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In Vitro Culture of BMSCs on VEGF-SF-CS Three-Dimensional Scaffolds for Bone Tissue Engineering

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Abstract: The objective of the study was to discuss the biocompatibility of VEGF-silk fibroin-chitosan (VEGF-SF-CS) three-dimensional scaffolds in order to offer an ideal scaffold for bone tissue engineering. Bone marrow derived mesenchymal stem cells (BMSCs) were seeded on the VEGF-SF-CS three-dimensional scaffolds and silk fibroin-chitosan (SF-CS) three-dimensional scaffolds. On VEGF-SF-CS and SF-CS three-dimensional scaffolds, the cell adhesion rate was increased as time went on. Scanning electron microscopy: The cells grew actively and had normal multiple fissions, granular and filamentous substrates could be seen around the cells, and cell microfilaments were closely connected with the scaffolds. The cells could not only show attached growth on surfaces of the scaffolds, but could also extend into the scaffolds. CCK-8 and ALP analysis proved that the vascular endothelial growth factor (VEGF) could significantly promote BMSCs growth and proliferation in the SF-CS scaffolds; however, the enhancement of BMSCs cell proliferation and activity by VEGF is dependent on time. Thus, VEGF-SF-CS composite scaffolds represent a promising new type of scaffold for bone tissue engineering.

Key words: Silk fibroin, Chitosan, Bone tissue engineering, Bone marrow derived mesenchymal stem cells, Vascular endothelial growth factor

Introduction

Repair of bone defects resulting from trauma, congenital malformations, and surgical resections remains a challenge that is being addressed by advanced bone tissue engineering approaches^{1,2}. A combinatorial approach for treating large bone defects include seeding cells, growth factors and biomaterials used in bone tissue engineering, can further enhance the success of cell therapy in this scenario³⁻⁷.

BMSCs derived from bone marrow are an obvious source of autologous stem cells, and used as seeding cells for cell therapy and tissue engineering⁸⁻¹¹. A number of studies have demonstrated that autologous BMSCs cultured in scaffolds can induce new bone formation *in vivo* and lead to improved healing of critical-size defects¹²⁻¹⁵. In some studies, BMSCs were induced into osteoblast-like cells with the osteogenic medium before transplantation. Osteoblast-like cells derived from BMSCs shared similar characters with osteoblasts. Osteoblast-like cells had a good ability of growth and proliferation and kept their biological characteristics constant after several passages. In addition,

osteoblast-like cells could secrete much bone matrix to form mature bone with deposition of calcium salts, and played an important role in osteogenesis and bone regeneration¹⁶. BMSCs can differentiate in bone, chondrogenic and osteogenic lineages upon specific induction with appropriate chemicals and factors¹⁷. The main advantage of using BMSCs for transplantation is their innate plasticity¹⁸. *In vitro* bone differentiation of BMSCs prior to transplantation may enhance engraftment efficiency. The present study evaluated the reconstruction effects of BMSCs and osteoblast-like cells differentiated from BMSCs in VEGF-SF-CS scaffolds and SF-CS scaffolds, and analyzed the effects of VEGF in SF-CS scaffold and *in vitro* biocompatibility for the first time.

Cell delivery vehicles using biomaterials have been under development over the past decades. The most common approach has been to seed cells onto porous scaffolds¹⁹. To guide the organization, growth, and differentiation of cells in tissue engineered constructs, the biomaterial scaffold should be able to provide not only a physical support for the cells but also the chemical and biological cues needed in forming functional tissues²⁰. With the advancements in the field of bone tissue engineering, it has now become possible to BMSCs to multiple mesenchymal tissue lineages such as: osteogenic, chondrogenic and adipogenic tissues with the proper stimulation²¹.

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Scaffold materials are often critical for successful bone tissue regeneration using bone tissue engineering techniques. During the last few years, SF-CS scaffold has been recognized as a revolutionary material for applications in orthopedics and maxillofacial surgery due to it being biodegradable, biocompatible, and having osteoconductive properties²²⁻²⁴. They provide a porous interconnected environment for cells seeding and by-product circulation²⁵. In addition, they have been demonstrated to be non-cytotoxic in vitro and to exhibit a good in vivo biocompatibility without inducing a strong leukocytic inflammatory host tissue response after implantation²⁶. In our previous study, we mixed SF and CS into a 3D scaffold provide unique chemical, structural, and mechanical properties, which could be utilized for bone tissue engineering and regeneration applications. We previously found that the most appropriate proportion between chitosan and silk fibroin is 5:5 for bone tissue engineering.

In addition to scaffold design, delivery of drugs or bone healing promoting factors is commonly employed in bone tissue engineering strategies to accomplish several functions required for bone tissue regeneration²⁷⁻³². A group of growth factors known as VEGF has been studied extensively for applications related to bone regeneration. VEGF is a major regulator of angiogenesis. It is importance in triggering desired immune response and thus supports the bone healing process³³. VEGF may couple angiogenesis to osteogenesis both indirectly through its effects on endothelial cells and directly by modulating chondrocytes, osteoblasts and osteoclasts that all express VEGF receptors³⁴. Earlier findings established that VEGF functions as a key inducer of bone vascularization but also indicated that the various cell types involved in bone metabolism, such as perichondrial progenitor cells, osteoblasts and osteoclasts can respond to VEGF signalling³⁵. A direct effect of VEGF on osteogenic cells was suggested earlier by the increased proliferation, migration, differentiation and survival observed in human mesenchymal stem cells in vitro³⁶.

Bone tissue engineering aims to address problems caused by bone trauma and disease. This newly emerging therapy usually involved the culture of a single cell type (such as the osteoblast) on a scaffold prior to patient implantation³⁷. Until now, the effects of VEGF in SF-CS scaffold and in vitro biocompatibility have not been analyzed. In order to offer an ideal scaffold for bone tissue engineering, we added VEGF into SF-CS scaffold to reconstruct a 3D scaffold instead for the first time. This composite scaffold was hypothesized to be a useful carrier for BMSCs and growth factors that are relevant to bone regeneration. Finally, the in vitro approaches to the attachment, proliferation, and differentiation of rabbit BMSCs using VEGF-SF-CS scaffolds are described. The aim of the present study was to analyze the biocompatibility of the VEGF-SF-CS three-dimensional scaffolds in order to offer an ideal scaffold for bone tissue engineering.

Materials and Methods

Materials

Three healthy New Zealand white rabbits of either gender, aged 3 months, weighting about 3.0 kg, were obtained from the Center for Experimental Animals at China Medical University (Shenyang, China) with a National Animal Use License number of SCXK-LN2011-0009. Animal use has been approved by Animal Use and Care Committee at China Medical University with a protocol number of CMU62043006. All experiments and surgical procedures were approved by the Animal Care and Use Committee at China Medical University, which complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. Silk fibroin and Chitosan were chosen as they are widely used natural biomaterials in bone tissue engineering. Raw silk was purchased from nancong (Sichuan, China). Chitosan was purchased from tongxing company (Jiangsu, China). VEGF (121a.a.Human, 0.7mg/bottle) was purchased from PROSPEC company (Israel). CCK-8 (0.5 mg/ml) and ALP measurement kit(96T) was purchased from Jiancheng Biotechnology Institute(Nanjing, China). NaHCO₃, CaCl₂ and ethanol were bought from Nanjing (China). All other chemicals and reagents were of analytical grade unless specifically mentioned.

BMSC isolation, culture and differentiation

For the surgical procedures, the rabbits were anesthetized with 10% chloral hydrate (0.35 ml/100 g) intraperitoneally. Bone marrow (3-5 ml) was extracted from rabbit femurs using a heparinized syringe, then centrifuged and washed with PBS. The cell suspension was centrifuged with Percoll lymphocyte separation medium, and the ivory white layer in the middle was collected, PBS washed and cultured in complete low-glucose DMEM. Cells were seeded in culture flasks at 1.0×10^4 cells/cm² and incubated at 37 °C in a humidified atmosphere with 5 % CO₂. Half of the culture medium was exchanged after 24 h, and completely exchanged after 48 h, followed by medium changes every 2-3 days. Unattached cells were discarded by refreshing the medium. Cells at 85 % confluency after 10-12 days, then trypsinized, centrifuged and passaged at a 1:2 ratio after 10-12 days. Third passage cells were induced to differentiate with osteogenesis induced liquid (0.1 μMol/l dexamethasone, 50 μmol/ml vitamin C, 10mmol/L β-glycerophosphate and containing 10% fetal bovine serum glucose DMEM), followed by medium changes every 2-3 days.

Alkaline phosphatase staining

BMSCs were evaluated for alkaline phosphatase activity 14 days after osteogenesis induction and in those without osteogenesis induction(control group). Cells were fixed for 10 min at 4 °C and

incubated with a 2 % cobalt nitrate and 1% ammonium sulfide (ALP kit, Hongqiao, Shanghai, China³⁸). Areas stained tan were designated as positive.

Von Kossa assay

BMSCs plated in 6-well plates were fixed in 70 % ethanol 18 days after osteogenesis induction in those without osteogenesis induction (control group). Cells were stained with von Kossa silver and placed under ultraviolet light for 10 min. Cells were then treated with 5 % NaS₂O₃ for 2 min, and washed with distilled water³⁹. Calcium nodules with a diameter greater than 1 mm were visualized by light-inverted microscope.

Preparation of VEGF-SF-CS and SF-CS scaffold

Bombyx mori silk fibers were treated twice with 0.5 % (w/w) NaHCO₃ solution at 100 °C for 30 min and then rinsed with 70 °C distilled water to remove sericin for 30 min. Then constant temperature drying with 50°C in the incubators overnight. Degummed silk was dissolved in a mix solvent system of CaCl₂(44.4g)/CH₃CH₂OH(46ml)/H₂O(57.5ml) (mole ratio, 1:2:8) at 70 °C for 6 h and filtered to get a SF solution. After dialysis in a cellulose dialysis tubing (MWCO=50000) against distilled water for 3 days with water change every 12 h). The final concentration of SF used was 8 %.

CS solutions were prepared by dissolving high molecular-weight CS (82.7 % deacetylation; Tongxing China) at 3.66 % (w/v) in 2% acetic acid. The final concentration of CS used was 2 %.

VEGF solution was prepared by dissolving 0.7 mg VEGF in 7ml deionised sterile water (0.1mg/ml).

SF/CS blend solutions(100ml) with SF/CS weight ratio 5:5 was prepared in the same solvent system at 10 wt% (combined weight of CS and SF). After dialysis in a cellulose dialysis tubing (MWCO=50,000) against distilled water for 3 days with water change every 12h, CS and SF solutions were obtained. Then added/7ml VEGF solution, stirred in a magnetic stirrer for 50 minutes. Poured the CS, SF and VEGF solution into the 24 hole teflon culture plate. The CS, SF and VEGF solution was lyophilized (VFD-2000, Boyikang, PRChina) to obtain VEGF-SF-CS scaffold. Then we prepared SF-CS scaffolds by the same method.

To improve water stability, the dry VEGF-SF-CS and SF-CS scaffold were treated in methanol solution (concentration<10 %) for 2 h to crystallize the SF content and neutralize the CS content. The VEGF-SF-CS and SF-CS scaffold was lyophilized to remove the excess methanol.

BMSCs seeding

The scaffolds were sterilized with 75 % alcohol under ultraviolet light overnight and then rinsed extensively three times with sterile PBS. Before cell culturing, scaffolds were pre-wetted by immersion in DMEM for 12 h in the 37 °C incubator.

The BMSCs were cultured onto VEGF-SF-CS scaffolds, SF-CS scaffolds and 24-well tissue culture plate wells (control group; n = 3; diameter 15 mm; height 13mm) for 3 and 6 days after osteogenesis induction at 37 °C under an atmosphere of 5 % CO₂ and 95 % air, with the original cell culture density of 4,000 cells/well.

BMSCs Morphology on scaffolds

The morphology of BMSCs after osteogenesis induction on the VEGF-SF-CS scaffold and SF-CS scaffold was observed on day1, 3 and 7 by SEM. The samples were washed with PBS and fixed with 3.0% glutaraldehyde at 4 °C for 4 h. Subsequently, they were dehydrated through a series of graded ethanol (30, 50, 70, 80, 90, 95 and 100 %), air-dried overnight, and sputtered with gold for SEM observation. Adherent cell morphology, patterns of cellular adherence with respect to structural features of the VEGF-SF-CS scaffold and SF-CS scaffold, cell-cell interactions, and adherent cell density were assessed.

The adhesion of BMSCs on scaffolds

BMSCs after osteogenesis induction on the VEGF-SF-CS scaffold, SF-CS scaffold and control group were harvested and the concentration adjusted to 1×10⁴/ml. Cells were placed in cell culture plates precoated with VEGF-SF-CS scaffold and SF-CS scaffold, 1 ml per well. Cells cultured in culture plates with no scaffold served as controls. 6 parallel wells were used for each group. Cells were cultured in an incubator at 37 °C with 5 % CO₂. The number of non-adhered cells was quantified at 1, 2, 4, 8 and 24 h.

The cell adhesion rate (%) = (number of seeded cells - non-adhered cells)/number of seeded cells × 100 %.

CCK-8 Test of BMSCs on scaffolds

In order to examine whether grafted cells were survived, cell viability and proliferation was determined by using CCK-8. Briefly, after samples were cultured 1, 3, 5 and 7 days, the culture medium was replaced with serum free culture medium containing CCK-8 (0.5 mg/ml). Cultured for 4 h, the samples were transferred to a 96-well plate. The absorbance was measured at 450 nm using an ELISA reader (Bio-Rad Model 550,USA).

Alkaline Phosphatase (ALP) Activity of BMSCs on scaffolds

The intracellular ALP activity in the scaffolds and control group was measured using an ALP measurement kit. Briefly, after samples were cultured 1, 4, 7 and 10 days, then washed with PBS for 3 times. Samples and control group with BMSCs were immersed in 500 microlitre cell lysis solution containing 0.1 % Triton X-100 and 5 mM MgCl₂ through overnight. 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

Table 1 CCK-8 Assay of BMSCs on Different Scaffolds and Control Group ($\bar{x} \pm s$, $n=3$)

Time	VEGF-SF-CS	SF-CS	Control Group	F ^a	P ^a
1d	0.476 $\pm$ 0.016	0.469 $\pm$ 0.014	0.484 $\pm$ 0.023	0.535	0.611
3d	0.967 $\pm$ 0.133	0.921 $\pm$ 0.051	0.881 $\pm$ 0.030	15.038	0.005
5d	1.324 $\pm$ 0.330	1.215 $\pm$ 0.424	1.164 $\pm$ 0.037	13.991	0.006
7d	1.521 $\pm$ 0.599	1.390 $\pm$ 0.737	1.264 $\pm$ 0.456	13.393	0.006
F ^b	82.746	99.080	174.859		
P ^b	0.001	0.000	0.000		

^aThe comparison of different groups at the same time; ^bThe comparison of same groups at the different time.

Table 2. ALP Assay of BMSCs on Different Scaffolds and Control Group (U/ g prot, 4000 cells/wel, $n=4$)

Time	VEGF-SF-CS	SF-CS	Control Group	F ^a	P ^a
1d	2.465 $\pm$ 0.049	2.449 $\pm$ 0.035	2.441 $\pm$ 0.026	0.385	0.691
4d	5.317 $\pm$ 0.242	4.303 $\pm$ 0.162	3.874 $\pm$ 0.145	63.254	0.001
7d	9.833 $\pm$ 0.516	7.871 $\pm$ 0.434	6.036 $\pm$ 0.387	71.712	0.000
10d	12.766 $\pm$ 0.514	9.594 $\pm$ 0.533	7.452 $\pm$ 0.495	108.157	0.000
F ^b	573.059	339.679	191.119		
P ^b	0.001	0.000	0.000		

^aThe comparison of different groups at the same time; ^bThe comparison of same groups at the different time.

supernatant with 50 microlitre p-nitrophenyl phosphate (5mM) in 150 mM 2-amino-2-methyl-1-propanol buffer solution at room temperature for 30 min in dark. Following the incubation period, the reaction was stopped by adding 50 μ l of 0.2N NaOH to denature ALP and the OD was measured at 520 nm using an ELISA reader (Bio-Rad Model 550, USA).

Statistical analysis

All quantitative data were expressed as the means $\pm$ SD. Statistical analyses were performed using the statistic program SPSS 17.0. Results were analyzed by one-way ANOVA with a Student's t-test. Probability values of less than 0.05 were considered statistically significant. Before results analyzed by one-way ANOVA, all quantitative data obeyed the normality distribution.

Results

Cell morphology

Cell morphology was studied using a light-inverted microscope (Figure 1), BMSCs demonstrated good survival, and rapid proliferation.

In primary cells, BMSCs were a round morphology (Fig.1a). After 12 hour, BMSCs were attached to the culture flasks sparsely and displayed a fibroblast-like, spindle-shaped morphology (Fig.1b). After 3-4 days of incubation, cell number increased and cells continued to grow, proliferation started and the cells gradually grew into small colonies, displayed an elongated spindle shape, cells grew well and actively proliferated, they assumed a more

spindle-shaped, fibroblastic morphology (Fig.1c). This pattern continued and was more evident after 5 days, The hematopoietic cells exhibited a typical cobblestone morphology at high magnification (Fig.1d). After 10-12 days majority of the cells were attached and acquired large, flattened or multilayer growth morphology (Fig.1e) and did not change throughout different passages (Fig.1f).

Osteogenic differentiation of BMSCs

Two weeks latter, alkaline phosphatase staining was greater in BMSCs after osteogenesis induction (Fig.2a) than in those without osteogenesis induction (Fig.2b). In addition, von Kossa staining 2 weeks after osteogenesis induction (Fig.2c) revealed a significant increase in calcium nodules when compared to those without osteogenesis induction groups (Fig.2d).

Adhesion rate of BMSCs

After 1h, 3h and 6h, cell adhesion rate was increased with time up in three groups. The adhesion rate in VEGF-SF-CS group and SF-CS group were higher than that in control group ($P<0.01$). In 1h, the major populations of adhesion rate in SF-CS scaffolds were nearly the same as those in VEGF-SF-CS scaffolds, there were no significant differences between scaffolds groups ($P>0.05$). After 3h and 6h, there was a significant increase in adherent cells as compared to 1 h. The adhesion rate in VEGF-SF-CS group and SF-CS group was significantly higher than that in control group ($P<0.01$), but there were still no significant differences between scaffolds groups ($P>0.05$). We proved that the SF-CS

Figure 1. Cell morphology of BMSCs by a light-inverted microscope. a: Primary cells; b: Cultured 12 h; c: Cultured 3 day; d: Hematopoietic cells exhibited a typical cobblestone morphology at high magnification; e: Cultured 12 day; f: Third passage BMSCs.

scaffolds could increase adhesion of BMSCs. It also proved that the VEGF unable to promote BMSCs adhesion in the SF-CS scaffolds.

SEM images of the BMSCs cultured with the VEGF-SF-CS scaffolds

Growth of osteogenically induced BMSCs on scaffold cellular attachment and interaction within the VEGF-SF-CS scaffold and SF-CS scaffolds were evaluated by electron microscopy.

Growth of cells on scaffold at 1 day, scattered round cells adhered to the scaffold and a large number of particles were observed on the cell surface. Cell microfilaments were connected

Figure 2 *In vitro* analysis of osteogenic differentiation after BMSCs osteogenesis induction and in those without osteogenesis induction.

(a) Alkaline phosphatase expression 14 days after BMSCs osteogenesis induction.

(b) Alkaline phosphatase expression 14 days after BMSCs without osteogenesis induction.

(c) and (d) Von Kossa assay comparing calcium nodules between BMSCs osteogenesis induction(c) and BMSCs without osteogenesis induction (d) after 18 days culture. (e) Quantitative analysis of calcium nodules present in the two groups. A significant increase is seen in the BMSCs osteogenesis induction group when compared with BMSCs without osteogenesis induction groups, *p < 0.05.

with the scaffold material (Fig 4a).

For 3 days cultured in osteogenic media, a large number of cells had adhered to the surface and pores of the scaffold. The cell aggregates became enlarged and actively proliferated. SEM results showed that single round cells became polygonal or triangular. Granular and filiform substances were observed around the cells. Cell microfilaments and pseudopodia were tightly connected to the scaffold (Fig 4b).

At 7 days, an increased number of cells had adhered to the surface and pores of the scaffold compared with 3 days, and cells grew and proliferated well. They exhibited typical osteoblast cell morphology and adhered tightly on the scaffolds surface or inside by formation of lamellipodia and filopodia, regarded as cells spreading, there were a number of cells present that are extending towards other cells in the culture. Cells were attached well on VEGF-SF-CS scaffolds and appeared spreaded and most cells were collected together to form cellular aggregations, which is crucial

for the viability and function of BMSCs in vitro. The SF-CS scaffolds showed sparse or less attachment of cells as compared to VEGF-SF-CS scaffolds (Fig. 4c).

CCK-8 test

The proliferation of BMSCs on VEGF-SF-CS scaffolds, SF-CS scaffolds and control group cultured for 1, 3, 5 and 7 days were compared by CCK-8 assay. The absorbance value for both the VEGF-SF-CS scaffolds and SF-CS scaffolds gradually increased, indicating significant cell growth within both scaffolds. The data are shown in Ttable 1. Data of BMSCs cultured without scaffolds was also present. The cell number increased with culture time. The BMSCs proliferated well in both VEGF-SF-CS scaffolds and SF-CS scaffolds, there were no significant differences with the cells cultured in control group at the 1 day(p>0.05). But after 3 day, data of BMSCs cultured in the VEGF-SF-CS scaffolds high above the cells cultured in the SF-CS scaffolds, and data of BMSCs

Figure 3. Adhesion rate of BMSCs, * denote $P < 0.05$ compared with control group

cultured in both of scaffolds remarkably higher than those cultured without scaffolds, statistically significant differences ($p < 0.05$).

Alkaline phosphatase (ALP) activity

To evaluate the osteoblastic differentiation of the mesenchymal stem cells in the VEGF-SF-CS scaffolds, SF-CS scaffolds, we measured the alkaline phosphatase (ALPase) activity using spectrophotometry. The ALP activity of BMSCs cultured in the VEGF-SF-CS scaffolds, SF-CS scaffolds and control group was shown in Table 2. For total ALP activity measurement, during culture period from day 1, there was no significant difference in OD values between VEGF-SF-CS, SF-CS and control group ($P > 0.05$), but from day 4 to 10, a significant difference in OD values was found between scaffolds groups and the control group ($p < 0.01$). The OD values in VEGF-SF-CS groups was the highest. This suggests the effect of VEGF on the activity of BMSCs. As culture time from day 4 to 10, the growth trend of the OD values in three groups was increased rapidly. The OD values reached the peak at 10d. It means that the VEGF-SF-CS scaffolds are stable for prolonged culture system and the stabilized VEGF greatly enhance the ALPase activity.

Discussion

Osteoprogenitor cells, osteoconductive scaffolds and osteoinductive factors are the three main elements for forming tissue engineered bone. Highly porous scaffolds which performed the role of a temporary matrix for anchorage dependent cells are an important factor in the success of tissue engineering⁴⁰⁻⁴². Composite scaffold was hypothesized to be a useful carrier for seed cells and growth factors that are relevant to bone regeneration. To maximize the capacity of BMSCs to regenerate bone, VEGF are generally used to promote differentiation into osteoblasts. VEGF have been reported to be effective in enhancing bone formation in a variety of animal studies. Various strategies for

Figure 4. SEM images of BMSCs cultured with the VEGF-SF-CS scaffolds a: 1st day; b: 3rd day; c: 7th day

manipulating BMSCs in order to overcome the low cellular survival and transdifferentiation potency of BMSCs after implantation are pretreatment with growth factors or preconditioning such as VEGF or osteogenesis induction to over express antideath signals⁴³.

SF facilitates cell adhesion and growth, and has relatively low thrombogenicity, low inflammatory response, and low protease susceptibility when highly crystallized⁴⁴⁻⁴⁷. Chitosan is particularly attractive candidates in the scaffold composites due to their minimal inflammatory reactions or toxic degradation and good biodegradability coupled to excellent osteoconductivity in the case of bone graft⁴⁸⁻⁵⁰. A direct effect of VEGF on osteogenic cells was the increased proliferation, migration, differentiation⁵¹.

In the present study, a composite scaffold design was hypothesized to increase osteogenesis of BMSCs through incorporation of VEGF. We analyzed the VEGF-SF-CS composite scaffolds for bone tissue engineering applications. Such a scaffold may be particularly suited for implantation into bone defects due to its increased proliferation, migration, differentiation and osteoconductive. For this purpose, we added VEGF into SF-CS scaffold to reconstruct a 3D scaffold instead for the first time.

The BMSCs can be stimulated by Vitamin C, beta-glycerophosphate, and dexamethasone that be added in culture media to differentiate to preosteoblasts before the BMSCs cultured onto VEGF-SF-CS scaffolds, because that can avoid BMSCs being induced into vascular endothelial cells by VEGF after seeded. To establish safety of BMSCs-engineered bone scaffolds, it is critical to evaluate their phenotypic stability⁵²⁻⁵³).

Nominal differences in cellular adhesion and proliferation were observed between BMSCs after osteogenesis induction. Additionally these results suggested that the biomaterial was suitable for the proposed *in vitro* studies as it facilitated BMSCs initial attachment onto the surface, spreading and subsequent growth.

The time course of BMSCs-SF-CS adhesion clearly demonstrated adhesion of BMSCs to SF-CS scaffolds within minutes. The rapid time course of BMSCs adhesion to SF-CS scaffolds was significant, characterized by 29 % adhesion by 60 min post-seeding. The adhesion rate of BMSCs in VEGF-SF-CS group and in SF-CS group significantly exceeded those in control group ($P < 0.05$). Previous studies have indicated that SF-CS scaffolds increased adhesion and growth of BMSCs. These observations achieved our primary objective, namely, to determine if SF-CS scaffolds had the potential to establish an adhesive interface with BMSCs *in vitro*. It also proved that the VEGF unable to promote BMSCs adhesion in the SF-CS scaffolds.

7 days BMSCs morphology study by SEM was performed to further evaluate the biocompatibility of these scaffolds. The seeding of BMSCs on VEGF-SF-CS scaffolds up to 7 days resulted in completely polygonal or triangular cell morphology similar to what is typically observed in standard cell culture flasks. These observations confirmed the cell survival and proliferation on VEGF-SF-CS scaffolds, assuring its biocompatibility. The evidence of cell-to-cell interaction and cell spreading can be considered as signs of healthy cells and indicative for noncytotoxic response of the cells on VEGF-SF-CS scaffolds⁵⁴⁻⁵⁵).

The level of cell growth and proliferation on the scaffolds and control group were assessed using CCK-8 and ALP assay *in vitro*. It could be seen that the absorbance index of the tested groups increased with the increase of culture time. In the first day, statistically no significant differences ($p > 0.05$) were observed in the cell proliferation and differentiation among three groups. Data of BMSCs cultured in the VEGF-SF-CS scaffolds and SF-

CS scaffolds high above the cells cultured in the control group after 3-day culture, which implied that the scaffolds were beneficial to cell development. While the number of BMSCs on VEGF-SF-CS scaffolds exhibited remarkably higher than those on SF-CS scaffolds. These results indicated that VEGF promoted the proliferation and differentiation of BMSCs significantly under our conditions. Our study is also suggestive evidence from *in vitro* experiments that VEGF may regulate bone formation through a direct effect on BMSCs. It has been reported that human VEGF binds to BMSCs in culture and is capable of inducing migration and alkaline phosphatase activity⁵⁶). Our results indicate a difference in the proliferation and differentiation of BMSCs in the different types of scaffold. The reason for varied production of extracellular matrices in the different scaffolds may be due to the differences in chemical compositions of the matrices.

While fabricate the VEGF-SF-CS scaffold for bone tissue engineering, a critical part is to maintain bioactivity of VEGF in manufacturing VEGF-SF-CS scaffold, as during this manufacture course, VEGF bioactivity is mostly like to be destroyed in lyophilization and methanol crosslinking. In lyophilization course, usage of cryoprotector may reduce or even prevent influence of stress on activity of protein⁵⁷). Silk protein and chitsan can be used as cryoprotector to well maintain activity of VEGF^{58,59}). Alexander proved that conformation of protein treated with low concentration methanol was basically not changed and covalent structure of protein molecule was not destroyed⁶⁰). We took the following measures in manufacture VEGF-SF-CS scaffold to prevent VEGF bioactivity from destroyed: 1. Set lyophilization temperature at -40°C and used silk and chitsan as cryoprotector to further maintain VEGF activity. 2. Methanol concentration $< 10\%$. 3. The whole VEGF-SF-CS manufacture processes were designed as a physical course. Through ALP and CCK-8 testing result, we could fundamentally believe that there was no obvious degradation of VEGF bioactivity.

In this experiment, SF-CS scaffold was manufactured in aqueous solution and might wrap active VEGF molecule without damaging VEGF activity. Mandal and Kundu utilized lyophilization to prepare 3D silk fibron scaffold wrapped with bovine serum albumin and then inspected on its *in vitro* release and performance⁶¹). Kundu investigated VEGF loaded silk fibron protein continuously releasing within 3 weeks and proved that it could protect VEGF evenly and continuously releasing in silk fibron scaffold⁶²). Through ALP and CCK-8 test result, we could basically determine that in VEGF-SF-CS scaffold, VEGF could evenly and continuously release within certain period.

Interestingly, the enhancement of BMSCs proliferation and differentiation by VEGF is dependent on time. VEGF didn't promote the proliferation and differentiation of BMSCs significantly at early time (day 1), but had obvious effect when cultured at later time points (days 3-10), demonstrating that the

effect of VEGF which promotes the proliferation and differentiation of BMSCs is time dependent. These results indicate that correct VEGF delivery strategies greatly affect BMSCs proliferation and differentiation and should be considered when designing delivery system.

We compared, for the first time, the physiochemistry and osteogenesis of VEGF-SF-CS scaffolds and SF-CS scaffolds. Our results confirmed that VEGF-a bioactive growth factor-has a significant capacity to improve the *in vitro* bioactivity of SF-CS scaffolds. The results confirmed that the direct effect of VEGF on BMSCs was the increased proliferation, migration, differentiation and survival. The VEGF-SF-CS scaffolds are biocompatible and have no negative effects on the BMSCs *in vitro*. Our data supports the potential utility of SF-CS as a biocompatible scaffold. Our findings demonstrate that VEGF-SF-CS scaffolds are suitable substrates for bone tissue engineering and VEGF confers the potential physiological benefit of accelerated reconstruction. Future work will be conducted in a bone defect model of animals to investigate potential repaired reaction of the VEGF-SF-CS scaffolds, and to better understand the biomechanical properties and potential uses of VEGF-SF-CS scaffolds, as well as the bone-forming capacity of this type of scaffold in animal models.

In conclusion, VEGF-SF-CS composite scaffolds represent a promising new type of scaffold for bone tissue engineering.

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