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Original Article

Comparison studies of mineralized and non-mineralized SF/CS hybrid bone scaffolds co-cultured with the osteoblast cell line MC3T3-E1 *in vitro*

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Abstract: This work aimed to investigate the cytocompatibility and osteoinductivity of mineralized and non-mineralized silk fibroin/Chitosan (SF/CS) composite scaffolds co-cultured with the osteoblast cell line MC3T3-E1 *in vitro*. The mineralized (SF/CS/HA) and non-mineralized (SF/CS) scaffolds were fabricated utilizing freeze-drying method and biomimetic mineralization technique. The macrostructure and morphology as well as mechanical strength of two scaffolds were characterized. The MC3T3-E1 were cultured, expanded and seeded on SF/CS/HA and SF/CS scaffolds. The MC3T3-E1/scaffold constructs were cultured for 10 days and the adhesion, proliferation and osteogenic differentiation of MC3T3-E1 were determined using early adhesion ratio, cell counting kit-8 (CCK-8), alkaline phophatase (ALP) activity and scanning electronic microscopy (SEM). The two scaffolds had high porosity with interconnected porous architecture, and the mean porosity of SF/CS/HA, SF/CS scaffolds was 75.5% ± 5.9% and 86.1% ± 6.3%, respectively. The SF/CS/HA scaffold exhibited improved compressive strength and compressive modulus compatible with mechanical property of the cancellous bone. Significant proliferation on the SF/CS scaffold were observed while SF/CS/HA scaffold effectively promoted early cell attachment and enhanced osteogenic differentiation of MC3T3-E1 after 10 days of co-culture. The SF/CS/HA and SF/CS scaffolds were both biocompatible and had almost no negative effects on the MC3T3-E1 *in vitro*. However, the SF/CS/HA scaffold had better potential to be applied in bone tissue engineering for its good mechanical property and osteogenic differentiation.

Keywords: Silk fibrion, chitosan, hydroxyapatite, bone tissue engineering, scaffold

Introduction

The treatment of jaw bone tissue defects resulted from infection, cancer, trauma or other factors continues to be a major problem for oral and maxillofacial surgeons. Currently, the main approach is bone grafting, however a variety of bone grafting have certain disadvantage. The autogenous bone graft is likely to cause increasing pain and infection of patients, while allograft or xenograft has been severely limited due to the presence of immune system rejection and other issues of cross-infection virus. Bone tissue engineering is a new research area that has attracted much attention with the aim of repairing tissue defects and regenerating new tissue [1]. The approach of bone tissue engineering mainly includes three aspects: osteogenic cells, osteoinductive molecules and biomaterial scaffolds. Among these, development of porous biomaterial scaffolds plays a crucial role in guiding new tissue regeneration. As the synthetic temporary extracellular matrix, the scaffold provides the microenvironment for regenerative cells, supporting cell attachment, proliferation, differentiation and neo tissue genesis [2]. Natural bone is a biocomposite in which inorganic apatite crystals are deposited on collagen fiin w woven into a three-dimensional structure. In recent years, an important trend in research area of bone repair materials is to mimic the structure and main component of bone tissue.

Hydroxyapatite (HA) is among one of the widely used bioceramic material having similar composition and morphology to the inorganic component of natural bone. In addition, it can provide a favorable environment for cell adhesion,

osteoconduction, and osteoinduction [3]. However, pure hydroxyapatite is not used for scaffold fabrication due to poor mechanical characteristics, lack of porous structure, low biodegradation rate and brittleness [4]. On the other hand, natural polymers also have been regarded as the proper candidates for tissue engineered scaffolds. Chitosan (CS) is composed of repeating units of D-glucosamine and *N*-acetyl glucosamine linked in a β (1-4) manner [5]. Much attention has been paid to chitosan-based biomedical materials, with regard to their unique properties such as biodegradability, non-toxicity, antibacterial effects and biocompatibility [6]. It can be molded into various forms (gels, membranes, sponges, beads and scaffolds) and has an exceptional pore forming ability for potential applications in tissue engineering, drug delivery and wound healing [5]. Blending of CS with other natural biocompatible materials like silk fibrion (SF) is expected to provide enhanced tissue repair and regeneration [7]. Silks are common natural occurring fibrous proteins produced by insects and spiders, and also have demonstrated great biocompatibility along with outstanding mechanical properties and proteolytic degradation [8]. However, the main disadvantage of both natural polymers is low mechanical strength, so as to present the difficulties to use them as a scaffold material alone. To overcome these shortages above, Polymer blending can be used as an effective method to prepare material in order to meet the performance requirements for bone scaffold.

In this study, using freeze-drying method and biomimetic technique, we prepared scaffolds composed of the natural polymers CS and SF, supplemented or not with HA. To compare and characterize some physical and chemical properties of HA/CS/SF and CS/SF three-dimensional scaffolds, osteoblast (MC3T3-E1) cell line was seeded separately on HA/CS/SF and CS/SF composite scaffolds *in vitro* to investigate their effects on osteoblast adhesion, proliferation and differentiation, in order to provide theoretical and experimental basis for the design of bone tissue engineering scaffold.

Materials and methods

Materials

The mouse osteoblast cell line MC3T3-E1 was supplied by the Experimental Technology Sector of China Medical University (Liaoning, PR China). Chitosan with a degree of deacetylation of 82.7% and a weight-averaged molecular weight of 400,000 was purchased from Tongxing Company (Jiangsu, PR China). Raw silk was purchased from Nancong (Sichuan, PR China). All other chemicals and reagents were of analytical grade unless specifically mentioned.

Preparation of SF/CS and SF/CS/HA scaffold

Bombyx mori silk fibers were treated with 0.5% (w/w) $NaHCO_3$ solution twice at 100°C for 30 min, and then rinsed with distilled water for 30 min at room temperature to remove sericin (degumming), afterwards kept at 60°C to dry. Degummed silk was dissolved in a mixed solution system of $CaCl_2/CH_3CH_2OH/H_2O$ (molar ratio of 1:2:8) at 70°C for 6 hours and filtered to obtain a SF solution. A cellulose dialysis membrane tube (MWCO 8,000~12,000 Da) was used for the removal of impurities from the SF solution by soaking in ultrapure water for 3 days with water change every 3 hours. The final concentration of SF solution used was 8%.

Chitosan (82.7% deacetylation; Tongxing China) with high molecular weight was dissolved at 3.66% (w/v) in 2% acetic acid. The final concentration of CS used was 2%. In order to fabricate SF/CS scaffold, SF/CS blend solutions (100 ml) with SF/CS weight ratio 5:5 was prepared in the same solvent system at 10% (w/w) (combined weight of CS and SF) and then under magnetic stirring for 6 hours. The SF/CS solution was poured into the 24-well culture plate, frozen for 24 hours at -20°C and lyophilized for 48 hours at -54°C and 80 Pa. To improve water stability of scaffold, the dry SF/CS scaffold were immersed in methanol solution for 2 hours, and lyophilized to remove the excess methanol. To obtain the SF/CS/HA scaffold, the soaking mineralization of SF/CS was prepared by the alternate soaking method [9]. The SF/CS scaffold was immersed in 0.2 M $CaCl_2$ and 0.12 M Na_2HPO_4 solutions for 30 min each for 4 cycles. The size of each scaffold was approximately 10 mm in diameter and 2 mm in height.

Scanning electron microscopy (SEM) examination

The porous structure of the scaffolds was studied by SEM (JSM-TM3000, Japan). The scaf-

folds samples were cut into square pieces of 0.2 × 0.2 cm sizes and coated with gold using a JEOL JFC-110E Ion Sputter for 60 s at 20 mA before observation under the SEM. The SEM images and pore size were analyzed later using Image J software.

Porosity measurements

The pore size of SF/CS/HA and SF/CS can be measured by image analysis software (Smile View Ver 2.1). The porosity of scaffolds was evaluated by liquid displacement [10]. Briefly, scaffolds were immersed in a known volume (V_1) of ethanol, and then pressed to force all trapped air out of the scaffold. The total volume of ethanol and ethanol-impregnated scaffold was recorded as V_2. The ethanol-impregnated scaffold was removed and the residual ethanol volume recorded as V_3. The porosity of the scaffold was expressed as: $(V_1-V_3)/(V_2-V_3) \times 100\%$.

Swelling ratio and water absorption

Swelling property of scaffold was tested by immersion in distilled water for 24 hours at 37°C. The sample number for each scaffold was three. The wet volume of the samples (S_1, swollen volume) was measured and divided by the initial dry volume of each sample (S_2, dried at 37°C overnight). The swelling ratio of the scaffold was expressed as: $S=(S_1/S_2-1) \times 100\%$. Water absorption property of scaffold was determined by immersion in distilled water for 24 hours at 37°C. The sample number for each scaffold was three. After excess water was removed, the wet weight of the scaffold (W_1, swollen weight) was determined. Samples were then dried in an oven at 60°C under vacuum overnight and the dry weight of scaffolds (W_2, dried weight) was determined. The water absorption property of scaffold was expressed as: $W=(W_1/W_2-1) \times 100\%$.

Mechanical properties

The compression modulus of the scaffold was evaluated on a ZWICK Z2005 instrument (ZWICK, Germany) equipped with a 100 N at room temperature. The cross-head speed was set at 0.5 mm/min. Three samples were evaluated for each composition. Cylinder-shaped samples were 10 mm in diameter and 10 mm in height. The compressive stress and strain were graphed and the average compressive strength as well as the compressive modulus and standard deviation were determined [11].

The osteoblast cell line MC3T3-E1 culture

The osteoblast cell line MC3T3-E1 was cultured in alpha minimum essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (Hyclon, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were cultured in 25 cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2. The cells were subcultured every 2 or 3 days using 0.25% (w/w) trypsin and 0.02% (w/v) EDTA solution.

The samples were sterilized with 75% alcohol under ultraviolet light overnight and then rinsed extensively three times with PBS solution. Before cell co-culturing, scaffolds were prewetted by immersion in α-MEM for 24 hours in the 37°C incubator.

The osteoblast cell line MC3T3-E1 was seeded onto the tops of SF/CS/HA scaffolds (1.0×10^6 cells/scaffold), SF/CS scaffolds and 24-well culture plate (blank control group). The scaffolds were left undisturbed in an incubator for 3 h allow the cells to attach to them and then an additional 1 ml of culture medium was added into each well. The cell/scaffold constructs were cultured in a humidified incubator at 37°C with 5% CO_2 for up to 10 days. The medium was changed every 3 days.

Morphology observation

The inverted phase contrast microscope was used to observe the cell morphology, growth and proliferation, meanwhile the photograph was obtained. The morphology of osteoblast cells on the SF/CS/HA scaffold and SF/CS scaffold was observed on day 3 and 7 by SEM. The samples were washed with PBS and fixed with 2.5% (w/w) glutaraldehyde/cacodylate for 4 hours at 4°C, rinsed three times with PBS and dehydrated in a grade ethanol series (30%, 50%, 70%, 80%, 90%, 95% and 100%). Samples were dried at a critical point of carbon dioxide, and sputtered with gold for SEM observation.

Cell adhesion

For cell adhesion experiments, cells were seeded onto the SF/CS/HA scaffold group, SF/CS scaffold group and blank control group at a

density of 1 × 10^6 cells /mL. Cells were placed in cell culture plates precoated with SF/CS/HA and SF/CS, 1 mL per well. Cells cultured in culture plates with no scaffold served as controls. 9 parallel wells were used for each group. Cells were cultured in an incubator at 37°C with 5% CO_2. After samples were co-cultured 1, 3, and 6 hours, three samples were taken from each group at each time point. The cell adhesion rate = the number of attached cells/the number of seeded cells × 100%.

CCK-8 assay

Cell proliferation was quantified by using a cell counting kit (CCK-8). Briefly, after samples were cultured 1, 3, 5 and 7 days, cell/scaffold samples were harvested at each time point, culture medium was replaced with serum free culture medium containing CCK-8 (0.5 mg/ml) at 37°C under 5% CO_2 for 4 h, the samples were transferred to a 96-well plate. Optical density (OD) was measured at 450 nm on an ELISA reader (Bio-Rad Model 550, USA).

Alkaline phosphatase (ALP) activity

The ALP activity of the MC3T3-E1 was evaluated with the expression of ALP by a standard procedure according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). Briefly, samples and control group were cultured 1, 4, 7 and 10 days, irrigated with PBS three times to remove as much residual serum as possible and then 1 ml of 0.1% Triton X-100 were placed on the samples and control group at 4°C through overnight to break up the cells. The whole solution was transferred to a tube and centrifuged at 13,000 rpm for 10 min at 4°C. ALP activity was measured by mixing 50 μL of supernatant with 50 μL p-nitrophenyl phosphate (5 mM) in 150 mM 2-amino-2-methyl-1-propanol buffer solution. After 30 min incubation at 37°C, the reaction was stopped by the addition of 50 μL of 0.2 N NaOH and the OD was measured at 520 nm using an ELISA reader (Bio-Rad Model 550, USA). Total protein content was determined using Bicinchoninic Acid (BCA) Protein Assay Kit. The ALP activity was expressed as unit perg protein.

Statistical analysis

All data were expressed as means ± standard deviation (SD). Statistical analysis was conducted using SPSS 17.0 (SPSS Inc, Chicago, IL, USA). Results were analyzed by using one-way ANOVA. *P*-values of less than 0.05 were considered significant.

Results

Characterization of the SF/CS/HA scaffold and SF/CS scaffold

Scaffold morphology: Two kinds of the scaffolds exhibited highly three-dimensional porous structures as revealed by SEM and the pores showed a large degree of interconnection. The SF/CS scaffolds showed smooth surface. The porosity can be reached 80% above, with sizes ranging from 50 to 300 μm. After crystallization, hydroxyapatite particules could be observed in the SEM images of HA/SF/CS scaffolds, and it were homogeneously dispersed within the composite scaffold. Compared to the SF/CS scaffolds, the SF/CS/HA scaffolds were similar in the microscopic morphology, which explained that the addition of HA did not influence the porous structure. The pore size and porosity of the HA/SF/CS scaffolds both less than that of SF-CS scaffolds due to adhesion of hydroxyapatite particules (**Figure 1A** and **1B**).

Swelling ratio and water absorption: Swelling ratio and water absorption of the scaffolds are important features to evaluate biomaterial properties for tissue engineering. The swelling capability is responsible for the polymeric matrix expansion, which leads to an increase of the pore size and to the subsequent variation of the scaffold's morphology. During the process of cell culture, the better water absorption means the more nutrient solution attach to the scaffolds, but in the meantime, the scaffolds are more likely to become deformed. The SF/CS scaffolds had a swelling ratio of (12.3 ± 0.6)% and a water absorption of (273 ± 12)%. It was found that the swelling ratio and water absorption of the scaffolds decreased with the HA content. The SF/CS/HA scaffolds had a swelling ratio of (7.2 ± 0.5)% and a water absorption of (169 ± 8)% (**Table 1**).

Mechanical properties: Mechanical performance, especially compressive strength, is very important for bone tissue engineering or porous scaffolds [12]. The compressive modulus and compressive strength of the scaffolds

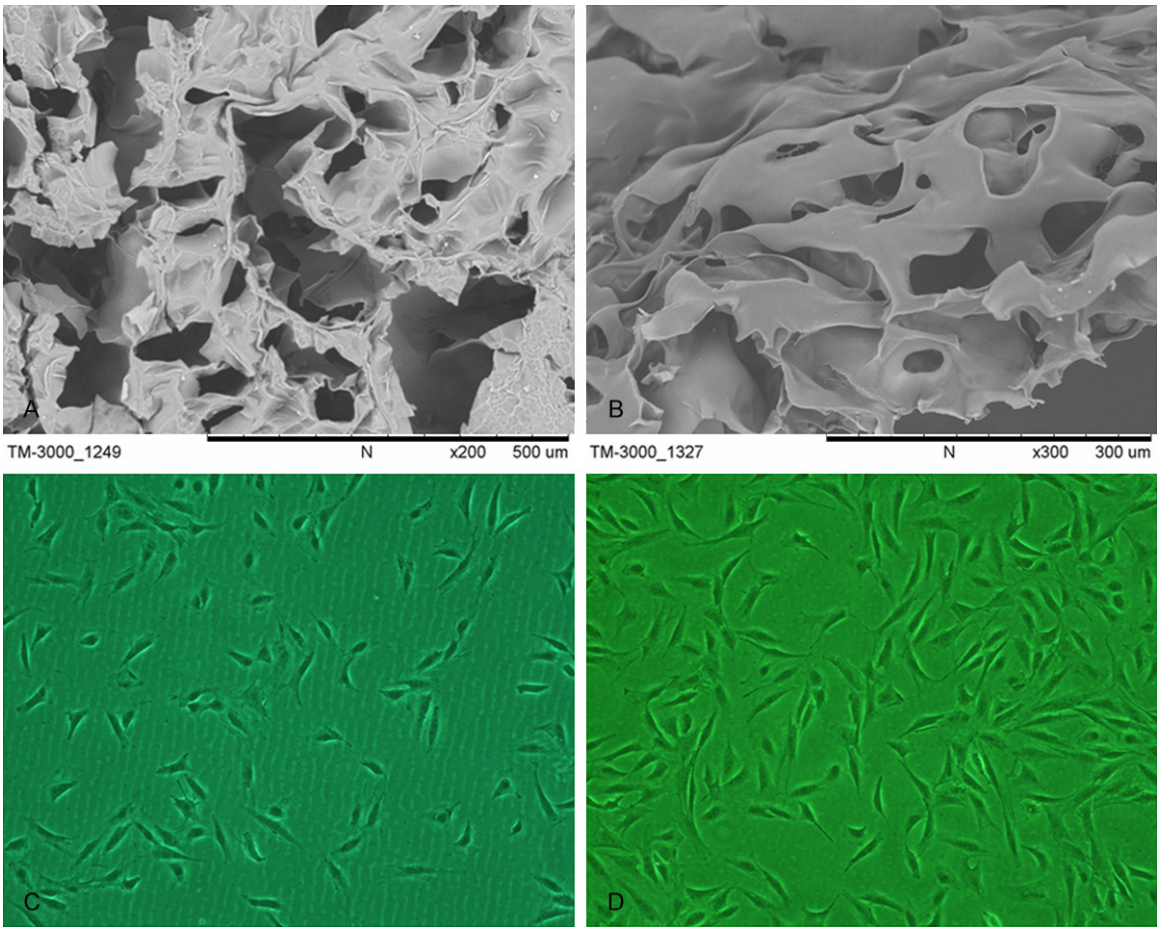

Figure 1. A: SEM photograph of the cross-sectioned SF/CS/HA scaffold; B: SEM photograph of the cross-sectioned SF/CS scaffold; C: Inverted phase contrast microscope morphologies and quantity of MC3T3-E1 on culture flask under normal culture conditions on day 1; D: Inverted phase contrast microscope morphologies and quantity of MC3T3-E1 on culture flask under normal culture conditions on day 2.

increased with HA content. The SF/CS scaffolds had a compressive modulus of 16.35 ± 2.89 MPa and a compressive strength of 0.67 ± 0.13 MPa. The compressive modulus and compressive strength of HA/SF/CS scaffolds were 23.19 ± 2.53 MPa and 1.03 ± 0.11 MPa, respectively (**Table 1**).

Cell morphology

Cell morphology was observed by using a light-inverted microscope (**Figure 2**). When suspended in the culture medium, the osteoblast cells MC3T3-E1 were in round morphology. After 1 hour, cells were attached to the wall of culture plate and appeared filopodia at 6 hours. At 1 day, cells adhered to the surface of the culture flask totally, majority of which were irregular and became polygonal or triangular. After the 3-day's culture, Cells grew well and proliferated actively, the spread cells maintained physical contact with each other and exhibited a flattened and branched shape (**Figure 1C** and **1D**).

SEM images of the MC3T3-E1 cell co-cultured with scaffolds

SEM was used to observe cellular morphology and cell attachment on the scaffold surface. Three days after seeding, SEM images showed that a number of cells adhered to the surface and pores of the scaffold. It was observed that the cells had many filopodia anchored on many different pores simultaneously. After 7 days in culture, the cell density had increased in scaffolds compared with that on day 3. The osteogenic cells were polygonal or spindle-shaped

Table 1. Characterization of the hybrid scaffolds

Scaffold type	Pore size (μm)	Porosity (%)	Water absorption (%)	Swelling ratio (%)	Compressive strength (MPa)	Compressive modulus (MPa)
SF/CS	265 ± 71	86.1 ± 6.3	273 ± 12	12.3 ± 0.6	0.67 ± 0.13	16.35 ± 2.89
SF/CS/HA	210 ± 59	75.5 ± 5.9	169 ± 8	7.2 ± 0.5	1.03 ± 0.11	23.19 ± 2.53

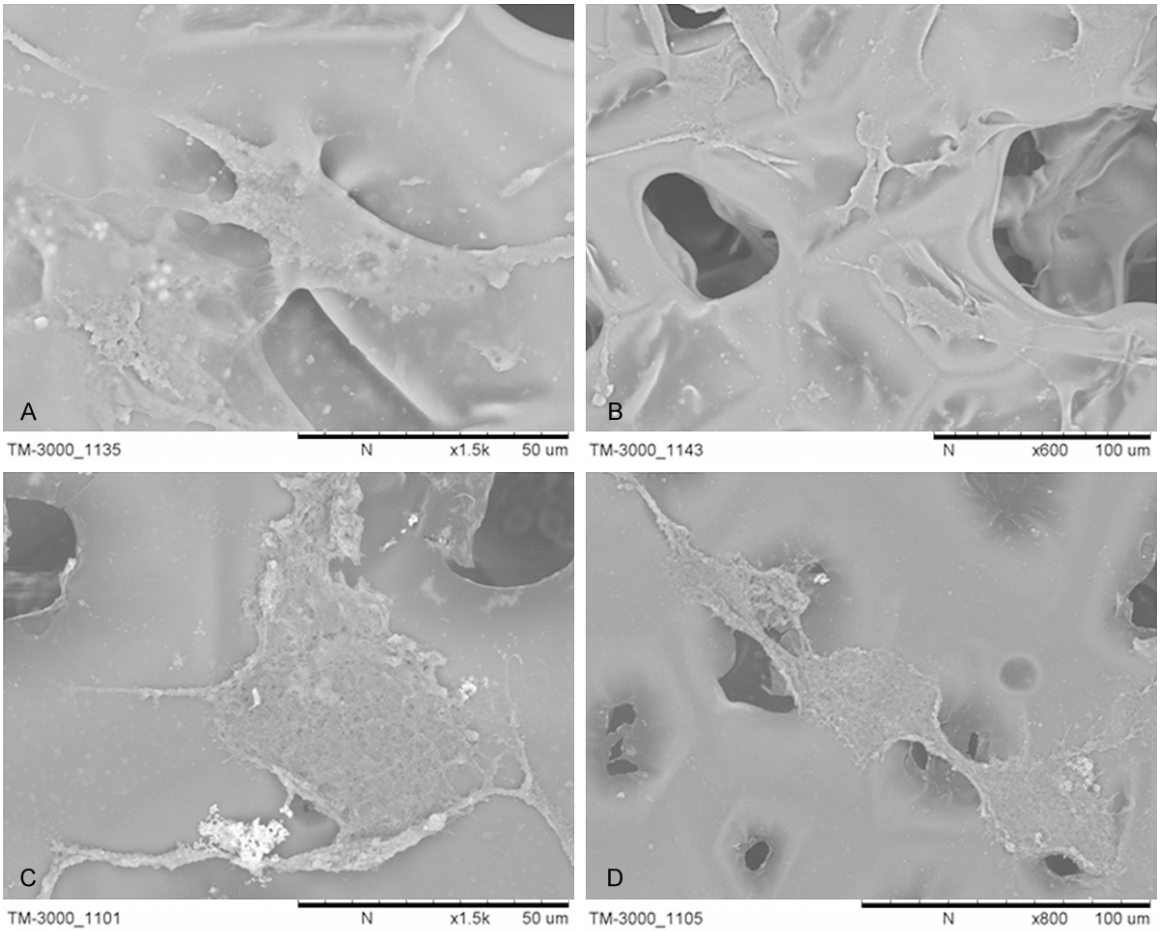

Figure 2. SEM micrographs of MC3T3-E1 cultured on the SF/CS/HA and SF/CS scaffold. After 3 days cell culture, fusiform-shaped cells attached to the pore walls of the SF/CS/HA (A) and SF/CS (C) scaffolds. after 7 days, cells proliferated in the SF/CS/HA (B) and SF/CS (D) scaffolds.

and adhered more tightly on the scaffolds surface or inside than that of day 3 (**Figure 2**).

Adhesion rate of osteoblast cell MC3T3-E1

After 1 h, 3 h and 6 h cultured, cell adhesion rate was increased in three groups with incubation period prolonging. The adhesion rate in HA/SF/CS group and SF/CS group were higher than that in control group and there was significant difference in cell numbers between scaffold groups and control group. More cells adhered to the SF/CS/HA scaffold than that on the SF/CS (**Figure 3**).

CCK-8 assay

The relative number of cells adherent to the different substrates was assessed using a CCK-8 assay. The detection sensitivity of CCK-8 is higher than that of other tetrazolium salts such as MTT, XTT, MTS or WST [13]. From day 1 to day 3, the proliferation of MC3T3-E1 cells on SF/CS/HA scaffolds, SF/CS scaffolds and control group increased rapidly, however, there was no significant difference between them. At day 5, a significant difference was observed between scaffold groups and control group. After 7 days of incubation, cell proliferation on

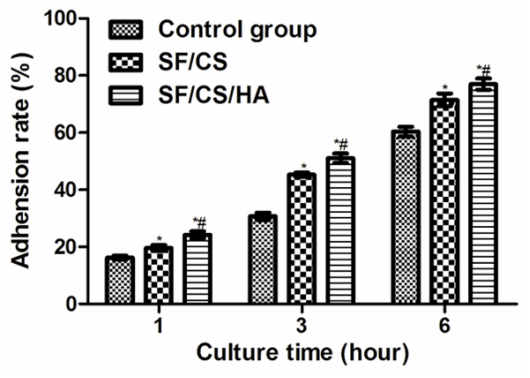

Figure 3. The adhesion rate of MC3T3-E1 seeded on the SF/CS/HA and SF/CS scaffold at 1 h, 3 h and 6 h period incubation. * and # represented significant difference compared between groups within the same culture period at $P<0.05$.

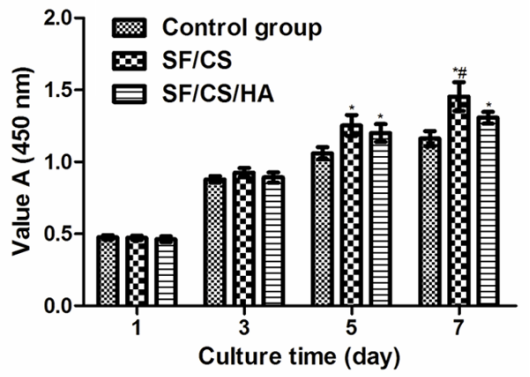

Figure 4. CCK-8 assay for proliferation of MC3T3-E1 cultured on the SF/CS/HA and SF/CS scaffold after a 7-day period incubation. * and # represented significant difference compared between groups within the same culture period at $P<0.05$.

SF/CS scaffolds was greater than that on the SF/CS/HA scaffolds (**Figure 4**).

Alkaline phosphatase (ALP) activity

Cell differentiation has been evaluated by measuring ALP activity of MC3T3-E1 cultured on the different scaffolds as this enzyme play an important role in the initiation of the mineralization process [14]. As time progresses, the ALP activity in three groups were increased. During culture period from day 1, there was no significant difference between SF/CS/HA, SF/CS and blank control group (P>0.05). After 7 days of culture, a significant difference appeared between scaffolds groups and the control group (P<0.01). After 10 days, SF/CS/HA scaffolds exhibited the higher ALP activity than the SF/CS possibly due to the presence of HA which is widely known to accelerate the mineralization process [15] (**Figure 5**).

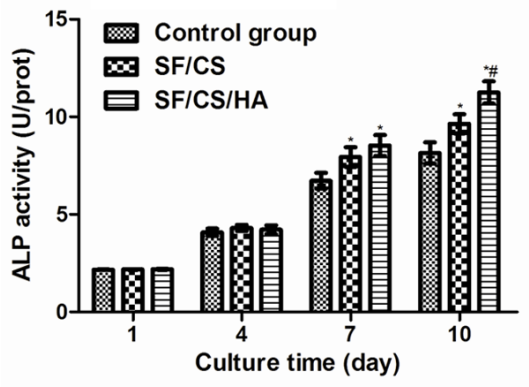

Figure 5. ALP activities of MC3T3-E1 cultured on the SF/CS/HA and SF/CS scaffold after a 10-day period incubation. * and # represented significant difference compared between groups within the same culture period at $P<0.05$.

Discussion

In bone tissue engineering, scaffold served as the matrices of tissue formation plays a pivotal role. Ideally, scaffolds should exhibit a number of properties, which enable to form complete bone tissue. These characteristics are the following: biocompatibility, osteoconductivity, osteoinductivity, bioactivity, good mechanical integrity throughout the bone healing process, a degradation rate such that the strength of the scaffold is maintained until the regenerated tissue can provide the necessary mechanical support, and interconnected porosity with a pore diameter of at least 100 μm, which is necessary for cell penetration, vascularization of the ingrown tissue and transport of nutrient and wastes [16]. However, it is actually impossible to fulfill the various requirements for scaffold materials by using a single material. Therefore, biocomposite system, which combines the advantages of different materials, is becoming more and more promising. Freeze-drying is a well-established method for making porous materials of controllable architecture for use in regenerative medicine applications [17]. Based on a biomimetic mechanism, we presented scaffolds composed of the natural polymer chitosan (CS) and silk fibrion (SF), supplemented or not with hydroxyapatite (HA). The osteoblast cell line MC3T3-E1 were seeded on

SF/CS/HA and SF/CS composite scaffolds *in vitro* tests to investigate their effects on osteoblast adhesion, proliferation and differentiation.

The regeneration of bone tissue aided by synthetic materials mostly depends on the porosity, pore size, and pore distribution (interconnection between the pores) of the scaffolds. Generally, a high porosity with a high interconnectivity between the pores is necessary to allow the growth of cells, vascularization, and diffusion of nutrients and wastes [18, 19]. There is no general consensus as to the optimal pore size for cell growth and tissue formation. In general, scaffold for tissue engineering should expresses three-dimensional porous structure with porosity no less than 70% and pore size ranging from 50 to 500 μm [20]. However, the size of pores and the degree of porosity always influence the mechanical property of the scaffold. Therefore, it is necessary to enhance the mechanical property as much as possible and meet the requirement of pores and porosity simultaneously. Cerron [21] found that bone regeneration in scaffolds with pore sizes between 15 and 50 μm help fibrovascular colonization, pores between 50 and 150 μm determine osteoid growth and pores higher than 150 μm facilitate internal mineralized bone formation. In our study, the SF/CS/HA scaffold and SF/CS scaffold had interconnected pore network and the porosity both beyond 70%. The inner pore sizes of two kinds of scaffold were about ten to three hundreds micron, which satisfy to spatial cell distribution and nutrients or wastes metabolism. Scaffolds for bone tissue engineering must have adequate mechanical strength to support bone tissue regeneration at the areas of implantation. The SF/CS scaffolds had a compressive modulus of 16.35 ± 2.89 MPa and a compressive strength of 0.67 ± 0.13 MPa. With the addition of hydroxyapatite, the compressive modulus and compressive strength of SF/CS/HA scaffolds were reached 23.19 ± 2.53 MPa and 1.03 ± 0.11 MPa, respectively. Although there is no clearly defined standard of the balance between porosity and mechanical strength required by bone tissue engineering, it is generally considered that the scaffold material should have mechanical strength as close as possible to the strength of natural bone tissue [18, 20, 22]. Based on these results, the CS/SF/HA scaffold show better mechanical property than the CS/SF scaffold, and is more suitable to be used in skin tissue engineering.

The scaffolds for bone tissue engineering not only need microporous structure and suitable mechanical property, but also should have an excellent cell compatibility which will be beneficial to the growth of the seed cells. It is acknowledged that material surface will contact and interact with the cells. Therefore, adhesion of cells on the scaffolds is the foundation of the bone tissue engineering. In this work, pre-osteoblastic cell lines MC3T3-E1, which were selected is well-known as osteogenic cells that can be easily induced to differentiate into osteoblasts [23]. After a period of incubation, the adhesion rate in SF/CS/HA group and SF/CS group were higher than that in control group and there was significant difference statistically. Furthermore, the osteoblasts adhered more to the SF/CS/HA scaffolds significantly than to the SF/CS scaffolds. It proved that cells grew well on the both of scaffolds. The SF/CS/HA scaffolds had the higher ratio of adhesion compared with the SF/CS scaffolds and the ratio increased with the addition of HA. This may result from the change of roughness of the surface with HA content. It has been reported that cellular adhesion is sensitive to surface roughness. In most cases, high surface roughness of a material leads to enhanced cell adhesion [24, 25]. In order to studied cellular morphology and cell attachment on the scaffold surface, SEM was used to assess it. The number of viable cells in scaffolds is indicative of compatibility and cell suitability for bone tissue application. The seeding of MC3T3-E1 on the SF/CS/HA scaffolds and the SF/CS scaffolds up to 7 days resulted in completely polygonal or triangular cellular morphology. The evidence of cell-to-cell interaction and cell spreading can be regarded as signs of healthy cells and indicative for non-cytotoxic response of the cells on these two scaffolds [26].

The level of cell growth and proliferation on the scaffolds and control group were assessed using CCK-8 assay in vitro. It can be observed the cell numbers increased with time on all the samples. Data of MC3T3-E1 cells cultured on the HA/SF/CS scaffolds and SF/CS scaffolds high above the cells cultured in the control group after 5-day culture, More importantly,

there was a higher number of cells on the SF/CS scaffolds than that on the HA/SF/CS scaffolds after 7-day culture, which implied that the scaffolds were beneficial to cell development, especially on the SF/CS scaffolds.

ALP, an early osteogenic marker for differentiation, is important for the construction of bone matrix [27]. ALP increases the phosphate concentration in local environment through the hydrolysis of phosphate esters and thus elevates the mineralization of ECM [28]. The normalized ALP activity of MC3T3-E1 cultured in SF/CS/HA and SF/CS are shown in **Figure 5**. According to the results, the SF/CS/HA composite scaffolds possessed a greater ability to promote the osteoblastic differentiation of MC3T3-E1 than that of the SF/CS scaffolds, which also demonstrate that the SF/CS/HA scaffold has comparatively good biocompatibility.

This study first demonstrates the possibility of preparing mineralized and non-mineralized 3D-scaffolds based on CS, SF and HA with interconnected pores. Two kinds of the scaffolds presented good biocompatibility indicating that the freeze-drying method and biomimetic technique chosen here did not induce any significant cytotoxic response. Furthermore, HA/SF/CS scaffolds show the better mechanical property, improving cell growth and osteogenic differentiation can be served as an ideal biomaterial and more suitable for bone tissue engineering.

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Disclosure of conflict of interest

None.

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