. Hosts of allogenic and synthetic materials are available, but they also have inherent disadvantages, including risk of infection, immunological issues, structural integrity, and contouring abnormalities [1, 2]. Bone regeneration by means of tissue engineering has attracted increased interest. The concept of tissue engineering is based on three pillars: scaffolds, cells, and signaling molecules [3]. The appearance of a novel field of science called tissue engineering has brought up some hope for the solution of the aforementioned issues. In this field, it is believed that a combination of a three-dimensional porous scaffold with an adequate osteogenic or chondrogenic cell population can lead to the development of bone or cartilage tissue for total regeneration of an affected area when implanted in vivo [4].

This study aims to evaluate the combination of a poly-(ε-caprolactone)/tricalcium phosphate scaffold seeded with autologous expanded bone marrow-derived osteoprogenitor cells (PCL/TCP/Cells) to repair rabbit segmental mandibular defect in a rabbit model compared with a poly-(ε-caprolactone)/tricalcium phosphate scaffold alone (PCL/TCP).
The results of this model may indicate a highly effective useful method in many situations of traumatic bone loss encountered in clinical practice.

2 Materials and methods

2.1 Animals

Male New Zealand white rabbits were used in the present study. The rabbits weighed 3.5–4 kg and were aged 5–7 months. The housing and feeding of the animals were performed according to standard animal care protocols. The study was approved by the animal experiment ethic committee of Prince of Songkla University.

2.2 Materials

PCL–TCP (ratio of 80:20) composite scaffolds were fabricated by fused deposition modeling (FDM) as previously described [5–8]. The newest FDM techniques (FDM 3000; Stratasys, Eden Prairie, MN) were used. The scaffolds were produced in a class 10K clean room environment. Each scaffold manifested a lay-down pattern of 0°/60°/120°, porosity of 70%, and average pore size of 0.515 mm [9]. The scaffolds had a compressive modulus of 23.1 ± 6.16 MPa and compressive strength of 6.38 ± 0.82 MPa. The PCL–TCP scaffolds revealed a typical honeycomb structure with interconnected equilateral triangles of regular porous morphology [10]. The specimens were cut into smaller cuboids with dimensions of 10 × 5 × 3 mm². Micro-computed tomography (SkyScan-1076, Belgium) analysis revealed that the TCP existed as non-uniformly distributed particles on the rods of PCL [11].

2.3 Bone marrow harvest and cell culture

In the group of cell–scaffold constructs, the animals were anesthetized with intravenous injections of pentobarbital sodium (25 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) into the lateral ear vein. An injection of 2% lidocaine containing 1:100,000 epinephrine was administered into the operating site [2]. Under sterile conditions, approximately 25 mm³ of cancellous bone from the iliac crest was harvested and placed into the transport medium that consisted of Dulbecco’s Modified Eagle Medium (Gibco, USA) and 100 IU/mL penicillin and streptomycin. Having cleared away the periosteum and soft tissue, the bone piece was cut into small pieces [1]. Bone marrow–derived osteoprogenitor cells were isolated from the cancellous bone particles by the explant technique. The bone was manually morcellized into about 2–3 mm³ pieces using a blade and placed into 3.5 cm petri dishes. The small bone pieces containing bone marrow and trabecular bone were collected and placed into α-Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotics (penicillin/streptomycin) [12]. Non-adherent cells were removed from the cultures after 3 days by washing with phosphate-buffered saline solution, and fresh medium was replaced. Adherent cell clusters grew into colony-forming fibroblast-like cells in 10–14 days and the medium was changed every 3 days [13]. After the first passage, the medium was replaced with osteogenic medium (α-MEM with 10% FBS, 100 nmol/L dexamethasone, 50 μg/mL ascorbic acid, and 10 mmol/L β-glycerophosphate), which would stimulate bone marrow–derived osteoprogenitor cells to differentiate into osteoblasts. The osteoblastic nature of the cultured cells was confirmed by positive results of an alkaline phosphate assay and calcification nodule formation during the continuous culture. In the present study, cells in passage II were used for cell seeding.

2.4 Cell seeding into PCL/TCP scaffolds

When a confluent monolayer was reached, the cells were enzymatically released and concentrated by centrifugation (1200 × g, 10 min). Cells were counted and the viability was assessed by trypan blue staining and diluted to approximately 2 × 10⁷ cells/mL. Aliquots of 0.2-mL (1 × 10⁶) cell suspensions were seeded into the fabricated PCL/TCP scaffolds, which were placed in 48-well plates for culturing. The cell scaffold constructs were left undisturbed in an incubator for 3 days to allow the cells to attach. Subsequently, an additional 1.0 mL of complete media was added to each well for 3 days of incubation, which allowed the cells to proliferate. The scaffolds were then delivered to the operating room for implantation. Under the same conditions, the fabricated PCL/TCP scaffolds without seeding of cells were added to 1.5 mL of complete media for incubation. After 3 days in the culture environment, the assembled cell–scaffold constructs were implanted into the mandibular defect sites. The bone marrow–derived osteoprogenitor cell-seeded PCL–TCP scaffolds and PCL/TCP alone were randomly inserted into the defect sites.

2.5 Mandible defect model preparation and repair

Ten New Zealand white rabbits were randomly divided into 2 groups of 5 animals each according to the observation period of 4 and 8 weeks. Surgeries were carried out under aseptic conditions and were anesthetized with intravenous injections of pentobarbital sodium (25 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) into the lateral ear vein. An injection of 2% lidocaine containing 1:100,000 epinephrine was administered into the operating site [2]. A 2-cm incision was made approximately 1 cm lower to the lower edge of
the mandible body to expose the bone. A bicortical defect of \(10 \times 5 \text{ mm}^2\) was created at the lower border of the mandibular body using low speed rotary burs under continuous saline irrigation on both sides of the mandible in all animals. The periosteum was removed during surgery to exclude the interference of periosteum osteogenesis. Ten defects of five rabbits were randomly divided into three groups: cell–scaffold constructs (PCL/TCP/Cells) were inserted into three defects, PCL/TCP alone constructs (PCL/TCP) were inserted into three defects, and autogenous bone particles were inserted into three defects, with the last defect kept empty. Implant replacement was randomly placed into right and left sides and was secured in position by suturing the muscle, subcutaneous tissue, and skin in layers. All animals received intramuscular injection of penicillin 10,000 units per day immediately after surgery. The animals were killed by a lethal dose of barbiturate after a healing period of 4 and 8 weeks. The mandibles were removed, soft tissues were cleaned, and the mandibles were prepared for radiographic and histologic testing [14].

### 2.6 Sample evaluation

The mandibles were harvested en bloc and radiographs were taken according to the time interval for the radiographic evaluation. Specimens were then fixed in 10% buffer formaldehyde and decalcified with formic acid. After dehydration and paraffin embedding, the specimens were sectioned at 5 \(\mu\text{m}\) and stained with hematoxylin and eosin (H&E). The specimens were examined by light microscopy (Axiostar, Carl Zeiss, Germany) for the descriptive qualitative data. The percentage of new bone formation in the bone areas was quantitatively calculated by the histomorphometric technique using the digital image processing software (Image-Pro Plus 5.0, Media Cybernetics Inc., Silver Spring, MD, USA).

### 2.7 Statistical analysis

The data were analyzed using the total bony defect area and the area of new bone formation. The percentage of bone area was measured two times separately to reduce bias. Intergroup differences between treatment types of healing were compared using one-way analysis of variance. The level of statistically significant difference was considered at \(P < 0.05\).

### 3 Results

#### 3.1 Clinical observation

All surgical sites healed uneventfully. There were no marked signs of inflammation or rejection during the postoperative period. At 4 and 8 weeks post-operation, the mandibles were removed and macroscopically observed. The bone defects healed well and no necrosis or obvious inflammation was detected in any animal.

#### 3.2 Radiographic results

The radiographic appearances of the hemimandibles that focused on the created defect sites in the 4-week group are shown in Fig. 1. The PCL/TCP alone group (PCL/TCP) demonstrated that the defect had a persistent size with minimal radiopacity at the surrounding native bone (Fig. 1a).
The PCL/TCP scaffold seeded with autologous expanded bone marrow-derived osteoprogenitor cells (PCL/TCP/Cells) showed a reduction in size of the created defect with more radiopaque new bone formation at the surrounding native bone (Fig. 1b) compared to the PCL/TCP alone scaffold group. The group with autogenous bone particles showed fully calcified radiopaque areas that filled the defects (Fig. 1c).

On radiographic examination at 8-week interval after the operation of the PCL/TCP alone group (PCL/TCP), the defect is still demonstrated in the lower border of the mandible, which is nearly the same size as in the 4-week interval group. A slight increase in the radiopaque area in the surrounding native bone was observed (Fig. 1d). The group of PCL/TCP scaffolds seeded with autologous expanded bone marrow-derived osteoprogenitor cells (PCL/TCP/Cells) showed a marked increase of radiodense areas in the created defect (Fig. 1e) compared to the group with autogenous bone particles (Fig. 1f) except in the cortical bone structure of the lower border of the mandible.

3.3 Histologic results

The defects of the bony specimens were analyzed descriptively by histological sections and quantitatively by the histomorphological method. The groups of PCL/TCP unseeded scaffold at 4 weeks showed deposition of acellular osteoid material and mineralization of newly developed bone only in the periphery region of the surgical defect. The center of scaffold showed only small amounts of mineral substance and a higher proportion of osteocytes. Direct contact between the scaffold and newly formed tissue was observed; w: woven bone, f: fibrous tissue (collagen bundles), and p: particles of PCL/TCP scaffolds. (H&E stain, magnification ×10).

The percentage of bone area (PBA) calculated by the image processing software was 28.02% ± 4.95%.

The group of PCL/TCP/Cells at 4 weeks (Fig. 3a, b) revealed more areas of new bone formation compared to the PCL/TCP alone group where it spread to both the peripheral...
and middle areas of the surgical defect, suggesting the deposition of acellular osteoid material and mineralization of newly developed bone that seemed to bridge the bone defect. Direct contact between the scaffold and newly formed tissue was observed. This implied good integration and non-restricted infiltration of the scaffold with the surrounding tissue. New bone formation in this stage presented as a disorganized bone matrix and a higher proportion of osteocytes without a lamellar architecture or Haversian system that are the characteristics of woven bone. The PBA calculated by the image processing software was 43.50% ± 1.24%.

The groups of autogenous bone particles at 4 weeks (Fig. 4a, b) showed areas of immature lamellar bone pattern of new bone formation that filled most of the created defect. Some fibrous tissue-like areas were also noticed in the histologic specimen. The PBA calculated by the image processing software was 58.98% ± 2.60%.

The groups of PCL/TCP unseeded scaffolds at 8 weeks showed more areas of new bone regeneration compared to the 4-week group. Nevertheless, these areas of new bone formation were still located near the peripheral native bone region. Fewer areas of new bone formation were found in the middle zone of the defect, which was predominantly occupied by dense fibrous-like tissue and the remaining particles of scaffold (Fig. 5a, b). The PBA calculated by the image processing software was 38.78% ± 4.15%.

The groups of PCL/TCP/Cells at 8 weeks (Fig. 6a, b) showed increased new bone formation and more newly formed bone maturation compared to the 4-week group. The new bone formation areas were spread out all over the defect area that nearly bridged the defect gap. The newly formed bone areas were found in the peripheral regions that were in contact with the native bone areas and especially in the middle region of the defect, which was similar to the autogenous bone particle group. Mature bone formations were also noticed in most of the pore areas of the scaffolds. However, there were some areas where remaining particles of the scaffold could be detected. The PBA calculated by the image processing software was 50.79% ± 1.80%.

The groups of autogenous bone particles at 8 weeks (Fig. 7a, b) compared to the 4-week group showed more...
areas of more mature lamellar pattern of new bone formation that almost filled the whole defect. A thin peripheral cortical bone area, that included the fatty marrow spaces from the remodeling process, was also observed. The PBA calculated by the image processing software was 49.72% ± 2.12%.

The histomorphometric analysis results of the new bone formation areas that filled the created defects at 4- and 8-week intervals in each group are shown in Fig. 8. The PBA in the defect in the PCL/TCP/Cells group was significantly higher than in the PCL/TCP scaffold alone group in both the 4- and 8-week intervals (P < 0.05).

Fig. 6 Histologic micrograph of the 8-week PCL/TCP/Cells group. Panel a shows increased new bone formation in the peripheral (arrows) areas and in the middle parts (arrow head), which nearly bridged the defect area. However, there were remaining particles of scaffold that were detected in some regions. Mature bone formations can be seen in most of the pore areas of the scaffolds compared to the 4-week group. (H&E stain, magnification ×4). b Histologic picture from the middle section shows increased newly formed bony trabeculae and more bone maturation in the defect area and a small amount of fibrous connective tissue compared to the 4-week group; f: fibrous tissue (collagen bundles), b: new bone formation, and p: particles of PCL/TCP scaffolds (H&E stain, magnification ×10)

Fig. 7 Histologic micrograph of 8-week autogenous bone particle group. Panel a shows new bone formation that almost filled the whole defect area (arrows). The newly formed bone has a mature lamella pattern. Areas of remodeling of fatty marrow space and a slim outer cortex structure were also observed. (H&E stain, magnification ×4). b Histologic picture from the middle section showed more mature lamellar bone pattern of the newly formed bone including the initial marrow spaces from the remodeling process; b: new bone formation and m: marrow cavities. (H&E stain, magnification ×10)

Fig. 8 Histomorphometric data of the percentage of bone area (PBA) in each group revealed a higher percentage of bone area in the PCL/TCP/Cells group than in the PCL/TCP group in both time intervals (P < 0.05)

4 Discussion
A maxillofacial defect can result from various etiologies, such as congenital, infection, trauma, or tumor. Reconstruction of these defects is the crucial part of treatment to restore both the anatomical structure and function of the tissue or organ of the patient. These techniques range from traditional bone grafting through modern tissue engineering techniques using stem cells.

Bone marrow has been widely used in bone grafting procedures. Previous studies confirmed that bone marrow contains osteoprogenitor cells and can induce bone formation in both in vivo and in vitro [15]. Furthermore, bone marrow-derived osteoprogenitor cells can be isolated and
expanded in vitro and are capable of forming bone tissue. The present study aimed to demonstrate the effects of a combination of PCL/TCP seeded with autologous expanded bone marrow-derived osteoprogenitor cells to repair marginal mandibular defect in rabbit model compared to PCL/TCP scaffolds alone. Rabbit is a suitable animal model for the study due to ease of handling and the proper level of craniofacial growth to test a hypothesis. Furthermore, the pattern of bone accretion and bone mass during skeletal maturation are highly similar to those of human situations, and physiological bone healing is similar to human but 3 times higher [16, 17].

The clinical evaluation of all experimental animals revealed no complications during the surgical operations and surgery was well tolerated. The clinical appearance, food intake, and weight-bearing in each animal gradually improved within 10 days. The surgical wounds showed normal contour and color without signs of infection. It can be stated that the PCL/TCP scaffolds had good biocompatibility with the local tissues [9, 18–22].

Based on the radiographic examination, the PCL/TCP/Cells group showed a reduction in the defect size as observed from the increased radiopaque areas compared to the PCL/TCL scaffold alone in both time intervals of 4 and 8 weeks. The radiodensity area of the 8-week PCL/TCP/Cells group was even quite similar to the autogenous bone particle group except for the lower border of the cortical bone area. Thus the expanded bone marrow-derived osteoprogenitor cells seeded in the scaffold effectively enhanced new bone formation in the experimental animals. This was also confirmed by the histological examination. The PCL/TCP scaffold alone group demonstrated a low quantity of new bone formation. The majority of newly formed bone was limited to the nearby adjacent native bone margins. The central region of the defect was filled with predominantly reparative fibrous-like tissue and residual particles of the scaffold with very few areas of new bone formation. This probably resulted from the only mode of osteoconductive property of the PCL/TCP scaffold, which promoted bone formation by acting as a physical matrix suitable for deposition of new bone and allowed apposition from the adjacent existing bone. On the other hand, the PCL/TCP/Cells showed increased amounts of newly formed bone with more bone maturation compared with the PCL/TCP alone group of the same interval. Moreover, the newly formed bone tissue was not located only at the peripheral parts near the native bone margin but also scattered throughout in the central region. This possibly occurred from the osteogenesis mode of new bone formation of the seeded autologous expanded bone marrow-derived osteoprogenitor cells into the scaffold. The quantitative assessment of histomorphometric analysis also confirmed a higher percentage of bone area in the PCL/TCP/Cells groups than in the PCL/TCP alone groups in the 4- and 8-week groups.

The results of the present study tend to indicate that the scaffold/Cells construct by this method provided more new bone formation enhancement properties than the PCL/TCP scaffold alone with more bone maturation that was comparable with autogenous bone particles for bone regeneration in the surgically created defect.

5 Conclusions

The present study demonstrated the efficiency of PCL/TCP scaffolds seeded with bone marrow-derived osteoprogenitor cells for treatment of rabbit marginal mandibular bony defects. The defects treated with scaffolds that were seeded with bone marrow-derived osteoprogenitor cells displayed enhanced bone volume fraction that was superior to the PCL/TCP unseeded scaffolds at 4 and 8 weeks and was closely comparable with the autogenous bone particle group. This will be a promising technique for new bone regeneration to treat maxillofacial defects instead of the routine autologous grafting method with donor site morbidity. However, more extensive work with longer time periods, larger sample sizes, and controls should be carried out to verify this method of treatment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

7. Schantz JT, Hutmacher DW, Chim H, Ng KW, Lim TC, Teoh SH. Induction of ectopic bone formation by using human periosteal

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