เป็นหนังสือภาษาอังกฤษ

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ชื่อโครงการ

The Effects of Collagen on Properties of Chitosan-collagen Sponges as Scaffolds for Bone Tissue Engineering

(ผลของคอลลาเจนต่อคุณสมบัติของฟองน้ำไคโตซาน-คอลลาเจน
ในการเป็นตัวรองรับในขบวนการวิศวกรรมเนื้อเยื่อกระดูก)

โดย

รศ. เปรมจิต อาภรณ์แม่กลอง และ

รศ. ปริศนา ปริพัฒนานนท์

ทพ. นริศร สุวัฒน์วิโรจฒน์

ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ธันวาคม 2553

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณหน่วยงาน ผู้ทรงคุณวุฒิ เพื่อนร่วมงาน และเจ้าหน้าที่ทุกท่านที่ได้ให้การสนับสนุนการดำเนินงานวิจัยนี้ทั้งในรูปของทุนการดำเนินการวิจัย การเอื้อเฟื้ออุปกรณ์วิทยาศาสตร์ ให้คำแนะนำที่เป็นประโยชน์ และช่วยเหลือในการดำเนินงานต่างๆให้สำเร็จลุล่วงไปด้วยดีดังมีรายนามดังต่อไปนี้

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3. คุณพรพจน์ หนูทอง นักวิทยาศาสตร์ประจำหน่วยวิเคราะห์โครงสร้างระดับจุลภาค ศูนย์เครื่องมือวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์
4. ศูนย์วิจัยกลางคณะทันตแพทยศาสตร์ เจ้าหน้าที่ประจำศูนย์วิจัยกลางฯ และผู้ช่วยวิจัย
5. ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

คณะผู้วิจัยขอขอบพระคุณทุกท่านมา ณ. โอกาสนี้ด้วย

(ลายเซ็น) ปรมจิต อาภรณ์แม่กลอง

(นางสาวปรมจิต อาภรณ์แม่กลอง)

หัวหน้าโครงการวิจัย

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การศึกษานี้มีวัตถุประสงค์ที่จะทำการศึกษาผลของคอลลาเจนต่อคุณสมบัติของสารผสมไคโตซาน-คอลลาเจนต่อการส่งเสริมการเจริญเติบโตไปเป็นเซลล์สร้างกระดูกของหนูและต่อคุณสมบัติทางกายภาพและคุณสมบัติเชิงกลของตัวรองรับฟองน้ำไคโตซาน-คอลลาเจน การศึกษานี้แบ่งเป็น 4 กลุ่มการศึกษา กลุ่มที่ 1 คอลลาเจน กลุ่มที่ 2 ไคโตซาน กลุ่มที่ 3 1:1 ไคโตซาน-คอลลาเจน และกลุ่มที่ 4 1:2 ไคโตซาน-คอลลาเจน ตัวรองรับฟองน้ำไคโตซาน-คอลลาเจนได้รับการเตรียมโดยการใช้วิธีทำให้แห้งโดยใช้ความเย็น (Freeze-drying technique) เซลล์ไขกระดูกของหนูได้ถูกนำมาเลี้ยงบนตัวรองรับฟองน้ำในน้ำเลี้ยงเซลล์สำหรับการเหนี่ยวนำให้พัฒนาเป็นเซลล์สร้างกระดูก (osteogenic medium) เป็นเวลา 27 วัน การเจริญเติบโตและการเกาะติดของเซลล์บนตัวโครงร่างฟองน้ำได้รับการตรวจภายใต้กล่องอิเลตรอนแบบส่องกราด (scanning electron microscope, SEM) และทำการตรวจวัดระดับของการสังเคราะห์เอนไซม์อัลคาไลน์ฟอสฟาเตส (alkaline phosphatase activity, ALP) และออสติโอคัลซิน (osteocalcin) นอกจากนี้ยังทำการทดสอบคุณสมบัติในการอุ้มน้ำ (swelling test) การสลายตัวของตัวรองรับฟองน้ำโดยเอนไซม์คอลลาจิเนต (Collagenase) และไลโซไซม์ (Lysozyme) และคุณสมบัติในการรับแรง (mechanical test) จากการศึกษาพบว่าเซลล์ไขกระดูกของหนูมีการเกาะตัวที่ดีบนตัวรองรับฟองน้ำไคโตซาน-คอลลาเจนและมีระดับของ ALP และ ออสติโอคัลซินสูงกว่าเซลล์ที่ถูกเพาะเลี้ยงบนตัวรองรับฟองน้ำไคโตซาน หรือ คอลลาเจน ตัวรองรับฟองน้ำทุกกลุ่มมีความสามารถในการเก็บอุ้มน้ำได้ดี ตัวรองรับฟองน้ำไคโตซานและตัวรองรับฟองน้ำไคโตซาน-คอลลาเจนมีความทนทานต่อการย่อยสลายของเอนไซม์ที่สูงกว่าตัวรองรับฟองน้ำคอลลาเจน ตัวรองรับฟองน้ำไคโตซาน-คอลลาเจนในอัตราส่วน 1:1 มีความสามารถทนต่อแรงอัด (compressive strength) ได้สูงกว่ากลุ่มอื่นๆ ผลการศึกษาแสดงให้เห็นว่าการผสมคอลลาเจนกับไคโตซานทำให้ได้สารผสมไคโตซาน-คอลลาเจน (chitosan-collagen composite) ที่มีคุณสมบัติในการส่งเสริมการพัฒนาเป็นเซลล์สร้างกระดูกของเซลล์ไขกระดูกของหนูและยังส่งผลให้เกิดการพัฒนาคุณสมบัติทางกลและกายภาพของตัวรองรับไคโตซาน-คอลลาเจนอีกด้วย

คำหลัก

ตัวรองรับไคโตซาน-คอลลาเจน การพัฒนาเป็นเซลล์สร้างกระดูก คุณสมบัติทางกลและกายภาพของตัวรองรับไคโตซาน-คอลลาเจน

ABSTRACT

The study aimed to further investigate effects of chitosan-collagen blended on osteogenic differentiation of rat bone marrow stromal cells (RBMSc) including physical and mechanical properties of the sponges. The study was categorized into 4 groups, Group I: collagen, Group II: chitosan, Group III: 1:1 chitosan-collagen and Group IV: 1:2 chitosan-collagen sponges. Chitosan/collagen sponges were fabricated using the freeze-drying technique. Rat bone marrow stromal cells (RBMSCs) were seeded on the sponges and cultivated in mineralized culture medium for 27 days. Attachment and growth of cells on the sponges were examined under a scanning electron microscope (SEM). Expressions of ALP activity and levels of osteocalcin were monitored. Swelling property, collagenase and lysozyme enzymatic degradation and mechanical testing were performed. It was found that RBMSCs attached well on the structure of the sponges and expressions of ALP activity and osteocalcin on collagen and chitosan-collagen composite sponges were greater than chitosan sponges. All sponges expressed a high degree of water uptake. Chitosan and chitosan-collagen sponges showed a higher resistance to enzymatic degradation than collagen sponges. A 1:1 chitosan-collagen sponge demonstrated the highest compressive strength. The results indicated that blending of chitosan-collagen matrixes promoted osteoblastic differentiation of RBMSCs and improved mechanical and physical properties of the chitosan-collagen sponges.

Keywords

chitosan-collagen scaffold, osteoblastic differentiation, bone marrow stromal cells, physical and mechanical properties

INTRODUCTION

One of the approaches in cell-based bone tissue engineering is to regenerate new bone by culturing living cells directly on three-dimensional scaffolds[17]. Mesenchymal stem cells presenting in bone marrow (BMSCs) are an excellent source of bone progenitor cells[19,39]. MSCs can be isolated from the bone marrow mononuclear cells by their tendency to adhere to plastic culture dishes and be inducible to differentiate into many cell types in a mesenchymal lineage, including osteoblasts[15,30]. Progress of osteoblastic differentiation of BMSCs can be determined by analyzing expressions of ALP activity and osteocalcin, which are characteristic phenotypes of pre- and mature osteoblasts, respectively[3]. Under appropriate culture conditions, BMSCs can be expanded and seeded onto the three-dimensional scaffold to form tissue engineered constructs[13]. Autologous and allogenic BMSCs seeded directly into porous biomaterial scaffolds have presented the capacity to regenerate bone within segmental and craniofacial defects[7,11,44]. The method of transferring BMSCs with an appropriate scaffold shows potential to become an alternative for autogenous bone grafting[44].

Scaffolds influence the capacity of seeded cells to regenerate bone within the defect site[6] by functioning as extracellular matrix providing surface contact and temporary mechanical support for functional cells and maintaining space for tissue development[28]. They should be biocompatible, porous and resorbable and have suitable surface chemistry for cell attachment, proliferation and differentiation[18]. In addition, mechanical strength, swelling property and degradation behavior of scaffolds play crucial roles on the long-term performance of a tissue-engineered cell/material construct such as cell growth, cell adhesion, nutrient perfusion and tissue regeneration[18,27,31,38,44].

Natural biomaterials are widely used for scaffold fabrication in tissue engineering since they facilitate cell attachment and maintenance of differentiation function. Chitosan is a partially deacetylated derivative of chitin and is conducive to osteoblasts[32]. Collagen is a major component of ECM of bone and enhances proliferation, migration and differentiation of osteoblast-like cells[26]. To improve the mechanical and biological properties of scaffolds, collagen and chitosan were blended. A previous study reported that chitosan-collagen composite sponges were porous, biocompatible and able to support growth and differentiation of osteoblasts in a greater manner than chitosan sponges[2].

Thus, to further evaluate applicability of chitosan-collagen sponges in bone tissue engineering, this present study aimed to investigate the ability of the composite sponges to support osteoblastic differentiation of BMSCs and the effects of chitosan-collagen blending on mechanical strength and swelling and degradation properties of the chitosan-collagen composite sponges.

MATERIALS AND METHODS

GROUPS OF STUDY

The study was categorized into 4 groups, Group I: collagen, Group II: chitosan; Group III: 1:1 (by weight) chitosan:collagen, and Group IV: 1:2 (by weight) chitosan:collagen sponges. In all groups, 5 - 15 samples were analyzed for each investigated parameter at given time points.

PREPARATION OF CHITOSAN, COLLAGEN AND CHITOSAN-COLLAGEN SPONGES

Porous matrices of chitosan, collagen and chitosan-collagen composites were fabricated using the freezing and drying techniques as reported by Arpornmaeklong and co-workers[2]. To prepare 1% Chitosan suspension, chitosan powder (Medium molecular weight, Fluka, Switzerland) was dissolved in 1% acetic acid (Merck,

Germany) at RT. Chitosan and collagen sponges 1:1 and 1:2 ratios were prepared by pouring 1% chitosan suspension into 1% atelopeptide collagen (Succinyl Atelocollagen, Koken, Tokyo, Japan) under a constant gentle stiring at RT in ratios of 1:1 and 1:2 by weight, respectively.

The 1% chitosan, 1% atelopeptide collagen and chitosan-collagen composites suspensions were cross-linked with 0.05% glutaraldehyde (Merck, Germany) at RT. Then they were poured into 5.0 ml disposable injection syringes (NIPRO, Thailand) and kept at 4°C for 24 hours and -70°C for 12 hours. The frozen solutions were transferred to the freezing and drying machine (FD-20-55DMP1, F.T.S. System INC., USA) and dried at – 45°C for 48 hours. After that the cylindricle sponges were cut into 5 mm thickness (10x5 mm) for every investigation and 12 mm (12x5 mm) for mechanical testing.

BONE MARROW STROMAL CELL (BMSC) ISOLATION AND CULTURE

Male Spraque-Dawly rats, weight 300-350 g, were anesthetized with an intraperitoneal injection of 90 mg/kg Ketamine (Ketavet, Germany) and 7 mg/kg Xylazine (Xylaz, Germany). Bone marrow was aspirated from right and left femurs of the rats and cultivated in culture medium consisting of DMEM-F12 (Gibgo, USA), 10% FBS (Gibco, USA), 1% Penicilling/Streptomycin (Gibco, USA) and 0.01% Fungizone (Gibco, USA). The cell suspension from two femurs was placed in one T-75 flask (Costar, USA). The medium was changed on the fourth day of culture and every three days thereafter. At the subconfluent stage of 7-10 days, the cells were detached with 0.05% Trypsin and 0.02 wt% of Ethylenediaminetetraacetic acid (EDTA) (Gibco, USA) in PBS and subcultured at a density of 2x104 cells/cm^2. The cells in the first passage at 80% confluence were trypsinized and seeded on the sponges[39].

CELL SEEDING ONTO CHITOSAN-COLLAGEN SPONGES

Sponges, 10x5 mm, were placed in 24-well culture plates, one sponge per well. BMSCs were statically seeded into the sponges; $5x10^4$ cells in 100 µl / sponge. One hundred microliters of cell suspension was gradually seeded, using 200 µl pipette tip, on all surfaces of the sponge to enhance cell distribution. Cells were allowed to attach on the sponges in a minimal culture medium for three hours, then 1.5 ml of culture medium was added into each well. The sponges were incubated in a mineralized culture medium consisting of DMEM-F12 (Gibco), 10% FBS, 1% Penicillin/streptomycin, 0.1% Fungizone, 50 µg/ml ascorbic acid (Sigma, USA), 5 mM ß-glycerophosphate (Sigma, USA) and 10 nM Dexamethasone (Sigma, USA) at 37°C in a 5% CO2 and 95% air atmosphere for 27 days. Culture medium was changed every three days[1].

INVESTIGATED PARAMETERS

Swelling, enzymatic degradation and compressive strength tests were performed to examine mechanical and physical properties of the sponges. Cellular morphology and adhesion of cells on the sponges were examined using a scanning electron microscope (SEM). An expression of alkaline phosphatase activity and levels of osteocalcin in the culture medium were investigated to determine osteoblastic differentiation of bone marrow stromal cells.

Swelling test (Water adsorption test)

A known weight of chitosan-collagen sponges (Wo) were placed in distilled water for five minutes. After removal from the water, they were hung over a table for one minute until no dripping water was observed and then weighed immediately on an electronic balance (Ws)[33].

The content of the distilled water in the swollen sponges or percentage of water adsorption was calculated by the following formula.

Water uptake (%) = [(Ws-Wo)/Wo] x 100[27]

Each swelling experiment was repeated three times. A total of 15 sponges from each group were tested.

***In vitro* Collagenase and lysozyme degradation tests**

The biological stability of the chitosan-collagen sponges (5x10 mm) were evaluated by performing in vitro collagenase and lysozyme digestion tests. Each sponge was neutralized by immersing in distilled water and PBS and then by incubating the sponges in PBS over night. After that the sponges were incubated in each enzyme according to the study design.

Collagenase degradation test

A known weight of chitosan-collagen sponges (Wo) were immersed in 2 ml TESCA buffer (pH 7.4) containing 200, 400 and 800 µg/ml of collagenase (266 Unit/mg, Type I, Sigma) at 37°C for 24 hours or in 100 µg/ml collagenase type I (Sigma, USA) for 1 – 5 days[33].

Lysozyme degradation test

A known weight of chitosan-collagen sponges (Wo) were immersed in PBS containing three-time recrystallized egg-white lysozyme (Sigma, USA) (50,000 unit/mg) 4 – 16 mg/ml at 37°C for 24 hours and in 2 mg/ml lysozyme for 1 - 14 days[35].

At each time interval, the degradation was discontinued by removing the sponges from the enzymes and dipping the sponges into 50 ml of distilled water three times to remove any enzymes remaining and then blotting with filter paper to remove surface water. Then the sponges were lyophilized and weighed (Wt). The extent of an in vitro degradation was calculated as a percentage of weight loss according to the following equation[33,35].

Percent weight lose = [(Wo - Wt)/Wo]x100

Each biodegradation test was repeated two times. A total of 10 sponges from each group were tested.

Mechanical test (Compressive strength)

Mechanical properties of the sponges in a dry state (10x12 mm) were tested using a texture universal testing machine (Lloyd LRX, Ametek, USA), in a standard compression-testing mode. The cylindrical-shaped chitosan-collagen sponges in a dry state were cut into 12 mm thickness (10x12 mm). To ensure complete parallel of the cutting surface, the cut was performed in one stroke using a new razor blade and a guiding board and the sponges were stabilized on flat surface of a sample holder using custom design adhesive tape. The crosshead speed was set to 1 mm/min and compression was carried out until a maximum strain of 30% was achieved. Stress at a strain of 30% was employed as the compressive strength. A total of 12 sponges from each group were tested[23].

Cellular adhesion and morphology

In the cell-scaffold constructs, cellular morphology and adhesion to the sponges were assessed optically using SEM (5800LV, JEOL Ltd., Japan). The sponge-cell constructs were examined on culture-day 15. The examinations were performed on two sponges selected from each group (n=8). The constructs were fixed in glutaraldehyde and formaldehyde (Merck, Germany). They were then dehydrated in ethanol series of 30-100%, and then dried, fractured into two halves, mounted, gold sputter-coated and imaged[2].

Cell lysis and cellular protein analysis

At each given time, on culture-day three and every six days thereafter, the sponges were moved to a new well and washed two times in DPBS (Gibgo, USA). Cells were lysated by incubating on ice in 1% Triton X-100 (Sigma, USA) in DPBS for one and a half hours. Then protein solutions were centrifuged at 2000 g for 15 minutes at 4°C (Laborfuge 400R, Heraeus, Germany). The supernatants were kept at -20°C for the analysis of ALP activity and protein contents.

The quantification of the amount of protein in cell lysate was performed according to the manufacturer's instruction (Biorad Protein assay kit) based on Lawry's assay. The solutions were read at 650 nm absorbance in duplicate using a microplate reader (BiotrakTM II Amersham/Bioscience, Australia). A total cellular protein analysis was performed on the same samples as the ALP activity and osteocalcin measurements[1,12].

Alkaline phosphatase assay

The procedure in brief; the cellular protein solutions were mixed with 400µl of substrate solution (2 mg/ml p-nitrophenylphosphate disodium salt (Merck, Germany) in a lysis buffer, pH 10.3) and were incubated for one hour at 37°C. Then NaOH 50 mM (Merck, Germany) was added into each sample to stop the reaction. The solutions were read at 405 nm absorbance in triplicate using a microplate reader (BiotrakTM II Amersham/Bioscience, Australia). The analysis was performed on day three and every six days thereafter. Levels of the activity were neutralized with the amount of protein contents in the cell lysate solutions (µg p-nitrophenol/mg protein)[2]. A total of five sponges of each group were tested.

Osteocalcin assay

A quantification of osteocalcin in serum free cell culture medium was performed according to the manufacturer's instruction; the Osteocalcin EIA kit (Biomedical Technologies Inc., USA). The solutions were read at 450 nm absorbance in duplicate using a microplate reader (BiotrakTM II Amersham/Bioscience, Australia). A total cellular protein analysis was performed on the same samples as the ALP activity and calcium content measurements[12]. The assay was performed on culture-days 21 and 27. Levels of osteocalcin were reported as nano-gram per milligram protein (ng/mg protein). A total of three sponges of each group were tested.

DATA ANALYSIS

Data were expressed as the mean value ± the standard deviation of the mean (mean ± SD) and analyzed by oneway analysis of variance. Multiple comparisons with Tukey HSD and Dunnette T3 methods were used. The levels of statistical significance were set at $p<0.05$.

RESULTS

SWELLING TEST

All sponges absorbed and maintained a large volume of water within pore spaces. Average ratios of water uptake of all groups were 25 – 30 times higher than dry weight. The swelling ratios of all sponges were not significantly different. Chitosan-collagen sponges in a ratio of 1:1 (Group III, swelling ratio of 31.8±7.7) demonstrated a tendency to have a higher ability to preserve water than 1:2 chitosan-collagen sponges (Group IV, 30.0±8.8), chitosan (Group II, 28.8±6.5) and collagen sponges (Group I, 27.7±5.1), respectively, but there was no statistical difference among groups (Fig. 1).

DEGRADATION IN COLLAGENASE

Figures 2-5 demonstrate the percent of weight loss of chitosan/collagen sponges in collagenase and lysozyme solutions. In all concentrations of collagenase (200 – 800 μg/ml) solution, the percent of weight loss of collagen sponges (Group I, 51.22±26.79%) was higher, and chitosan sponges (Group II, 1.45±4.45%) was significantly lower than composite sponges (Groups III, 22.05±11.10 and IV, 24.90±13.84) (ANOVA, $F=43.76$; $df=3, 104$; $p<0.001$), but the degradation ratios of Groups III and IV were not significantly different (Tukey HSD, $MD=3.45$; $p>0.05$). In 200 μg/ml collagenase, the percentage of mass loss of Group I (45.19±20.40%) was significantly higher than Group II (0.94±5.81%), Group III (21.80±13.47%) and Group IV (22.54±14.41%) (ANOVA, $F=14.02$; $df=3, 32$; $p<0.001$). The mass loss of the composite sponges (Groups III and IV) in 400 and 800 μg/ml collagenase solutions (in 400 μg/ml, Group III: 22.28±9.84%; Group IV: 24.49±15.65% and 800 μg/ml, Group III: 22.07±11.06%; Group IV: 27.63±12.70%) were significantly higher than Group II (in 400 μg/ml, 1.44±5.32%, ANOVA, $F=11.68$; $df=3, 32$; $p<0.001$ and 800 μg/ml, 1.98±0.84%, ANOVA, $F=17.138$; $df=3, 30$; $p<0.01$) (Fig. 2).

When the sponges were incubated in a low concentration collagenase solution (100 μg/ml) for 1 – 5 days, the degradability of collagen (Group I) and the composite sponges (Groups III and IV) gradually increased over time. Percentages of mass loss of Group I, III and IV on incubation day five were significantly higher than on incubation-day one (Group I, $t=-11.361$; $df=9$; $p<0.001$; Group III, $t=-7.079$; $df=9$; $p<0.001$; Group IV, $t=-2.673$; $df=9$; $p<0.05$), except Group II ($t=-.554$; $df=9$; $p>0.05$). Group I had the highest and Group II the lowest degradation rates. By day five over 80% of the collagen sponges had degraded. It can be noticed that an increasing percentage mass loss of Group II, III and IV were minimal and markedly lower than Group I. The data showed that degradation degrees throughout five days of Groups

III (16.85±10.06%) and IV (20.13±11.26%) were constantly differing from Group I (66.98±19.76) and II (-0.89±5.80%), significantly (ANOVA, $F=257.209$; $df=3, 195$; $p<0.001$) (Fig. 3).

DEGRADATION IN LYSOZYME

The degradation degrees of the sponges in lysozyme solutions were lower than in collagenase solutions. Regarding the average degradation under the three concentrations of lysozyme solutions, 4, 8 and 16 mg/ml, average percentages of mass loss of all sponges were in a range of 15 – 30% of the original weight. A high degradation rate tended to be found on collagen (Group I, 25.33±7.53%), 1:2 chitosan-collagen (Group IV, 23.15±6.72%) and 1:1 chitosan-collagen (Group III, 19.88±5.28%) and chitosan sponges (Group II, 18.08±4.28%), respectively, but only Group I was statistically different from Group II (Tukey HSD, $MD=7.244$; $p<0.05$) (Fig. 4).

The dynamic degradation of the sponges in a low concentration lysozyme solution, 2 mg/ml, demonstrated that the percentage weight loss of collagen (Group I) and 1:1 and 1:2 chitosan-collagen sponges (Groups III and IV), but not chitosan sponges (Group II), were first found on incubation-day one. Throughout an incubation period of 14 days, the average percentage weight loss of Group I (25.40±7.54%) was consistently and significantly higher than Group II (-2.48±5.64%) (Tukey HSD, $MD=27.878$; $p<0.001$), Group III (13.0±7.49, Tukey HSD, $MD=12.405$; $p<0.001$) and Group IV (16.92±7.71) (Tukey HSD, day 1, $MD=8.483$; $p<0.01$). The percentage mass loss of Group I were significantly higher than Group III on days 5 and 14 (Tukey HSD, day 5, $MD=12.67$; $p<0.001$ and day 14, $MD=17.24$; $p<0.05$). The degradation degrees of Groups III and IV were not significantly different, except on day five, where the percentage weight loss of Group

IV was significantly higher than Group III (Tukey HSD, MD=7.25; $p<0.05$). On days three and five, the degradation of composite sponges in Groups III and IV were significantly higher than Group II (Tukey HSD, day 3: Group III, MD=18.66; $p<0.01$; Group IV, MD=20.85; $p<0.001$ and day 5: Group III, MD=18.99; $p<0.001$; Group IV, MD=26.24; $p<0.001$). The degradation of Group II was first detected on incubation-day 14. On day 14, the percentage weight loss of Group I was significantly higher than Group II and III (Tukey HSD, Group II, MD=25.22; $p<0.01$; Group III, MD=17.24; $p<0.05$), while Group IV was significantly higher than Group II only (Tukey HSD, Group II, MD=17.35; $p<0.05$) (Fig. 5).

MECHANICAL TESTING

The 1:1 chitosan-collagen (12.75±1.97) had significantly higher compressive strength than collagen (10.88±1.59) and 1:2 chitosan-collagen sponges (10.54±1.23) (ANOVA, F=5.003; df=3, 44; $p<0.01$). The differences of strength among chitosan (11.88±1.29) and other sponges (Groups I, III and IV) was not significantly different (Fig. 6).

GROWTH AND MORPHOLOGY OF CELLS ON THE SCAFFOLDS

Porous structure of collagen, chitosan and the composite sponges were demonstrated in our previous study[1]. After the 15 days post-seeding cultured-period, evidence of continued cell proliferation on the sponges could be detected. SEM images demonstrated that stellate shaped cells with multiple filopodia adhered to the scaffold surface and filled in pores (Fig. 7). Cells sprouted their cytoplasmic process on the surface of sponges and established intercellular contact forming a continuous sheet of cells and growth of cells in multilayers (Fig. 7A-D). Cellular attachment with cellular spread and cytoplasmic extension over the surface of the matrix interconnecting with adjacent cells are clearly shown on 1:1 chitosan-collagen

sponges (Fig. 7E). Qualitatively, size and numbers of cells on chitosan sponges (Fig. 7B) were relatively less than other groups (Fig. 7A, C and D).

ALKALINE PHOSPHATASE (ALP) ACTIVITY

ALP activity was analyzed to characterize osteoblastic differentiation in an early state. ALP activities of BMSCs on all sponges were gradually increased over culture-time. Significant differences of ALP activity was found early on culture-day three, when cells in each group showed different levels of activity (ANOVA, $F=48.689$; $df=3, 12$; $p<0.001$). The activity of cells on collagen sponges was consistently high. On culture-days 3, 6 and 15, it was significantly higher than other groups (Groups II, III and IV) (ANOVA, day 3: $F=48.69$; $df=3,12$; $p<0.001$; day 6: $F=71.77$; $df=3, 12$; $p<0.001$, day 15: $F=19.85$; $df=3,12$; $p<0.001$). On day 21, the ALP activity of collagen sponges (Group I) was not significantly different from 1:1 chitosan-collagen sponges (Group III) (Dunnett T3, $MD=130.97$; $p>0.05$) and on day 27, It was not significant difference from 1:1 and 1:2 chitosan-collagen sponges (Groups III and IV) (Dunnett T3, $MD=73.133$; $p>0.05$).

ALP activity of cells on chitosan sponges (Group II) was consistently lower than collagen sponges (Group I), but not markedly different from the composite sponges (Groups III and IV). It was significantly lower than 1:1 chitosan-collagen sponges (Group IV) on day three (Turkey HSD, $MD=-3.91$; $p<0.05$) and 1:2 chitosan-collagen sponges (Group III) on day 27 (Dunnett T3, $MD=-494.32$; $p<0.05$) only.

On culture-day 21, Group I had the highest level, followed by Group III: 1:1 chitosan-collagen sponges, Group IV: 1:2 chitosan-collagen sponges and Group II: chitosan sponges (ANOVA, $F=20.262$; $df=3, 12$, $p<0.001$). The highest level of activity of every group was found on culture-day 27. On culture-day 27, ALP activity of cells in Group I was significantly higher than Group II (Dunnett T3, $MD=1500.84$;

$p<0.05$). The activity of cells in Groups III and IV were at similar levels and were lower than collagen sponges (Group I), but not significantly, and only Group IV was significantly higher than chitosan (Dunnett T3, MD=494.32; $p<0.05$) (Fig. 8).

LEVELS OF OSTEOCALCIN IN CULTURE MEDIUM

Late stage osteoblastic differentiation was determined by measuring the amount of released osteocalcin in culture medium over a 24 hour period. During culture-days 21 to 27, levels of osteocalcin of cells on collagen (Group I, 8.224±1.15 ng/mg) and 1:2 chitosan-collagen sponges (Group IV, 3.35±0.22 ng/mg) significantly increased from culture-day 21 to day 27 (Group I, t=-6.968; df=2, $p<0.05$ and Group IV, t =-14.665; df=2; $p<0.01$), but the levels in groups of chitosan (Group II) and 1:1 chitosan-collagen sponges (Group III) were stable (Group II, t=-1.835; df=2; $p>0.05$ and Group III, t=-2.834; df=2; $p>0.05$). On culture-day 21, osteocalcin released in Group III (17.79±6.48 ng/mg) was higher than any other groups (Group I, 8.224±1.51; Group II, 3.57±0.23 ng/mg and Group IV, 3.35±0.22) but not significantly. On culture-day 27, levels of osteocalcin of cells on Group I (31.99±5.88 ng/mg) and Groups III (27.84±7.11 ng/mg) were significantly higher than Group II (5.81±2.33 ng/mg) and Group I was also significantly higher than Groups IV (18.29±1.71 ng/mg) (ANOVA, F=17.344; df=3, 8; $p<0.01$) (Fig. 9).

DISCUSSION

This study demonstrated the effects of blended chitosan and collagen on physical and mechanical properties of chitosan-collagen sponges and the ability of the chitosan-collagen sponges to support osteoblastic differentiation of BMSCs. The current study agree with previous report that high swelling ratio demonstrates ability of the scaffold to preserve high volume of water within the porous structure[27,33,36]. All sponges swelled and had high ratios of water uptake (Fig. 1). High ratios of water

uptake of sponges could be attributed to both their high hydrophilic and the maintenance of their three-dimenstional structure[27,36]. CLSM images of saturated chitosan-collagen sponges in our previous study[2] showed that porous structures were maintained and growth of cells on the sponges saturated with culture medium were clearly evident. The data suggested that a high water binding ability of the sponges could further enhance the penetration of cells into the inner area of the scaffolds and the swelling promote attachment and growth of cells on three-dimensional matrix by increasing pore size and the internal surface area[36].

In general, chitosan and collagen are degraded by enzymatic hydrolysis of lysozyme and collagenase enzymes[29,43]. In the current study, collagen sponges had the lowest biological stability in both lysozyme and collagenase enzymes, as it could be seen that collagen sponges experienced a very high degradation process, with the highest ratios of weight loss in both lysozyme and collagenase (Figs. 2-5). This finding indicates that collagen sponges had low biological stability[20,21,24,25].

Chitosan incubated in lysozyme and collagenase enzymes exhibited low degradation levels (Figs. 4-5). A high resistance to degradation of chitosan and influences of concentration of lysozyme and pH condition were clearly demonstrated at 24 hour incubation period, when percentage of weight loss in 2 mg/ml (Fig 5) was much lower than in 4 – 16 mg/ml (Fig 4). At optimum lysozyme activity in low pH conditions[14], the degradation of chitosan in a low level would be much enhanced and the differences of degradation rate of chitosan in 2 and 4 mg/ml lysozyme in a short incubation period would be decreased.

A dynamic degradation of chitosan sponges in a 2 mg/ml lysozyme solution started to be apparent on day 14 (Fig. 5). This might be due to a high hydrophilicity in chitosan sponges which makes bond cleavage and degradation of chitosan occur

after chitosan matrix is swollen. In a longer incubation period, when swelling reaches a maximum level, a degradation process overcomes the swelling process resulting in the detectable weight loss[35]. The findings suggested that a degradation of chitosan sponges would be more marked for over a longer incubation time.

Percentages in weight loss of the chitosan-collagen sponges (Groups III and IV) in both enzymes had low levels of 10-25% of original weight and were not markedly different from chitosan sponges. The results revealed that chitosan, followed by 1:1 and 1:2 chitosan-collagen sponges had a high resistance against enzymatic hydrolysis, while collagen sponges had the lowest resistance to degradation (Figs. 2-5). Hence, data suggested that a presence of chitosan in chitosan-collagen matrix enhanced resistance to the degradation of the chitosan-collagen composite sponges.

The mechanical properties of the matrix are of great importance due to the necessity of structural stability to withstand functional loading[22]. Although, collagen sponges showed the highest level of osteoblastic differentiation (Figs. 8-9) low compressive strength (Fig. 6), and a fast degradation rate (Figs. 2-5) have limited its application in bone tissue engineering[20,23,24,45].

In the present study, the findings suggested that mechanical strength and degradation resistance for the collagen sponges could be enhanced by blending collagen with chitosan matrixes. A high biomechanical compressive strength (Fig. 6) and resistance to enzymatic degradation were recorded for chitosan, 1:1 and 1:2 chitosan-collagen sponges, and were higher than collagen sponges (Figs. 2-5). This may attribute to an improved three-dimensional porous structure of 1:1 and 1:2 chitosan-collagen sponges1 and the presence of chitosan in chitosan-collagen matrix[27].

The increase of biological stability and mechanical strength of the chitosan-collagen sponges (Figs. 2-6) can be explained by structural change in the composite sponges[42]. The addition of chitosan changed the collagen fiber cross-linking and reinforced the structure and porosity of the composite sponges[27,40]. The finding supported a previous report[41] that collagen and chitosan formed new composite materials with improved osteoconductivity and biological stability.

Growth and differentiation of MSCs on scaffolds are influenced by cell attachment and the physical and biological properties of scaffolds[4,8]. Good cell adhesion and a high degree of cell spreading on the surface of all sponges shown on SEM images (Fig. 7) indicated that the chemical component of all sponges supported cell attachment[37] and the materials were biocompatible[5]. A tendency for greater numbers of cells and better intercellular network presenting on collagen and composite sponges (Fig. 7) suggests that collagen matrix in chitosan-collagen sponges improved favorability of surface materials to cell adhesion and promoted cell growth[5].

In the present study, collagen and chitosan-collagen composite sponges promoted osteoblastic differentiation of BMSCs into mature osteoblasts. Differentiated BMSCs sequentially expressed characteristic phenotypes of pre- and mature osteoblasts, ALP activity and osteocalcin[3], respectively (Figs. 8 and 9). According to levels of ALP activity and osteocalcin, the highest rate and degree of osteoblastic differentiation of BMSCs were found on collagen (Group I), followed by 1:1 chitosan-collagen (Group III), 1:2 chitosan-collagen (Group IV) and chitosan sponges (Group II) (Fig. 8). The ability of cells to spread well and attach onto material surfaces (Fig. 7) may contribute to acceleration of osteoblastic differentiation[4,12].

Taking into consideration that a level of osteocalcin in Group III, 1:1 chitosan-collagen sponges, on day 21 showed a tendency to be higher than other groups, and on day 27, a level of osteocalcin in Group III was not different from Group I, and the levels of Groups I, III and IV were higher than Group II (Fig. 9), the finding clearly demonstrated that the differentiation environment provided by collagen and chitosan-collagen sponges encouraged cell differentiation. It also revealed that the unique microstructure of 1:1 chitosan-collagen sponges provided the most optimal environment for osteoblastic differentiation of BMSCs.

It should be noticed that high levels of ALP activity of Groups I, III and IV were found in a late cell culture period on culture-days 21 and 27 at the same time as the increasing of osteocalcin levels (Figs 8 and 9), instead of an initial rising of the activity followed by a decrease corresponding to further increase of osteocalcin level in a higher differentiation stage as expected in general trend observed for osteoblastic differentiation[3,30]. The data indicate a beneficial effect of three-dimensional collagen and chitosan-collagen matrixes on osteoblastic differentiation enhancing robust osteoblastic differentiation of hBMSCs on the scaffolds. This observation is supported by a previous study which shows enhancing effects of extracellular matrix (ECM) on osteoblastic differentiation of rat BMSCs in osteogenic medium on titanium fiber meshes, where high levels of ALP activity and markers of mature osteoblast phenotypes, expression of osteopontin and calcium deposition, of BMSCs are found in a late cell culture period. While in a control group of titanium mesh without ECM, a peak of ALP activity was found on day 12 and calcium and osteopontin levels were relatively stable throughout a cell culture period[10].

Marked differences of osteoblastic differentiation of BMSCs on chitosan sponges and other sponges in Groups I, III and IV could be the result of differences in

sponge microstructures (Figs. 8 and 9). A better defined porous structure of 1:1 and 1:2 chitosan-collagen sponges could function as additional extracellular matrix enabling intercellular contact and promoting growth of cells in three-dimensions mimicking natural bone structure[2]. A porous structure of the composite sponges should further promote cell migration, spatial cell distribution and perfusion of nutrients deep into the inner structure of the scaffolds[23]. In addition, collagen matrix contains specific cell binding sites, particularly the RGD (Arg-Gly-Asp) amino acid sequences that induces cell adhesion and promotes differentiation on the surface of the biomaterial[16]. This is because interaction between RGD and integrin receptors on the cell surface regulates growth and differentiation of cells on the surface of scaffolds[34], and the strong binding of cell-matrix stabilizes the nucleo-cytoskeletal lattic and provides a resistance to mechanical detaching and cell traction force[9].

In conclusion, the results presented herein demonstrated that a blending of chitosan and collagen matrixes improved biostability and mechanical property of the chitosan-collagen sponges. The chitosan-collagen composite sponges provided supportive environment for osteoblastic differentiation of BMSCs. Basing on mechanical and physical properties and differentiation of BMSCs, 1:1 chitosan-collagen sponges were superior to 1:2 chitosan-collagen sponges as scaffolds for cell-based bone tissue engineering.

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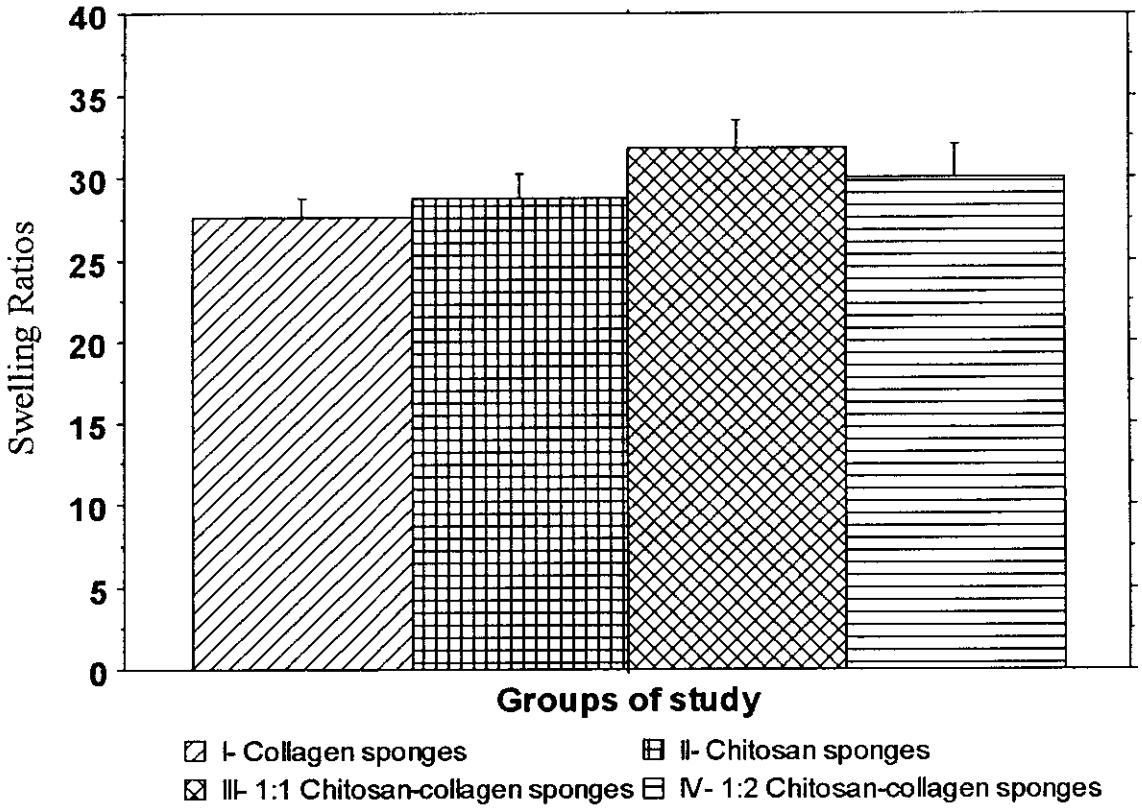

Fig. 1. Water uptake of the sponges. *P <0.05; significant against Groups I. †P <0.05; significant against Group II (mean ± SE, n = 15).

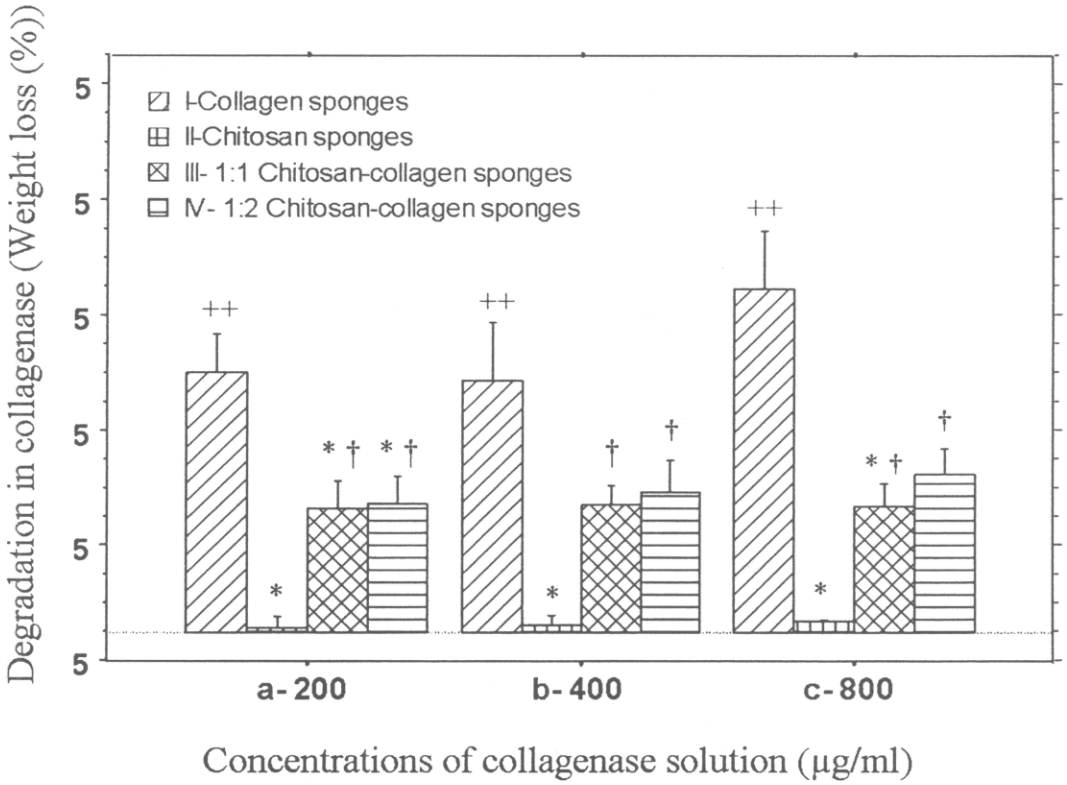

Fig. 2. The biodegradability of the sponges incubated in different concentrations of collagenase solution for 24 hours at 37°C. $^{*}P<0.05$; significant against Group I. $^{\dagger}P<0.05$; significant against Group II. $^{++}P<0.05$ significant against Groups II-IV (mean ± SEM, n = 10).

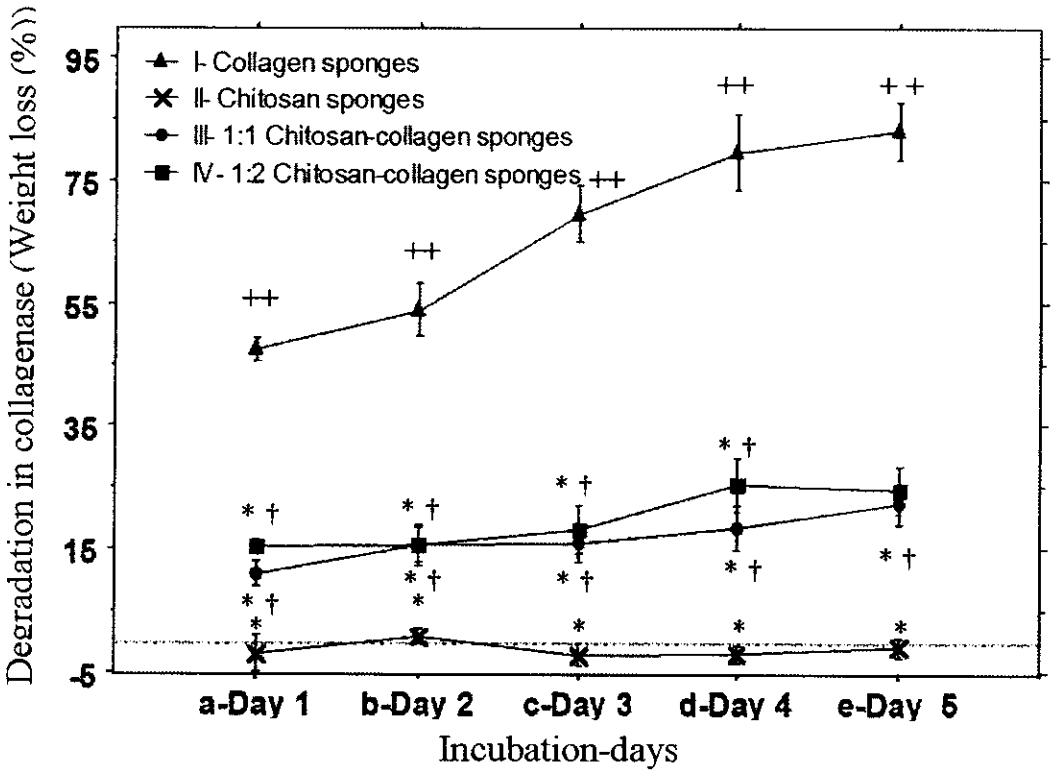

Fig. 3. The biodegradability of the sponges incubated in 100 μg/ml collagenase at 37°C for 1 – 5 days. $^{*}P<0.05$; significant against Groups I. $^{\dagger}P<0.05$; significant against Group II. $^{++}P<0.05$; significant against Groups II-IV (mean ± SE, n = 10).

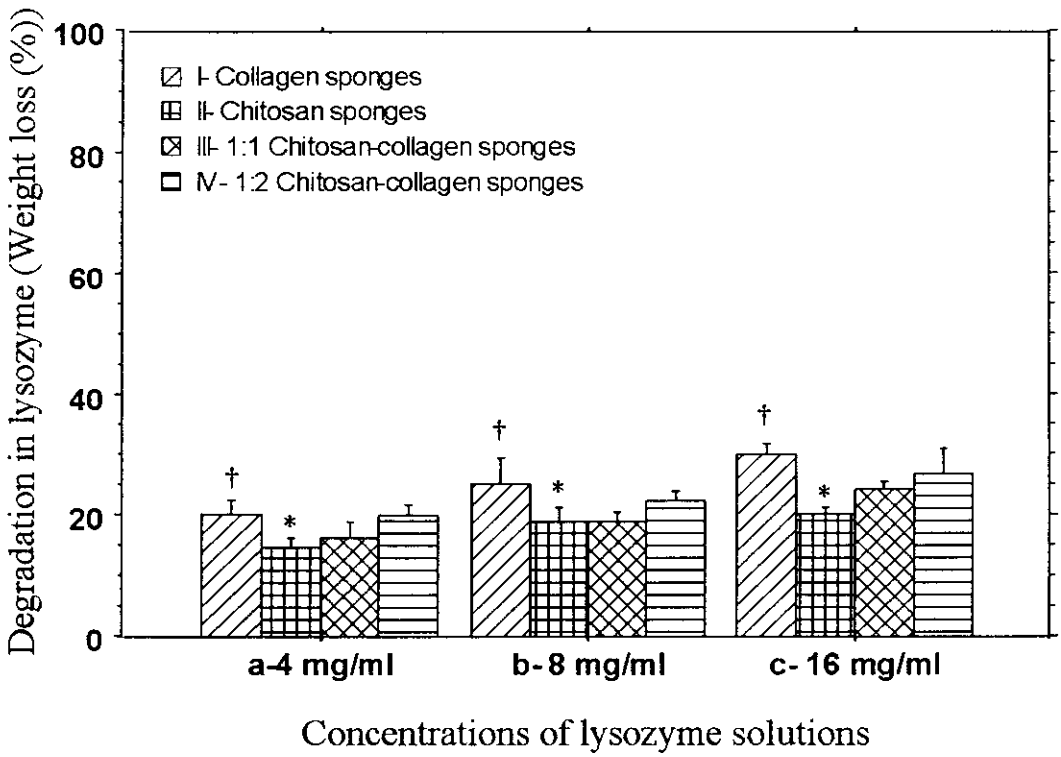

Fig. 4. The biodegradability of the sponges incubated in different concentrations of lysozyme for 24 hours at 37°C. $^{*}P<0.05$; significant against Groups I. $^{\dagger}P<0.05$; significant against Group II (mean ± SE, n = 5).

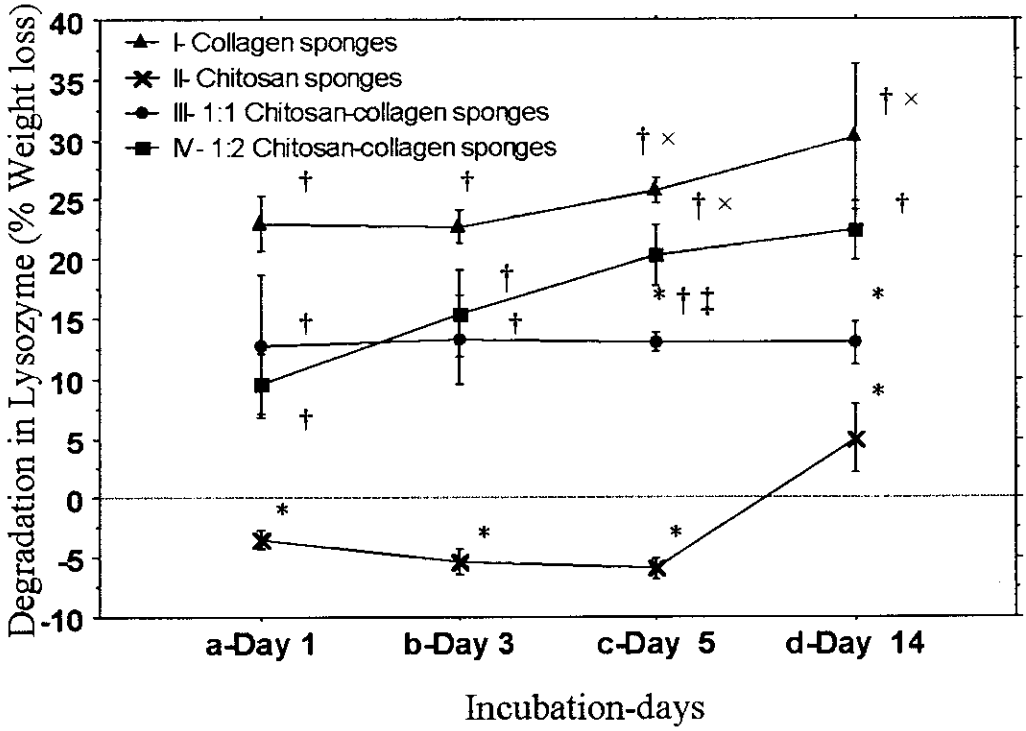

Fig. 5. The biodegradability of the sponges incubated in 2 mg/ml lysozyme at 37°C for 1 – 14 days. $^{*}P<0.05$; significant against Groups I. $^{\dagger}P<0.05$; significant against Group II. $^{\times}P<0.05$ significant against Group III. $^{\ddagger}P<0.05$; significant against Group IV (mean ± SE, n = 5).

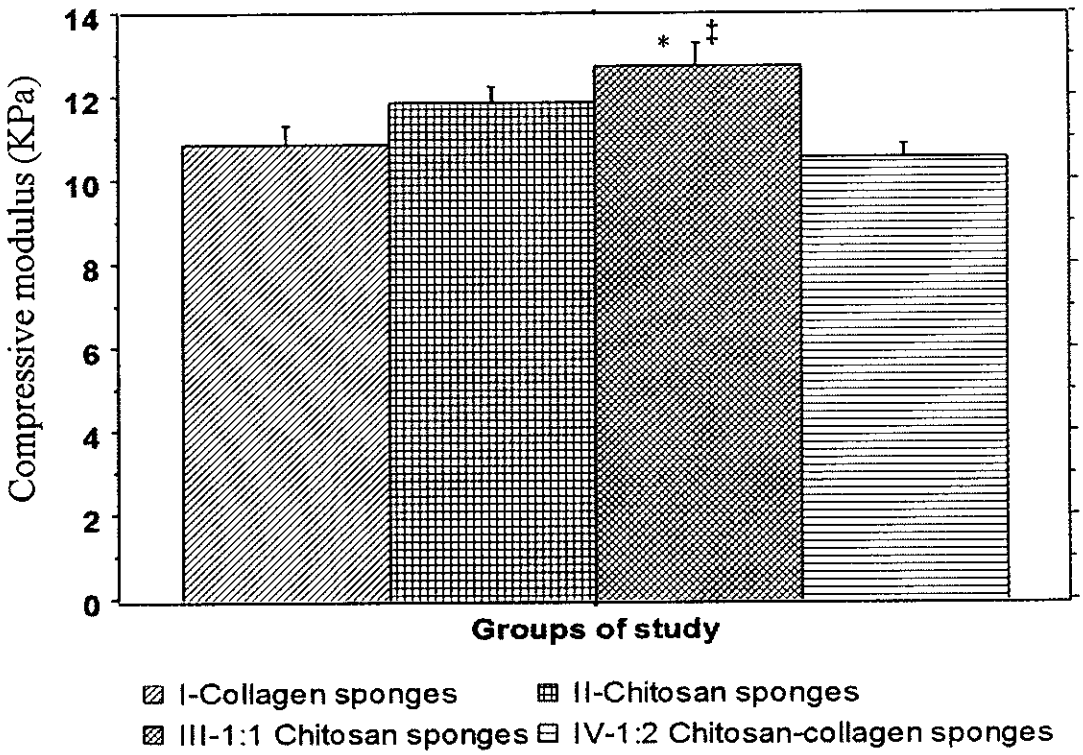

Fig. 6. Mechanical properties of the sponges. $^{*}P<0.05$; significant against Group I.

$^{\ddagger}P<0.01$; significant against Groups IV (mean ± SE, n = 12).

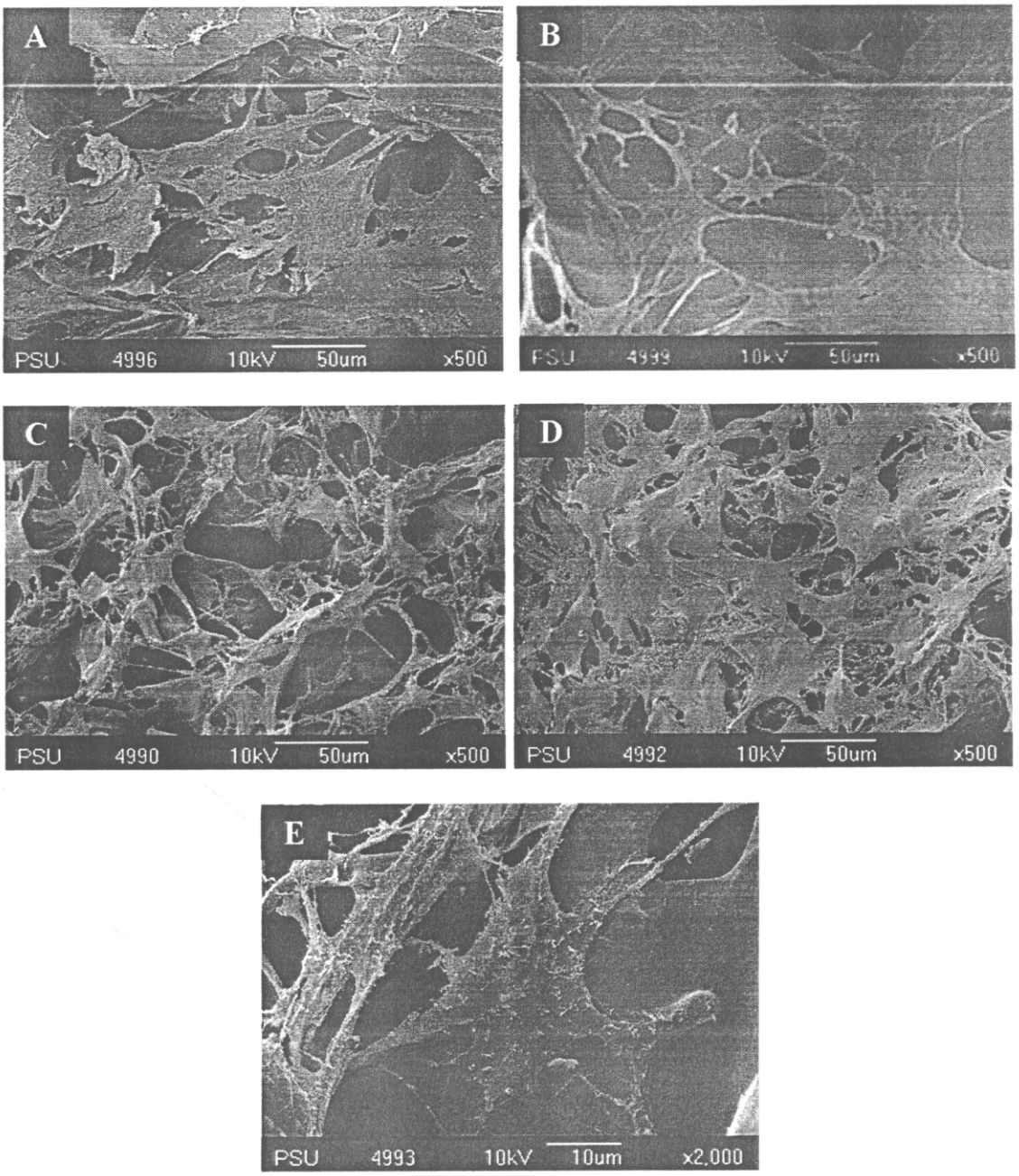

Fig. 7. SEM images show morphology, attachment and growth of BMSCs on the sponges on culture-day 15. (A) Group I: Collagen. (B) Group II: Chitosan. (C) Group III: 1:1 Chitosan-collagen. (D) Group IV: 1:2 Chitosan-collagen sponges. (E) Group III: 1:1 chitosan-collagen sponges at a higher magnification.

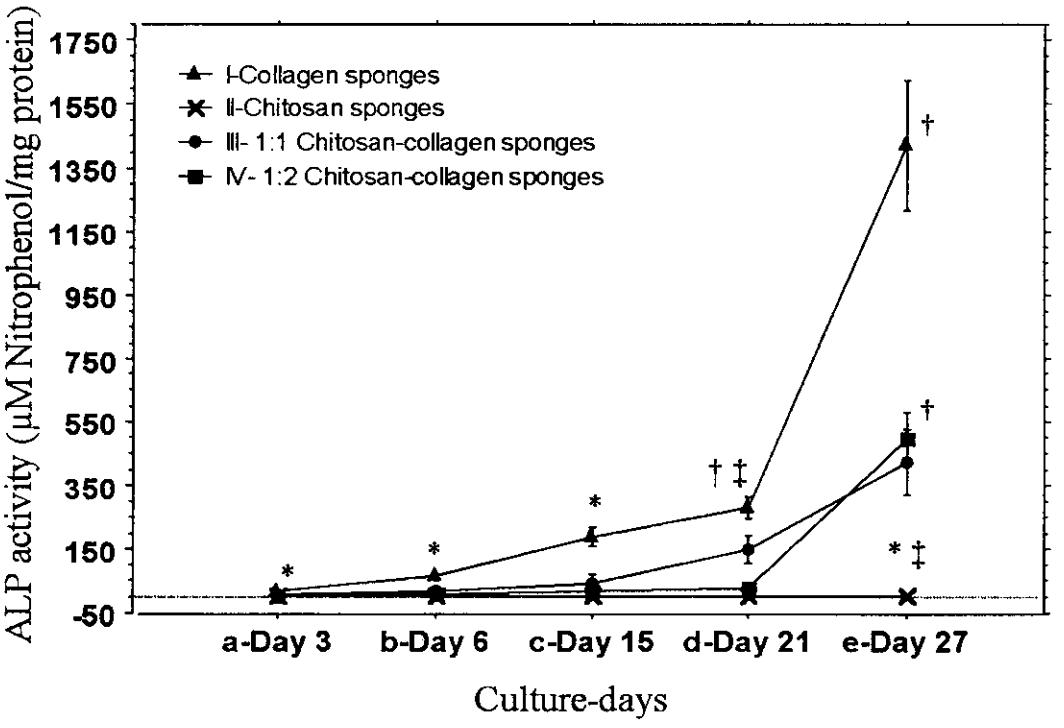

Fig. 8. Alkaline phosphatase (ALP) activity of BMSCs cultured for 27 days on the sponges. $^{*}P<0.05$; significant against Groups II, III and IV. $^{\dagger}P<0.05$; significant against Group II. $^{\ddagger}P<0.05$; significant against Group IV (mean ± SE, n = 5).

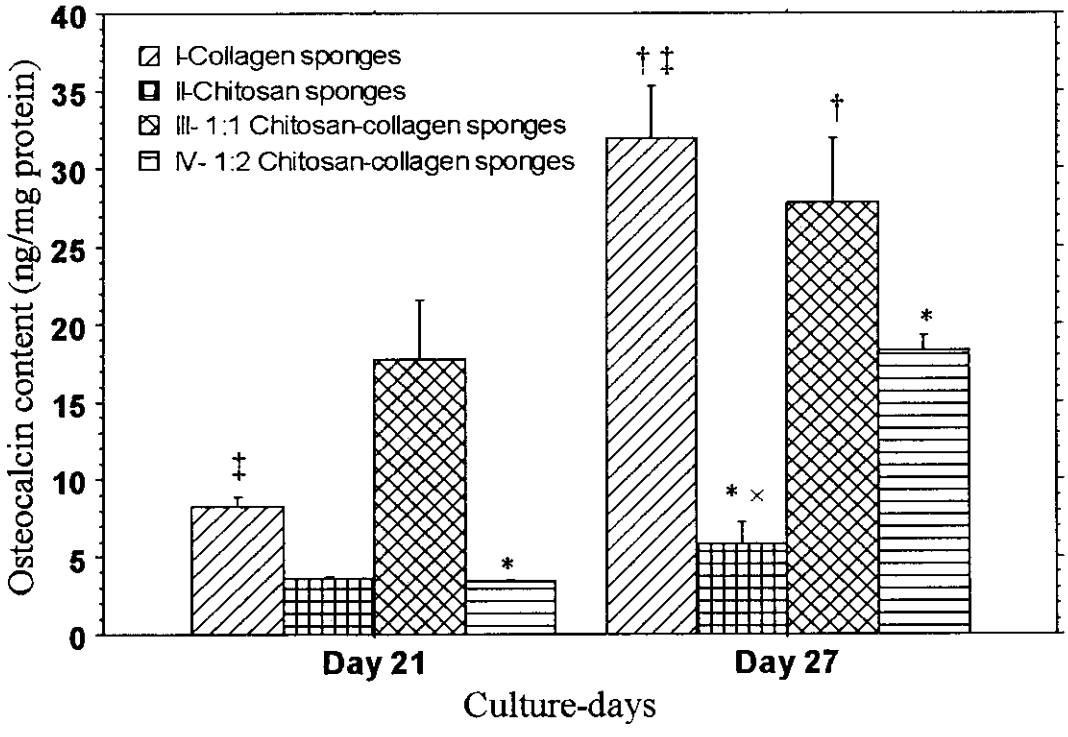

Fig. 9. Osteocalcin content of culture medium of BMSCs on the sponges on culture-days 21 and 27. $^{*}P<0.05$; significant against Groups I. $^{\dagger}P<0.05$; significant against Group II. $^{\times}P<0.05$; significant against Group III. $^{\ddagger}P<0.05$; significant against Group IV (mean ± SE, n = 3).