Original article

Preparation and characterization of demineralized bone matrix/chitosan composite scaffolds for bone tissue engineering

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Background: A propitious alternative to supply bone substitutes is to develop living tissue substitutes based on biodegradable materials. Demineralized bone matrix (DBM) can support and promote osteogenesis; scaffold is also attractive for use in bone tissue engineering. Chitosan scaffold has been shown to possess biological and mechanical properties suitable for tissue engineering and clinical applications.
Objectives: This study aimed to develop a novel DBM/chitosan composite scaffold and to investigate whether or not it has the ability to support the attachment and proliferation of human periosteal cells in vitro for bone tissue engineering.
Methods: Chitosan and DBM/chitosan scaffolds (ratios 1:1 and 1:2) were fabricated with a low-cost, freeze-drying technique via thermally induced phase separation. The microstructure, mechanical performance, and biological activity of the scaffolds were studied. Scanning electron microscopy was employed to monitor the surface variation of chitosan and DBM/chitosan porous scaffolds.
Results: Both scaffolds had porosities and pore sizes between 80 and 250 microns. The compressive modulus of DBM/chitosan composite scaffolds was significantly higher than chitosan scaffolds. The DBM/chitosan scaffolds have been developed with adequate pore structure and mechanical properties to serve as a support for periosteal cell growth.
Conclusion: DBM/chitosan composite scaffolds have mechanical properties and porosity sufficient to support ingrowth of new bone tissue. Cell attachment and proliferation findings indicate that DBM/chitosan composite scaffolds may be used as promising materials for bone tissue engineering application.

Keywords: Chitosan, demineralized bone matrix, human periosteal cells, scaffolds.

Biomaterial scaffolds play a principal role in bone tissue engineering. A composite that utilizes the osteoinductivity of demineralized bone matrix (DBM) and the attractive characteristics of chitosan may be potentially useful as a tissue-engineered bone substitute.[1] DBM contains bone morphogenic proteins (BMPs) and matrix proteins.[2 - 4] BMPs are potent osteoinductive glycoproteins, while matrix proteins, such as different collagens, provide an osteoconductive matrix.[5 - 7] Recently, 3-dimensional porous scaffolds loaded with specific human cells have been investigated in order to regenerate tissue in a natural manner. Bone tissue engineering substitutes have been known as an alternative strategy to regenerate bone.[8] The natural biodegradable polymer

chitosan is currently a subject of interest in bone tissue engineering.

Chitosan, a natural cationic biopolymer, is a linear polysaccharide originated from partially and completely deacetylated derivative of chitin. Chitin is composed of 2-acetamido-2-deoxy-(D-glucose) through a β (1-4) linkage commonly found in shells of marine crustaceans, insects, and cell walls of fungi. Chitosan-based materials can accelerate bone formation because of the similarity to glycosaminoglycans in structure.[9, 10] The ability of chitosan to support cell attachment and proliferation is attributable to its chemical properties. Major advantages of chitosan scaffolds for bone tissue engineering include the formation of highly porous scaffolds with interconnected pores, osteoconductivity and ability to enhance bone formation. [11] Due to its exclusive properties such as biodegradability, biocompatibility, nontoxicity, and anti-bacterial effect, chitosan-based biomedical materials have aroused much interest in biomedical field. [12, 13] However, the lack of bone-bonding bioactivity, low mechanical strength and loosening of structural integrity under wet conditions limit its use in bone tissue engineering. [14, 15] Therefore, it is preferable to develop a composite material with the advantageous properties of both DBM and chitosan. The designed composites are expected to show an increase in osteoconductivity together with sufficient mechanical strength, which will be of great significance for bone remodeling and growth.

A composite of DBM and chitosan therefore is expected to be a favorable biomaterial for bone tissue engineering. The goal of this study was to investigate and characterize the ability of DBM/chitosan scaffold to support the attachment and subsequent cellular proliferation of human periosteal cells *in vitro*. In the present study, DBM powder was mixed with chitosan solution by full agitation and then the mixture was freeze-dried into porous scaffolds. We hypothesized that incorporation of DBM into chitosan could be avoided the particle migration and handling difficulties in clinical applications. We have also investigated human periosteal cell (HPO) attachment in chitosan and DBM/chitosan matrices *in vitro* for potential use in bone tissue engineering applications.

Materials and methods
Study groups
There were three groups, namely: Group I: chitosan; Group II: 1:1 DBM: chitosan; Group III: 1:2 DBM: chitosan scaffolds. In all groups, at least five samples were analyzed for each investigated parameter at the given time points.

Ground bone matrix (size from 250 to 710 μm) provided by Bangkok Biomaterial Center was demineralized by exposure to 0.5 N HCl (Lab-Scan, Ireland), after which the ground demineralized bone matrices were washed, freeze dried, and stored at -80 $^{\circ}$C.

Preparation of DBM/chitosan composite scaffolds
Porous matrices of chitosan and DBM/chitosan composites were fabricated using simple freezing and drying techniques. To prepare 2.25% chitosan suspension, chitosan powder (medium molecular weight, Seafresh chitosan, Thailand) was dissolved in 1% acetic acid at room temperature. Chitosan and DBM composites were prepared by mixing DBM into 2.25% chitosan suspension with constant gentle stirring at room temperature in ratios of 1:1 and 1:2 by weight.

The chitosan and DBM/chitosan composites suspensions were cross-linked with 0.05% glutaraldehyde at room temperature. To obtain homogeneous blending, the solution was stirred and degassed under vacuum; 500 μl of the degassed suspensions was then pipetted into each well of 24-well cell culture plates and kept at 4 $^{\circ}$C for 24 h and -70 $^{\circ}$C for 12 h. The frozen samples were then transferred to be freeze-dried with lyophilizer (Christ freeze dryer Alpha 1-2, Osterode, Germany) and dried at -40 $^{\circ}$C for 48 h. For sterilization, the lyophilized porous scaffolds were irradiated by gamma ray (25-kGy)

Swelling test (plasma absorption test)
The chitosan and DBM/chitosan composites of known weight (Wo) were placed in plasma at 37 $^{\circ}$C for 5 min. After removal, they were hung for 1 min until no dripping fluid was observed and then weighed immediately on an analytical balance (Ws). The content of plasma in the swollen composites or percentage of plasma absorption was calculated by the following formula:

Swelling ratio (%) = (Ws-Wo)/Wo x 100

Each value was averaged from three parallel measurements.

Morphological observation

The microscopic structures of chitosan and DBM/chitosan scaffolds and cellular morphology and adhesion were visualized using a scanning electron microscope (SEM; Hitachi S-2360N, Hitachi Science System Ltd., Japan) at an accelerating voltage 12 kV. Dry scaffolds were sputter-coated with gold at 40 mA prior to microscopic investigation.

Mechanical properties of scaffolds (compressive modulus)

A universal testing machine (Instron 5567, USA) was utilized to evaluate compressive modulus of scaffolds by compressing the sample discs (13 mm in diameter and 3 mm in thickness) at constant rate of 0.5 mm/min. The reported values were the average of at least five specimens. Compressive modulus was calculated from the linear region of the compressive stress-strain curve at 5 to 35% deformation.

Cell culture

A human periosteal cell line HPO/CU-SH-01 was cultivated on a 2-dimensional cell culture plate in alpha-Minimum Essential Medium (α-MEM, Gibco, Invitrogen, California, USA) supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (50 μg/ml) and was incubated at 37 °C in humidified air with 5% CO_2. HPO cells were subcultured after reaching confluence. [16] The third to the fifth passages at 1.5 x 10^5 cells/scaffold were used in these experiments. Cultures were characterized for cell attachment, cell proliferation, and cell morphology.

HPO cells were statically seeded into the scaffolds: 1.5 x 10^5 cells in 100 μl/scaffold. One hundred microliters of cell suspension were gradually seeded, using 200-μl pipette tip, onto all surfaces of the scaffold to enhance cell distribution. Cells were allowed to attach to the scaffolds in a minimal culture medium for 3 h, and then 1.5 ml of culture medium was added to each well of 24-well culture plates. Cells were cultivated in 5% CO_2 at 37 °C in 95% relative atmospheric humidity for 28 days. The culture medium was changed every 2 - 3 days.

Cellular adhesion and morphology

In the cell-composite scaffolds, cellular morphology and adhesion to the scaffolds were analyzed optically using scanning electron microscope (SEM) (Hitachi S-2360N, Hitachi Science System Ltd., Japan). The scaffolds were assessed on culture-day 1, 7, and 21 using two scaffolds selected from each group.

The scaffolds were fixed in glutaraldehyde and formaldehyde. They were then dehydrated in an ethanol series of 30 - 100%, dried, fractured into halves, mounted, gold sputter-coated and imaged.

Cell viability assay

The MTT assay (reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to a purple formazan product) was used to estimate cell attachment and cell proliferation as previously described. [17] HPO cells were plated at 1.5 x 10^5 cells/well on test scaffolds and controls in 24 well plate. At 24 h after cell attachment and on days 3, 7, and 14 of culture, cells were rinsed with phosphate buffer saline solution (PBS), and incubated with 500 μl/well of MTT (0.5 mg/ml; Sigma, USA) for 2 h. This time period permitted the cellular conversion of MTT to insoluble formazan salts, which were dissolved in 500 μl/ well of dimethylsulfoxide (Sigma, USA). Absorbance was measured at 570 nm using an automated microplate reader. The optical densities (ODs) were calculated and presented as mean ± standard error of the mean. Data for each sample were collected from triplicate wells for each assay point and the experiments were performed in triplicate.

Cell viability (%) = Difference in absorbance (ODs-OD_0)/initial absorbance (OD_0) x100

Statistical analysis

All experiments were independently repeated at least three times, and the mean and standard error of the mean were determined. Significant differences were determined by analysis of variance (ANOVA) or unpaired *t*-test. Statistical analysis was performed using the statistical package for social sciences (SPSS) software, version 16.0 for Windows. A *P*-value refers to a comparison of a measured parameter in the experimental group with that of the appropriate control. Statistical difference was defined as $P < 0.05$.

Results
Compressive modulus

The compressive moduli of chitosan and DBM/chitosan scaffolds are demonstrated in Figure 1. The 1:1 (86.4 ± 8.7 kPa) and 1:2 (85.3 ± 4.1 kPa) DBM-chitosan had significantly higher compressive strength than chitosan (67.6 ± 5.5 kPa) scaffold ($P < 0.05$). The differences in strength between 1:1 and 1:2 DBM/chitosan composites were not statistically significant (Figure 1).
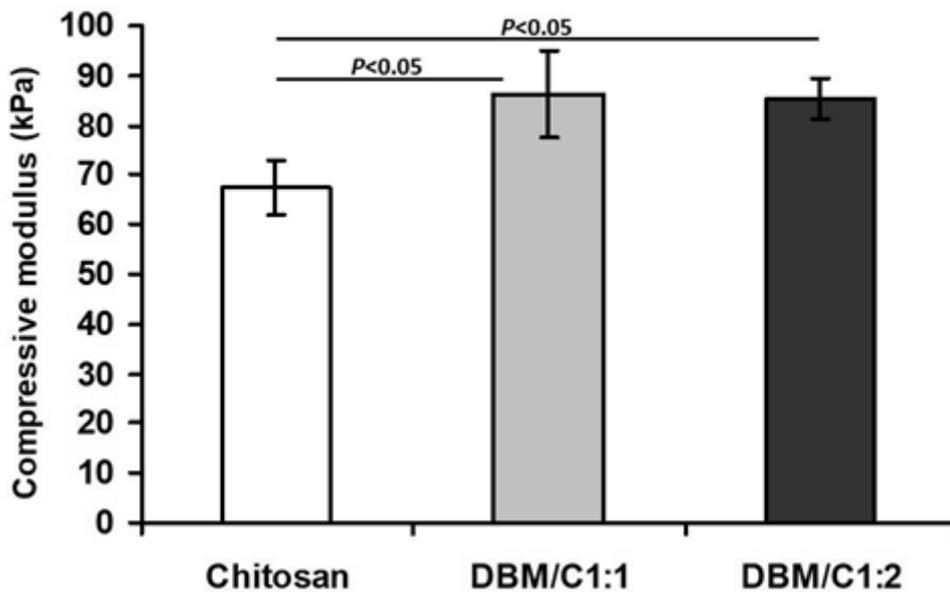
Figure 1. Compressive modulus of chitosan and demineralized bone matrix/chitosan (DBM/C) scaffolds.

Swelling test

The swelling ability of a scaffold is a crucial feature to determine its property for tissue engineering. The equilibrium swelling ratios were investigated by immersing scaffolds in plasma. The equilibrium swelling ratios of chitosan and DBM/chitosan scaffolds are demonstrated in Figure 2. All scaffolds absorbed and maintained a large volume of plasma within pore spaces. The swelling ratios of DBM/chitosan scaffolds could be clearly distinguished from chitosan scaffold. The first group containing pure chitosan displayed good swelling properties. The other groups containing chitosan and DBM had lower swelling ratios. The DBM/chitosan scaffold in a ratio of 1:1 showed a tendency to preserve less plasma than 1:2 DBM/chitosan scaffolds. This was attributable to the fact that they lost the gel-like structure after swelling in plasma.
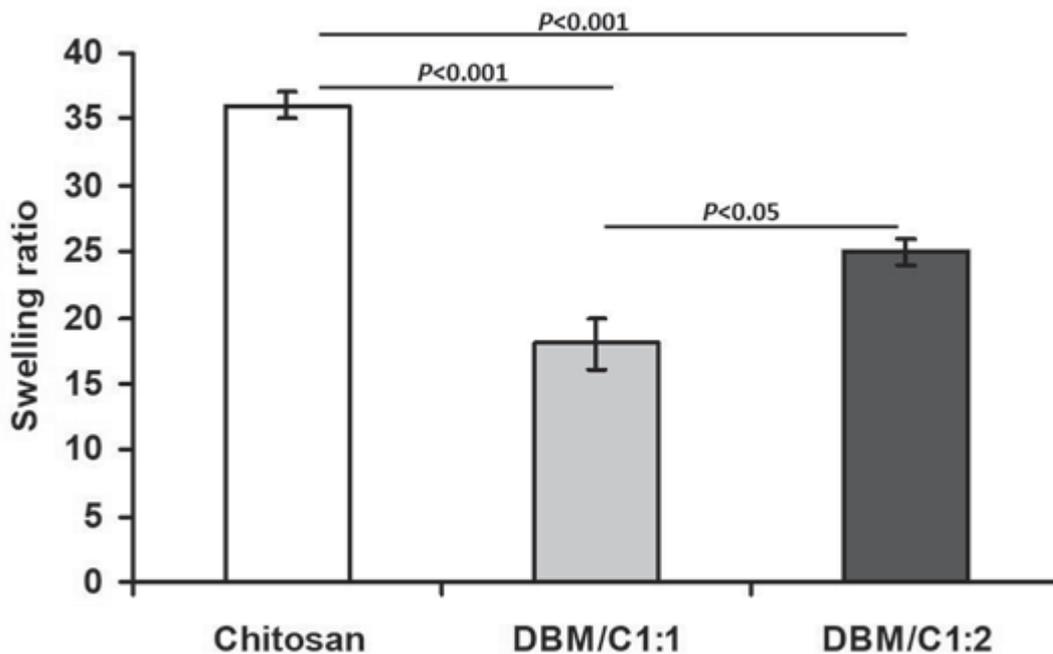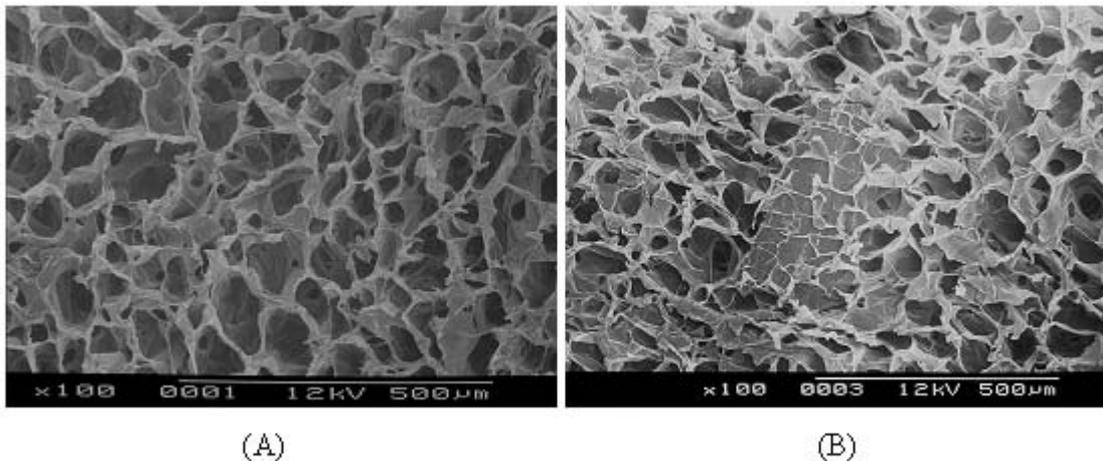

Figure 2. Equilibrium swelling ratio of chitosan and demineralized bone matrix/chitosan (DBM/C) scaffolds.
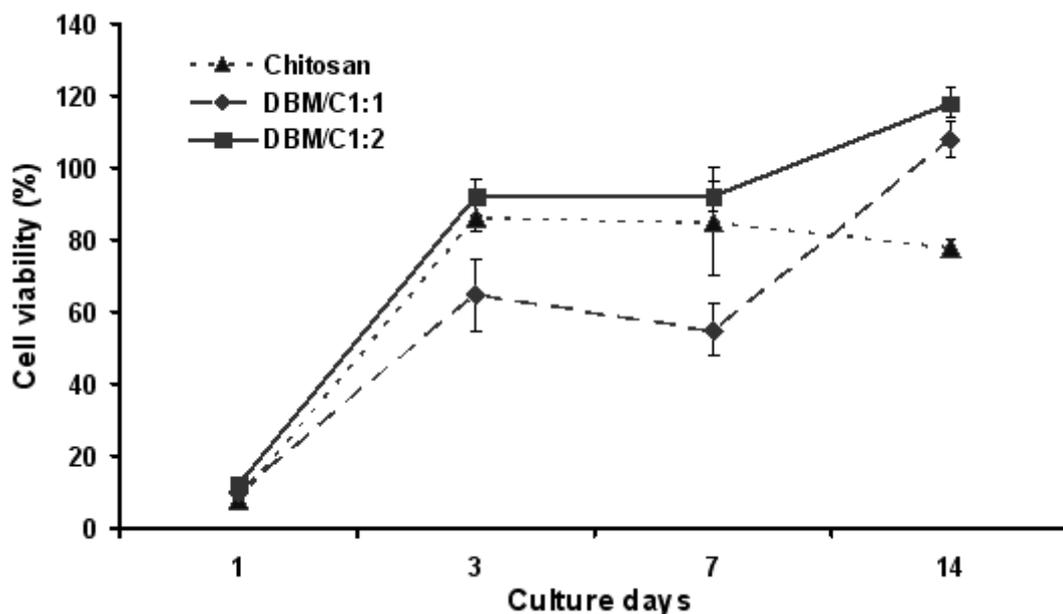
Microstructure of the scaffolds

The morphology of chitosan and DBM/chitosan scaffolds, as displayed by SEM in Figure 3, demonstrated the structure with an interconnected pore throughout the scaffolds. Pore size of the scaffolds ranged from 100 - 250 μm, which was favorable for cellular infiltration as documented by O'Brien and colleagues. [18] The interconnection of pores in DBM/ chitosan scaffold was similar to pure chitosan scaffold. There was no markedly significant difference in porous structure between chitosan and DBM/chitosan scaffolds, except for particles of DBM in the latter.

Cell viability assay

As illustrated in Figure 4, there was no significant difference in cell growth on chitosan scaffolds after cell seeding to culture-days 3, 7, and 14. Cell growth on 1:2 DBM/chitosan scaffolds were comparable to that of chitosan scaffolds during culture-days 3 and 7, and then sharply increased on culture-day 14. Growth of cells on 1:1 and 1:2 DBM/chitosan scaffolds had similar pattern throughout the cell-culture period and was significantly higher than on chitosan scaffold on culture-day 14 (Figure 4). The DBM/chitosan scaffolds at the mixing composition of 1:2 were selected to further evaluate the morphology and biological properties because of their better growth-promoting and swelling properties.

Figure 3. SEM photographs of cross-sectioned scaffolds: (A) chitosan and (B) demineralized bone matrix/chitosan.


Figure 4. Cell viability of human periosteal cells culture for 14 days on chitosan and demineralized bone matrix/chitosan (DBM/C) scaffolds.

Morphology of cells on structure of scaffolds

SEM micrographs showed that the porous three-dimensional structures of the scaffolds promoted intercellular contact and accumulation of extracellular matrix (Figure 5). Cells attached and sprouted their cytoplasmic processes on the surface and grew well into the porous structures of chitosan and DBM/chitosan scaffolds (Figure 5A, B). On the chitosan scaffolds, cells grew better on the outer surface because of the flabby microstructure of the scaffolds (Figure 5C). At a later stage, cells established intercellular contact, forming a continuous sheet of cells, proliferated in multiple layers, and secreted extracellular matrix. Cellular attachment, with cellular spread and cytoplasmic extension over the surface of matrix interconnecting with adjacent cells, is obviously shown on 1:2 DBM/chitosan scaffolds (Figure 5D). The matrix was dispersedly mineralized and mineralization nodules could be found in DBM/chitosan scaffolds more than chitosan scaffold on culture-day 21 (Figure 5E, F).
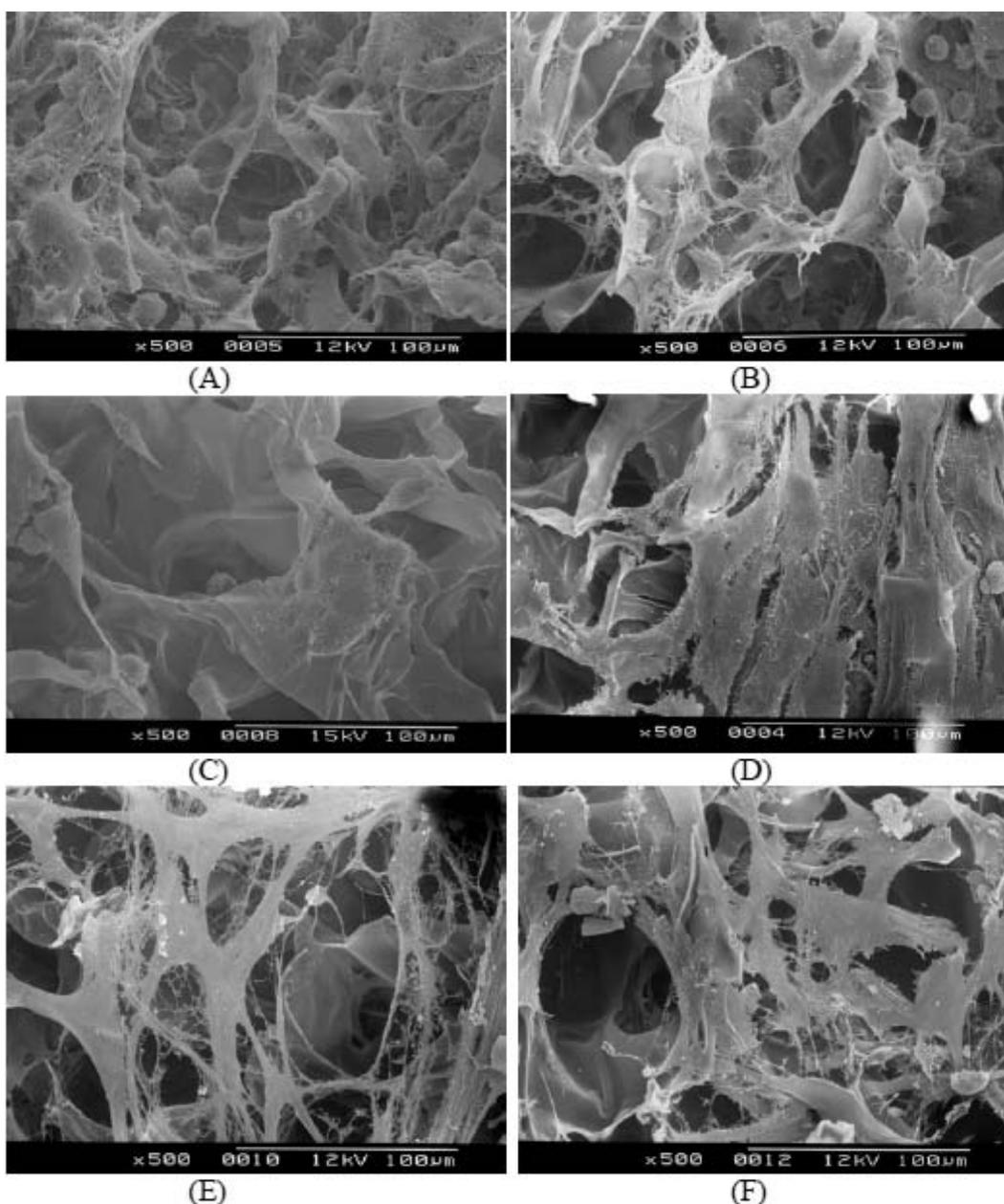
Figure 5. SEM photographs show morphology, attachment and growth of human periosteal cells on the scaffolds on culture-day 1, 7, and 21: (A, C, E) chitosan (B, D, F) demineralized bone matrix/chitosan, respectively.

Discussion

Tissue engineering provides new strategies to increase the utility of biomaterials for the development of novel composite scaffolds in bone regeneration. The incorporation of an osteoprogenitor cell source together with its *in vitro* osteogenic differentiation before implantation can accelerate the bone formation process within tissue-engineered biomaterials. In this study, we investigated the ability of demineralized bone matrix/chitosan scaffolds to support the attachment and subsequent *in vitro* proliferation of human periosteal cells. We first investigated the physical property of chitosan and DBM/chitosan scaffolds and examined their ability to support attachment and growth of human periosteal cells throughout DBM scaffolds in order to optimize a system for cell delivery.

Demineralized bone matrix is potentially attractive scaffold for use in bone tissue engineering because of their ability to support and promote osteogenesis of matrix-incorporated osteoprogenitors. Chitosan has played a critical role in bone tissue engineering over the last several decades, being a natural polymer obtained from chitin, which forms a principal component of crustacean exoskeleton. [9] Recently, considerable attention has been given to chitosan composite materials and their applications in the field of bone tissue engineering due to its minimal foreign body reactions, an intrinsic antibacterial nature, biocompatibility, biodegradability, and the ability to be molded into various geometries and forms such as porous structures, suitable for cell ingrowth and osteoconduction. [19] The composite of chitosan including demineralized bone is very popular because of the biodegradability and biocompatibility in nature. [20] This investigation showed the effects of combining DBM and chitosan on the physical, mechanical, and biological properties of scaffolds, and the capability of these composite scaffolds to support cellular proliferation, extracellular matrix production, and mineralization.

In the present study, we successfully prepared the DBM incorporated chitosan composite scaffolds using a simple method. There are several advantages that can derive from these composite scaffolds when they are applied to treat the bone. First, the DBM particle migration, immediate dispersion with blood or handling difficulties during the clinical applications can be avoided. Secondly, since demineralized bone matrix/chitosan composite is completely degradable, it could be implanted close to the site where it is needed, such as in the bone to treat bone fracture. Thirdly, osteoinductive and osteoconductive components in the composite scaffolds can be released in a controllable manner when patients are treated for long-term bone fractures. Another unique feature of these scaffolds is that it can be fabricated with different sizes and variety of shapes according to the target site.

In this study, homogenous composite scaffolds of chitosan and DBM were prepared and determined. The porous structure of the DBM/chitosan composite scaffolds was made by the lyophilization. The spongy scaffolds showed good porosity and some cells could grow in the pores of these three dimensional scaffolds. The DBM/chitosan composite scaffolds demonstrated better biocompatibility than chitosan scaffolds. Cells grown on the DBM/chitosan composite scaffolds were in a better state and had a higher proliferation. In agreement with this study, Yan et al. illustrated that preosteoblast cells adhered to the surface of chitosan/ hydroxyapatite/DBM scaffolds, and cell number increased with culture time. [21] The DBM particles bonded to the chitosan scaffold tightly and the combination prevented the DBM particles from diffusing to a certain extent. Some of the obstacles inherent in the use of DBM in tissue engineering could be overcome in additional studies.

In summary, it can be concluded that the DBM/ chitosan composite scaffolds had a superior microstructure and supported cell attachment and proliferation. Therefore, a combination of DBM and chitosan should create a suitable environment for proliferation and growth of osteoblasts. Furthermore, the porous structure of the DBM/chitosan scaffolds and good biocompatibility could combine with periosteal cells for bone tissue engineering, which is more useful, and the quality is easy to control. Accordingly, our findings suggest that the porous DBM/chitosan composite scaffolds may be used as promising materials for bone tissue engineering application.

Acknowledgements

Conflict of interest

None of the authors has any potential conflict of interest to disclose.

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