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In vivo biocompatibility and mechanical properties of porous zein scaffolds

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Abstract

In our previous study, a three-dimensional zein porous scaffold with a compressive Young's modulus of up to 86.6 ± 19.9 MPa and a compressive strength of up to 11.8 ± 1.7 MPa was prepared, and was suitable for culture of mesenchymal stem cells (MSCs) *in vitro*. In this study, we examined its tissue compatibility in a rabbit subcutaneous implantation model; histological analysis revealed a good tissue response and degradability. To improve its mechanical property (especially the brittleness), the scaffolds were prepared using the club-shaped mannitol as the porogen, and stearic acid or oleic acid was added. The scaffolds obtained had an interconnected tubular pore structure, 100–380 μm in pore size, and about 80% porosity. The maximum values of the compressive strength and modulus, the tensile strength and modulus, and the flexural strength and modulus were obtained at the lowest porosity, reaching 51.81 ± 8.70 and 563.8 ± 23.4 MPa; 3.91 ± 0.86 and 751.63 ± 58.85 MPa; and 17.71 ± 3.02 and 514.39 ± 19.02 MPa, respectively. Addition of 15% stearic acid or 20% oleic acid did not affect the proliferation and osteogenic differentiation of MSCs, and a successful improvement of mechanical properties, especially the brittleness of the zein scaffold could be achieved.

Keywords: Scaffold; Zein; Fatty acid; Brittleness; Mechanical properties; Subcutaneous implantation

1. Introduction

Bone tissue engineering has attracted much attention, with the hope of repairing tissue defects and regenerating new tissue [1]. The scaffold is one of the critical elements, and acts as a temporary substrate or template, providing the necessary support for the cell growth and maintenance of their differentiated functions; furthermore, its architecture defines the final shape of the new bone [2]. Thus, the success of bone tissue engineering applications depends, to a large extent, on the performance of the scaffold. Thus, in the design and manufacture of an appropriate scaffold, certain properties and factors must be taken into account. For example, in order to get three-dimensional (3-D) and highly interconnected macroporous network for cell growth and flow transport of nutrients and metabolic waste [3], scaffolds should have appropriate porosity, pore size and pore structure. Biodegradability and a suitable degradation rate are generally required to match the rate of neotissue formation. Of course, the scaffold should be also compatible with, and even stimulative towards, cell growth [4].

The mechanical property is also important for most materials used in the bone tissue engineering field, especially for natural materials such as proteins and

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polysaccharides. Although extensive efforts have been put into the development of porous scaffolds for bone regeneration with encouraging results, they still have a common limitation: the inherent lack of strength associated with porosity [5]. That is to say, they are not strong enough to meet the specific requirements of load-bearing applications.

Zein is a natural protein, and has been extensively utilized [6]. We have previously developed the zein microsphere drug delivery system of ivermectin, and prepared zein film and a 3-D zein scaffold to study its possible application in tissue engineering. Zein and its degraded product show good cell compatibility [7–9], which is the basis for further study, and their compatibility is significantly dose-dependent, owning to the existing active polypeptide structure [10]. As a 3-D porous scaffold for bone substitute, zein scaffold does not interrupt the adhesion, growth or proliferation of rat mesenchymal stem cells (MSCs), and zein porous scaffold had osteoconductive properties in the presence of dexamethasone [11]. As for the mechanical properties, the compression Young's modulus is 28.2 ± 6.7–86.6 ± 19.9 MPa and the compressive strength is 2.5 ± 1.2–11.8 ± 1.7 MPa, but, due to its relative brittleness, tensile and flexural data have not been obtained.

The aim of the present study was to examine the tissue compatibility of zein scaffolds *in vivo*, and to improve the mechanical properties of the 3-D zein porous scaffold, especially the tensile and flexural properties, by using fatty acids. In addition, the change in the mechanical properties of the scaffolds with an increase in porogen mass was analyzed. The microstructure (including pore size and interconnectivity), chemical bonding between the added fatty acid and zein, and the remaining fatty acid in the scaffold after degreasing were identified with a scanning electron microscope (SEM) and infrared spectroscopy.

2. Materials and methods

2.1. Materials

Zein of biochemical purity was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were analytically graded and used without further purification.

2.2. Animal test

The zein porous scaffolds for tissue compatibility assay were prepared according to our previous work [11]; the porosity of the obtained scaffolds was $75.2 \pm 1.3\%$. For implantation of the scaffolds (1 mm in thickness and 9 mm in diameter) in the rabbits, surgical procedures were performed on skeletally mature adult rabbits (white New Zealand rabbits, weighing 2.5–3.0 kg). Under general anesthesia, subcutaneous pockets were made to the right and left of three midline incisions of 5 mm on the back and parallel to the spine, and the cylinder scaffolds were implanted under sterile conditions at a distance of about 2.5 cm. A total of 90 scaffolds were implanted in 15 rabbits (45 for zein scaffolds on the right and 45 for fibrin scaffolds on the left), and all of the rabbits were monitored for signs of infection by diet, action and the condition of the healed would. At each of 7, 28, 91, 183 and 242 days post-implantation, three animals were euthanized and the scaffold area, including surrounding tissues, was retrieved.

After retrieval, implants with surrounding tissue were fixed in a 10% formalin solution (pH = 7.4), dehydrated in a graded series of ethanol (from 70% to 100% (v/v)), then hematoxylin and eosin (H&E) staining was used to analyze the degradation of scaffolds, inflammation and angiogenesis. Data analysis was performed using Image-Pro Discover 4.5 software (Mediacybernetics, USA).

2.3. Three-dimensional scaffolds fabrication

Samples were grouped and named according to the composition, including zein porous scaffold (ZPS), zein porous scaffold adding stearic acid (ZPSS) or oleic acid (ZPSO), and the scaffolds of DZPSS and DZPSO obtained from ZPSS and ZPSO by a degreasing process. The mannitol was recrystallized, and the club-shaped mannitol crystal was screened out using a sieve with a size of 224–355 μm.

ZPS control was prepared by packing a mixture of zein and mannitol into a stainless steel cylindrical-shaped mould (radius = 5 mm). The blocks were placed at 40 °C for 3 weeks. The samples were leached against water and lyophilized for 12 h, then stored in a desiccator for future use. ZPSS and ZPSO were fabricated using the same method as described above, and the concentration of stearic acid or oleic acid ranged from 15% to 50%. ZPSO or ZPSS was further degreased with hexane for 36 h, which yielded DZPSO and DZPSS.

2.4. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was performed to characterize the scaffolds before and after adding fatty acids. The dried sample was whetted carefully and mixed with KBr, and pressed into a pellet directly for FTIR determination (Bio-Rad FTS-185, USA). The FTIR spectra were recorded with 16 scans at 4 cm^{-1} resolution from 4000 to 400 cm^{-1}.

2.5. Porosity and water uptake [12]

Porosity was measured according to the apparent densities method described in our previous work [11]. In brief, the porosities of the scaffolds were calculated from the apparent densities of the scaffolds and the density of the solid polymeric material. The open porosity, P_a, was

$$P_a = (m_3 - m_1)/(m_3 - m_2) \times 100\%. \quad (1)$$

The total porosity, P_t, was obtained by

$$P_t = (D_t - D_b)/D_t, \quad (2)$$

$$D_b = m_1 D_1/(m_3 - m_2). \quad (3)$$

The swelling ratio of the scaffold and the water content in the scaffold were calculated as follows:

$$\text{water uptake}\,(\%) = [(m_3 - m_1)/m_1] \times 100, \quad (4)$$

where m_1, m_2, and m_3 are the mass of dry samples, apparent mass of saturated samples and mass of wet samples, respectively. D_b is the volume density of the samples, D_t is the density of zein (1.22 g/cm^3), and D_1 is the density of the test liquid at testing temperature.

2.6. Scanning electron microscopy (SEM)

SEM (S-450, Hitachi, Japan) was used to observe the surface and the inner microstructure of the scaffolds and the configuration of their inner pores. Before observation, one specimen from each group was split and coated with a gold layer using a sputter coater.

2.7. Mechanical properties assessment

Four groups of scaffolds (diameter = 10 mm and height = 20–40 mm, $n > 5$) were used for compressive and tensile tests. At the same time, a three-point bending test was also adopted, while the sample ratio of

length to diameter was 5:1 (diameter = 10 mm and height = 50–60 mm, $n > 5$).

2.7.1. Compressive properties

Compressive tests were carried out using a speed-controlled Electronic Universal Material Testing Machine (Zwick Z020 KN, Germany) in surroundings with same temperature and humidity. The minimum possible recorded force was 1 N.

2.7.2. Tensile properties

Tensile tests were carried out using a crosshead speed of 1.0 mm/min; maximum stress was determined as the stress values corresponding to 20% strain if, at this step, the samples were not damaged.

2.7.3. Flexural properties

The bending test was performed using the three-point bending method [13]. The crosshead speed of the material testing machine was 1.0 mm/min. The effective length in the bending test was fixed at 30 mm, and the definition of maximum stress corresponds to that for the tensile test.

2.8. Cell culture on three-dimensional zein porous scaffolds

Each scaffold from four groups (ZPS, ZPSS, ZPSO and DZPSS) with 10 mm in diameter and 2 mm in thickness was sterilized by an ethylene oxide sterilization method. The sterilized scaffolds were immersed into LG-DMEM complete medium (containing 20% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin) for 12 h before cell seeding.

2.8.1. Cell proliferation

The isolation and expansion of mesenchymal stem cells (MSCs) from rat bone were performed according to our previous work [11]. MSCs at passage 2 were seeded at 2.4×10^4 cell/ml onto the top of zein porous scaffold, placed in a 48-well polystyrene plate, and incubated at 37 °C. Cell culture was performed with medium changing every 3 days. The number of scaffolds used for each experimental group was three. The relative number of MSCs attached to the scaffolds was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) at an OD of 490 nm ($n = 3$); 630 nm was chosen as the reference wavelength.

2.8.2. Alkaline phosphatase (ALP) activity

ALP activity of the cells on the scaffolds was measured in triplicate cultures at preset intervals using a colorimetric method. Briefly, 375 μl of 4-nitrophenyl-phosphate reactive solution was added to each well and placed at 37 °C for 5–20 min. The absorption was determined at a wavelength of 405 nm on a microplate reader (Stat Fax 2100, USA) after stopping the reaction with 250 μl of 2 N NaOH.

2.9. Statistical analysis

The number of independent replicates is listed individually for each experiment. All data are expressed as mean ± standard deviation and analyzed by Fisher's method for multiple comparisons. Statistical significance was accepted at $p < 0.05$.

3. Results and discussion

3.1. Animal experiment

Tissue compatibility of zein porous scaffolds was assessed *in vivo* by implanting them under the skin of rabbits, and histological sections from 7 to 242 days post-implantation were observed.

A gross analysis of scaffolds was performed. Overall, the scaffolds maintained their original shape (shown in Fig. 1). The fibrous capsule formed after 7 days of implantation, and the surface of scaffolds stayed smooth over 28 days. At the 91st day post-implantation, the scraggly structure on the surface of scaffolds could be observed, meaning that the materials were being obviously degraded. At 242 days, zein scaffolds were degraded completely and only the scar could be identified.

Histological analysis via light microscopy was performed. Fig. 2 shows optical images of H&E histological staining of zein scaffolds after implantation. Both degradation and tissue response within and surrounding the scaffold could be observed. It was seen that acidophilic leukocytes and neutrophils migrated from the blood into the scaffolds at the 7th day, but the cellular reaction was mainly restricted to the periphery (Fig. $2A_1$ and A_2). Fibrocytes appeared and formed a thin capsule around the scaffold, with a thickness of about 84.97 ± 18.35 μm (Fig. 3). At the same time, the internal skeleton structure still remained integrated; no significant absorption could be observed (Fig. 4A). At the 28th day, fibrosis was present; a fibrotic layer of 220.67 ± 122.18 μm thickness could be observed around the scaffold (Fig. 3). Hemangiectasia occurred, which was caused by the operation; a large number of leukocytes gathered between the fibrotic layer and the scaffold (Fig. $2A_1$ and A_2). With the degradation of the scaffold, more cells migrated into the scaffolds, accelerating this degradation (Fig. 4B). At the 91st day, the degree of fibrosis began to decrease to 130.48 ± 55.05 μm (Fig. 3), the volume of the implants significant decreased, and fibrous tissue appeared in the scaffold (Fig. $2A_1$ and A_2). With the increase of cellular infiltrations, the rate of scaffold degradation was accelerated, which resulted in collapsed scaffold structures. Many small blood vessels could be observed, predominantly at the periphery of the scaffold, although some new growing vascular structures appeared within deeper parts of the scaffolds (Fig. 4C). At the 183rd day, only fragments were present, and the scaffolds were massively degraded; more blood vessels obviously occurred in the scaffold (Fig. 4D). Moreover, the fibrotic layer decreased to 58.48 ± 16.3 μm (Fig. 3) and the inflammatory corpuscle was reduced. At the 242nd day, the scaffolds were degraded completely and only laminar and ordered collagen at the implant site remained.

The scaffold is an important factor to prompt the development of bone tissue engineering. The objective of the current study was to assess the degradation and tissue response of zein porous scaffolds. The *in vivo* environment is very complex, and the degradation of the implant was affected by many factors (e.g. implant size and location, health of the animal, and enzymatic and local cellular activity) that could all influence the rate of degradation of a given scaffold. Generally speaking, the migration of cells was affected by the air exchange and nutritional supply after implantation. The existence of pores greatly increases

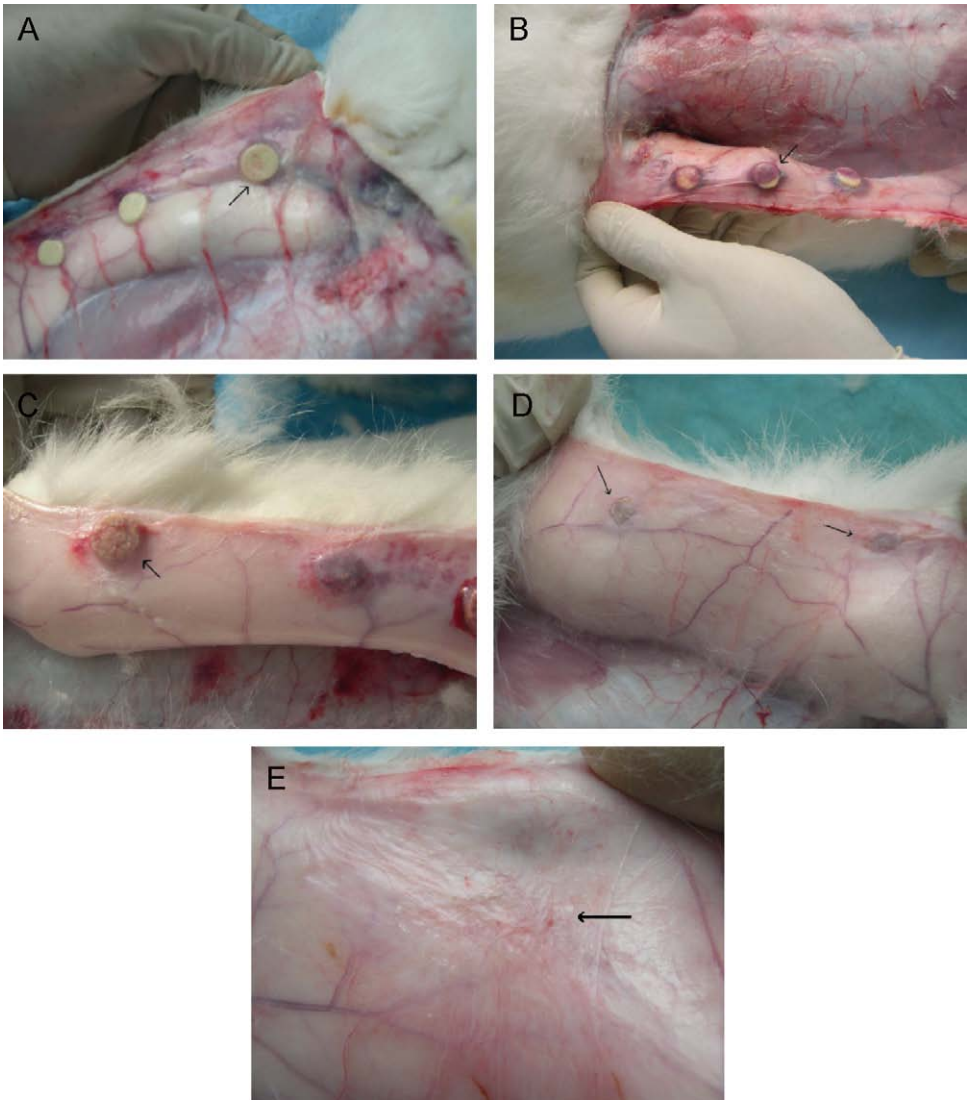

Fig. 1. Gross analysis of scaffolds after subcutaneous implantation in rabbits for (A) 7 days; (B) 28 days; (C) 91 days; (D) 183 days; (E) 242 days.

the surface area of materials, which is favorable for the growth of cells. It is relatively easy for blood vessels and tissue to grow into the scaffold directly. Cells migrated into zein porous scaffolds at an early stage, as the results show in Figs. 2 and 4, because of the adaptable pores, which was the basis for the degradation of the scaffold and angiogenesis. The new blood vessels and collagen tissue in the pores of the materials prove that the zein scaffold had a good compatibility at 91 days post-implantation.

3.2. Fabrication and physical properties of 3-D scaffolds

Besides the compatibility, the 3-D construction and mechanical properties are also key factors for bone tissue engineering substitutes. Therefore, on the basis of the results of animal experiments, we prepared zein porous scaffolds using a porogen leaching method, which had been further improved by choosing club-shaped mannitol as the porogen. Until now, several methods have been developed to prepare porous 3-D biodegradable scaffolds, including phase separation, gas foaming, fiber extrusion and bonding, emulsion freeze-drying, three-dimensional printing, and so on [14,15]. Compared to the other methods, the porogen leaching method provides easy control of the pore structure, and the process is reproducible [16].

Fig. 5 shows SEM images of the inner structure of zein scaffolds ZPS and ZPSO with different mass fractions of porogens and with/without OA addition. After the leaching and lyophilization processes, mannitol was removed from the scaffolds, and pores in shape of micro-tubes were formed. The ratio and the connectivity degree of pores in the scaffold obviously increased with the increase in porogen content, and SEM revealed that the scaffold with 70% porogen had a highly interconnected macroporous network structure.

Ideal scaffolds for bone tissue engineering should have appropriate porosity, pore size and pore structure, to ensure a biological environment conducive to cell

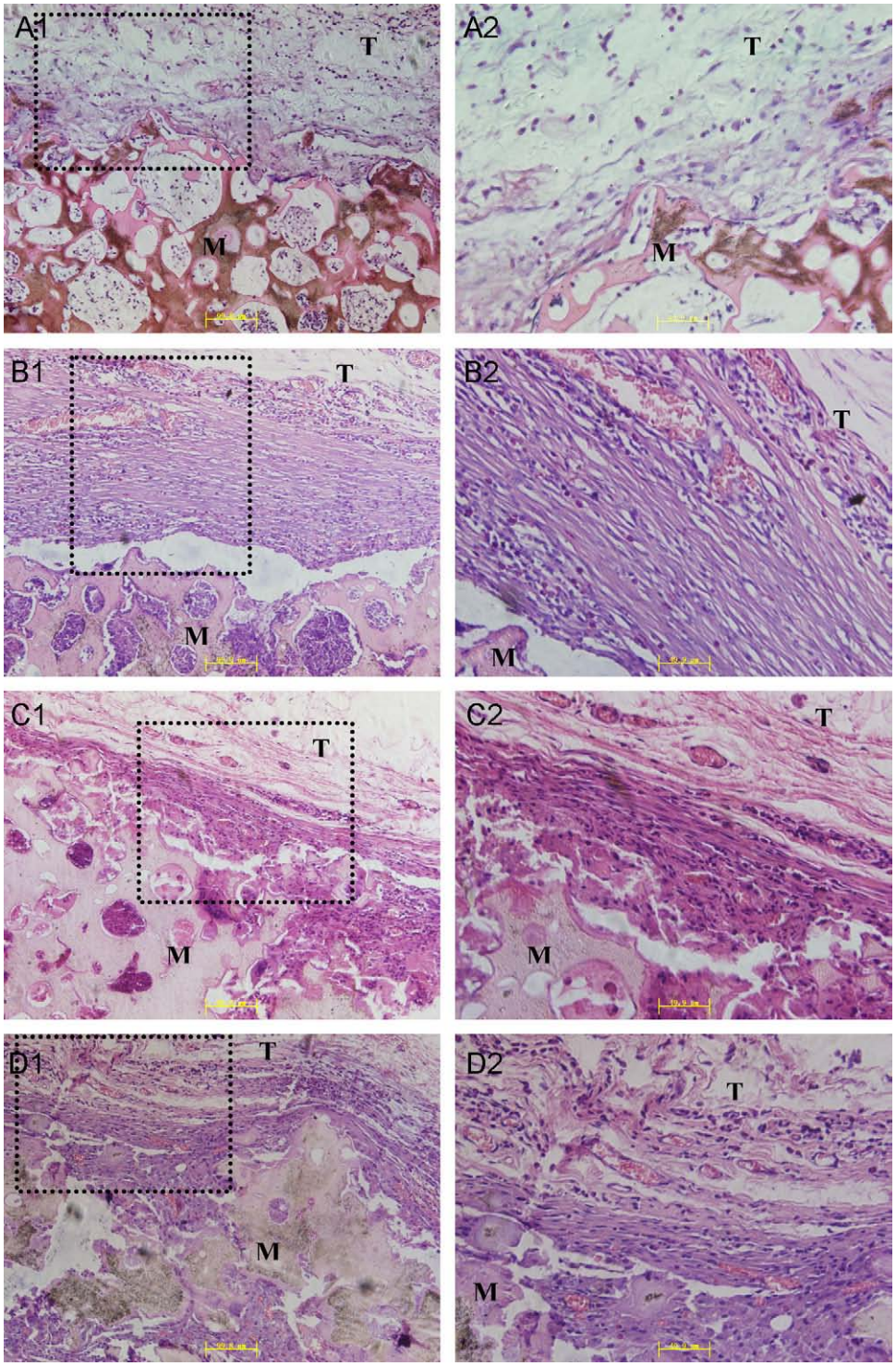

Fig. 2. Images of hematoxylin–eosin stained sections of specimens in the subcutaneous site after the implantation for (A) 7 days; (B) 28 days; (C) 91 days; (D) 183 days. "M" means material and "T" means tissue. Bars in the left and right columns represent 99.8 and 49.9 μm, respectively.

attachment, proliferation and flow transport of nutrients and metabolic waste [17–19]. The pore size should be large enough to support cell migration and bone ingrowth against being covered by the cells, forming pore bridging and occlusion; the optimal pore size required for bone ingrowth has been suggested to be from 100 to 300 μm by some researchers. The club-shaped mannitol was filtrated through mesh screens with 224–355 μm pore size. As shown in Fig. 5, pore sizes of the scaffolds ranged from 100 to 380 μm, and there were some pores relatively smaller than the size of porogen that might have resulted from the leaching process. Furthermore, the pores were

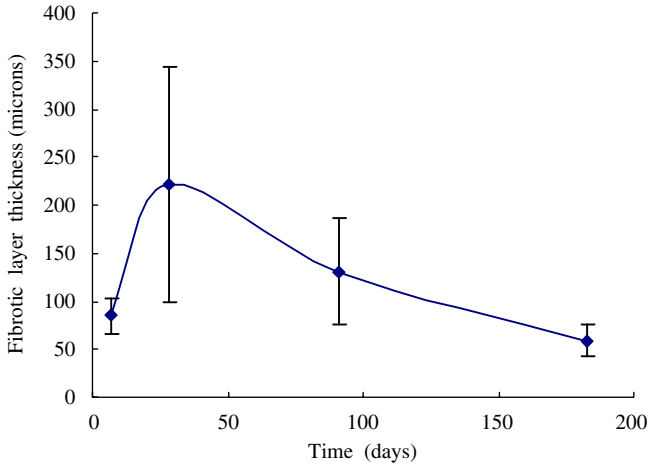

Fig. 3. The thickness change of the fibrotic layer around the scaffold following subcutaneous implantation analyzed by using Image-Pro Discover 4.5 software.

interconnected by a number of smaller pores with a size of 30–60 μm. The plasticizer of both SA and OA had no significant effect on the pore size of the scaffolds.

The salt leaching method has been used in the preparation of porous scaffolds of other natural polymers, such as silk fibroin [20]; sodium chloride was the most common porogen. Our previous study indicated that the mass fraction of porogen (at the same particle size) had significant influence on the porosities of scaffolds, but the particle size of porogen (at the same mass fraction) had no significant effects on the porosities of scaffolds [11]. We guess that the water absorptability of sodium chloride made the adjustment of pore size difficult for zein scaffold preparation, and its physical shape determines that the macropores cannot interconnect well under low concentrations of this particle. Thus, the present study indicated that mannitol is superior to sodium chloride.

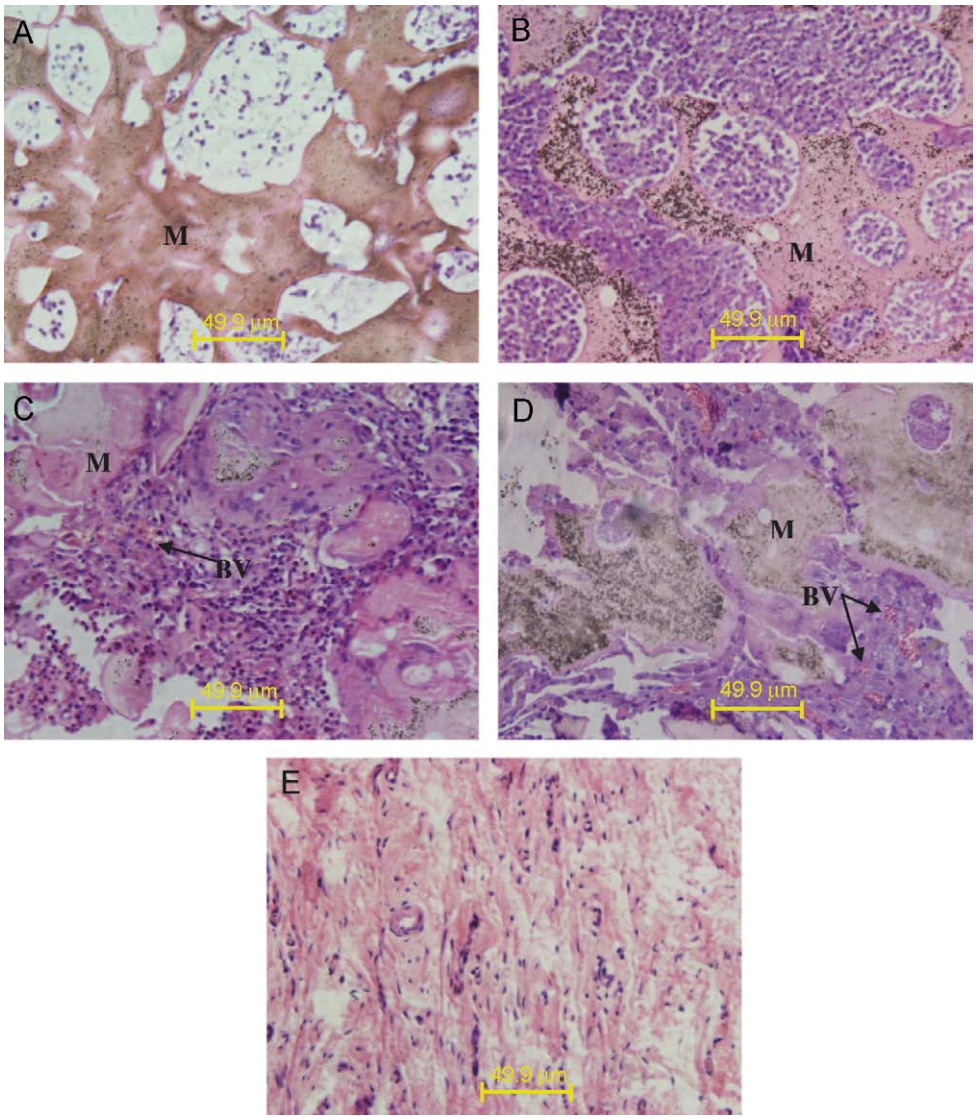

Fig. 4. Images of hematoxylin–eosin stained sections of specimens in the subcutaneous site after implantation for (A) 7 days; (B) 28 days; (C) 91 days; (D) 183 days; (E) 242 days. "M" means material and "BV" means blood vessels. Bar = 49.9 μm.

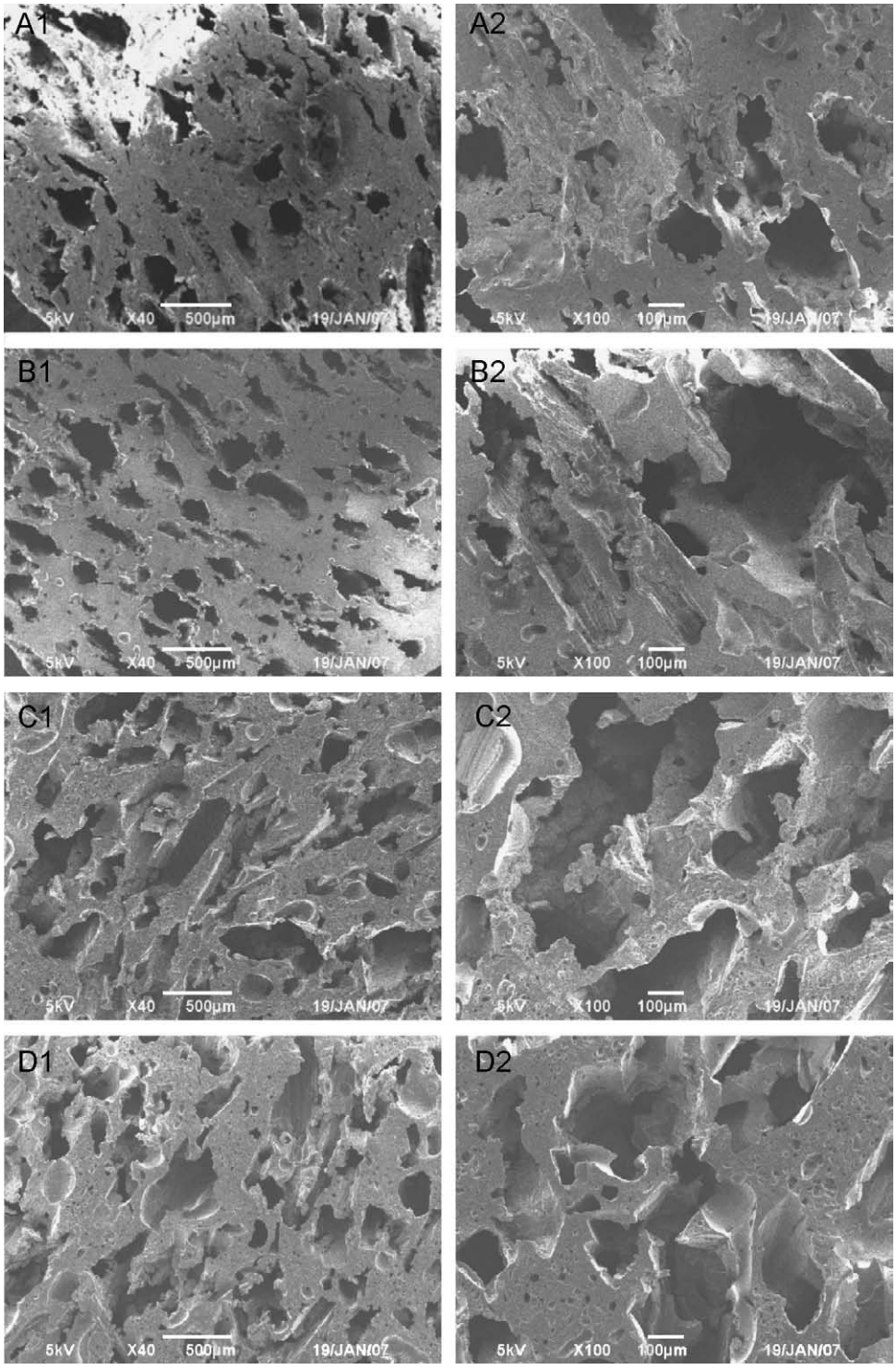

Fig. 5. Scanning electron micrographs of the microstructure of ZPS at a mass fraction of 30% (A), 50% (B), 70% (C), and ZPSO at a mass fraction of 70% (D).

Table 1 showed the porosity change of the scaffolds as a function of the mass fraction of mannitol. The porosity was determined by the apparent densities method. Increasing the mass of mannitol from 30% to 70% caused the porosity to gradually increase. This result is consistent with the previous finding that scaffold porosity can be controlled by the amount of incorporated porogen [21].

Porosity is defined as the percentage of void space in a solid; it is necessary for bone tissue formation because it allows migration and proliferation of osteoblasts and mesenchymal cells, as well as vascularization [22].

Table 1
Open porosity and percent of water uptake of 3-D biodegradable zein porous scaffolds with different mannitol mass fraction ranging from 30% to 70% (w/w) determined by the apparent densities method

Mass fraction of mannitol (%)	30	40	50	60	70
Open porosity (%)					
ZPS	50.5±3.3	56.9±2.9	65.3±2.4	73.7±3.1	79.1±1.6
ZPSS	47.1±2.6	56.4±4.4	68.9±3.1	69.1±3.9	77.8±0.6
DZPSS	50.4±0.7	60.1±4.4	66.2±4.6	67.1±1.4	77.7±1.3
ZPSO	34.1±2.1	53.7±3.4	56.3±2.6	73.6±2.7	73.4±0.8
Water uptake (%)					
ZPS	87.6±14.9	114.7±13.5	172.6±16.1	241.8±32.1	309.4±21.7
ZPSS	75.8±9.0	112.3±20.6	190.0±33.7	189.5±36.8	297.1±15.0
DZPSS	83.2±2.6	130.0±27.7	177.1±41.9	179.4±6.2	305.0±25.8
ZPSO	45.0±4.2	129.9±39.1	121.0±16.5	264.3±29.9	263.2±10.0

The porosity of ZPSO at lower porogen content (⩽50%) was much smaller than other groups, and no significant difference among the four groups was observed at higher porogen content (>50%). Porosities of 65.3%±2.4%, 56.3%±2.6%, 68.9%±3.1%, and 66.2%±4.6% were found for ZPS, ZPSO, ZPSS, DZPSS, respectively, and were considered to be suitable for a scaffold (porogen mass of 50%).

The ability of the scaffold to adsorb water is one of the important factors that affects the penetration of nutrients into the scaffold and the exudation of the products of metabolism. The results in Table 1 give the water uptake ratios of four groups of scaffolds. The prepared 3-D biodegradable scaffolds based on zein showed significant water uptake ability; the values were over 100%, and the highest value reached 309.4±21.7%. Generally speaking, the water uptake ratio would be weakened with a decrease in the porosity. ZPSO had a relatively lower water uptake ratio than those of the other groups, while the water uptake ratio still reached from 121.0±16.5 to 263.2±10.0 with a change in the porosity from 56.3±2.6 to 73.4±0.8. Shi et al. reported a collagen/chitosan porous scaffold with a suitable water uptake ratio of about 84.8% [23]. According to the report of Lawton, the type of plasticizer had a great effect on the water absorption of the zein film; the water uptake of the zein film plasticized with OA was the worst compared with other plasticizers such as glycerol, levulinic acid, triethylene glycol, and so on [24]. This result also was observed in the present study. Addition of another fatty acid, SA, in the scaffold also lowered the amount of water uptake of the scaffold.

3.3. Mechanical properties

Our previous study using sodium chloride, with a mass fraction of 1.4, as the porogen showed the highest Young's modulus of 87 MPa and the highest compressive strength of 12 MPa, but we failed to get the flexural and tensile properties due to the brittleness of the zein scaffold [11]. Stearic acid (SA) and oleic acid (OA) have been chosen as plasticizers in order to resolve this problem and to enhance the flexibility of the zein film [24,25]. In the present study, zein porous scaffolds with different concentrations of fatty acid ranging from 20% to 50% (w/w) were prepared. Fig. 6 clearly shows that the strength (both compression and bending) of OA-added zein scaffold (ZPSO) reached a maximum value at the 30% point (mass ratio of plasticizer), and then decreased following the increase of OA content after this point. However, addition of fatty acids implied a possible cytotoxic effect and reduction in biocompatibility, although it has been shown that OA had rather weak effect on cell death and gene expression (Jurkat cells, Raji cells or human lymphocytes) than other fatty acids such as arachidonic acid and linoleic acid [26]. In any case, a rather lower OA addition should be preferred. As a result, the compressive strength and modulus and flexural strength and modulus still retained significant values of 10 and 160 MPa, 7.5 and 125 MPa, respectively, at low level of OA content of 20%. Therefore, 20% OA becomes a suitable content, and was selected for performing the following study.

Fig. 7 shows the mechanical properties for four different scaffolds: ZPS, ZPSO, ZPSS and DZPSS. The mass fraction of porogen (mannitol) changed from 30% to 70%, where the concentration of SA and OA were fixed as 15% and 20%, respectively. The compressive strength (Fig. 7A$_1$) shows high values for ZPSO compared to ZPS at low porogen content (⩽50%), and the maximum value of ZPSO reached 51.81±8.79 MPa, which was 1.8 times that of ZPS. Following an increase in the porogen concentration to over 50%, the difference between ZPSO and ZPS disappeared. Compressive strength for ZPSS varied from 19.95±1.36 MPa to 4.52±1.27 MPa, which shows lower values compared with those of ZPS. The degreasing process had no influence on compressive strength for ZPSS. The compressive moduli for ZPS, ZPSO, ZPSS and DZPSS are shown in Fig. 7A$_2$. In the figure, ZPSS and DZPSS show lower values compared with the others, but have nearly the same values as each other. Moreover, addition of 20% oleic acid made the compressive modulus of ZPSO not significantly different than that of with ZPS, although

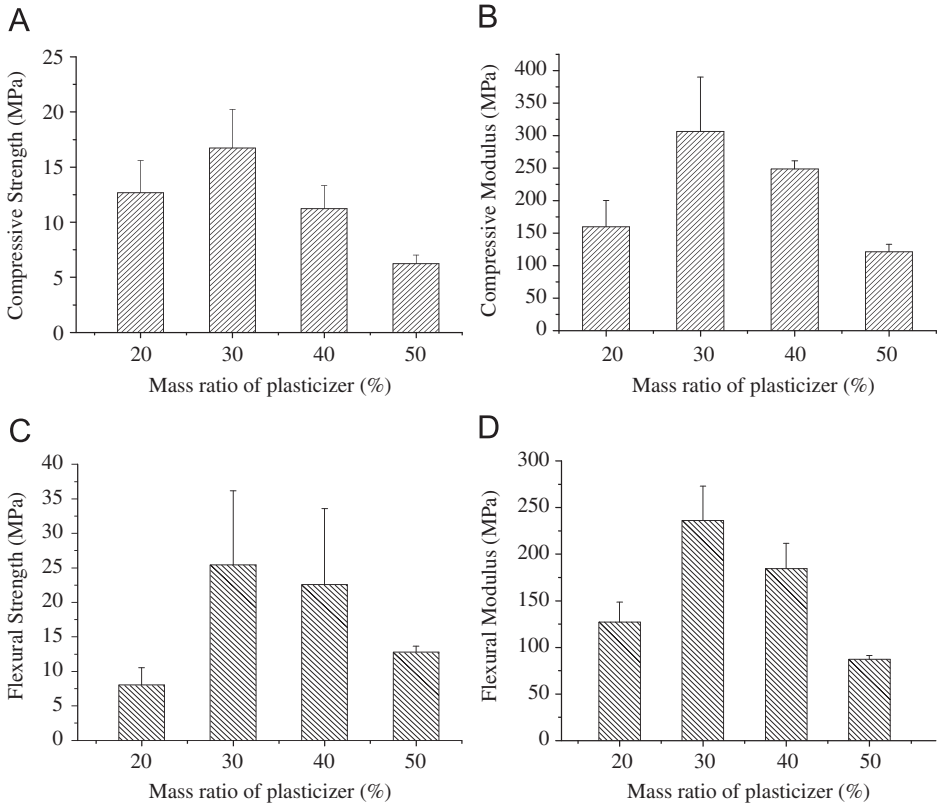

Fig. 6. Effects of oleic acid (ZPSO) content ranging from 20% to 50% on the mechanical properties, including compressive strength (A) and modulus (B), flexural strength (C) and modulus (D) of scaffolds.

it was significantly higher than those of ZPSS and DZPSS.

As templates to guide tissue regeneration, scaffolds also need to have an elastic modulus in order to retain the shape with which they were designed [27]. Because of the brittleness of ZPS, experiments for tensile and flexural strength could not be performed in the range of porogen concentration from 30% to 70%. In other words, it is clear that the brittleness was significantly improved, allowing performance of the tensile and flexural tests for ZPSS, DZPSS and ZPSO in this study. Fig. $7B_1$ indicates that the tensile strength for ZPSS ranged from 2.53 ± 0.41 MPa to 0.16 ± 0.03 MPa, which was lower than ZPSO ($n > 5$). The degreasing process had no influence on the tensile strength of ZPSS. For the tensile modulus shown in Fig. $7B_2$, three groups of scaffolds show similar tendencies as those of the tensile strength in Fig. $7B_1$. ZPSO shows a higher tensile modulus than other groups when the porogen concentration was lower than 50%, and the difference disappeared thereafter. The modulus (Fig. $7B_2$) of ZPSO remained high and varied from 751.6 ± 58.8 to 195.6 ± 46.3 MPa, and no difference could be observed between ZPSS and DZPSS when the porogen content was more than 40%.

The flexural strength of the scaffolds in Fig. 7C shows nearly the same tendency as that obtained from tensile strength in Fig. 7B. The values of flexural strength for ZPSO in Fig. $7C_1$ are twice that of ZPSS or DZPSS, and the maximum value reached 17.71 ± 3.02 MPa. ZPSO had a maximum value of 514.4 ± 19.0 MPa of flexural modulus, which was also the highest one among all. It should be noted that the mechanical properties of ZPSS were not affected by the degreasing process. This process was performed to reduce the possible cytotoxic effect of fatty acids to as low a level as possible, and the reflux with hexane for over 36 h could remove free fatty acids in scaffold thoroughly (data not shown).

Mechanical properties are those that involve a relationship between stress and strain or provide a reaction to an applied physical force. The three-dimensional scaffolds derived from the biomaterials for bone tissue engineering require suitable mechanical properties (a compressive modulus of 10–2000 MPa for trabecular bone) [28]. Therefore, the scaffold could maintain structural support for cellular proliferation and bone matrix secretion to leave space for new bone tissue growth during scaffold degradation. Because of their low mechanical strength, natural polymers are limited in their use, although they have the advantage of biocompatibility and biodegradability. Nazarov fabricated 3-D silk-based porous scaffolds; the mechanical stress was 250–280 KPa and the compressive modulus was 790–1000 KPa [29]. In our previous work, we also obtained porous scaffolds using zein as the matrix material and sodium chloride as the porogen. The tensile strength and modulus, and the flexural strength and

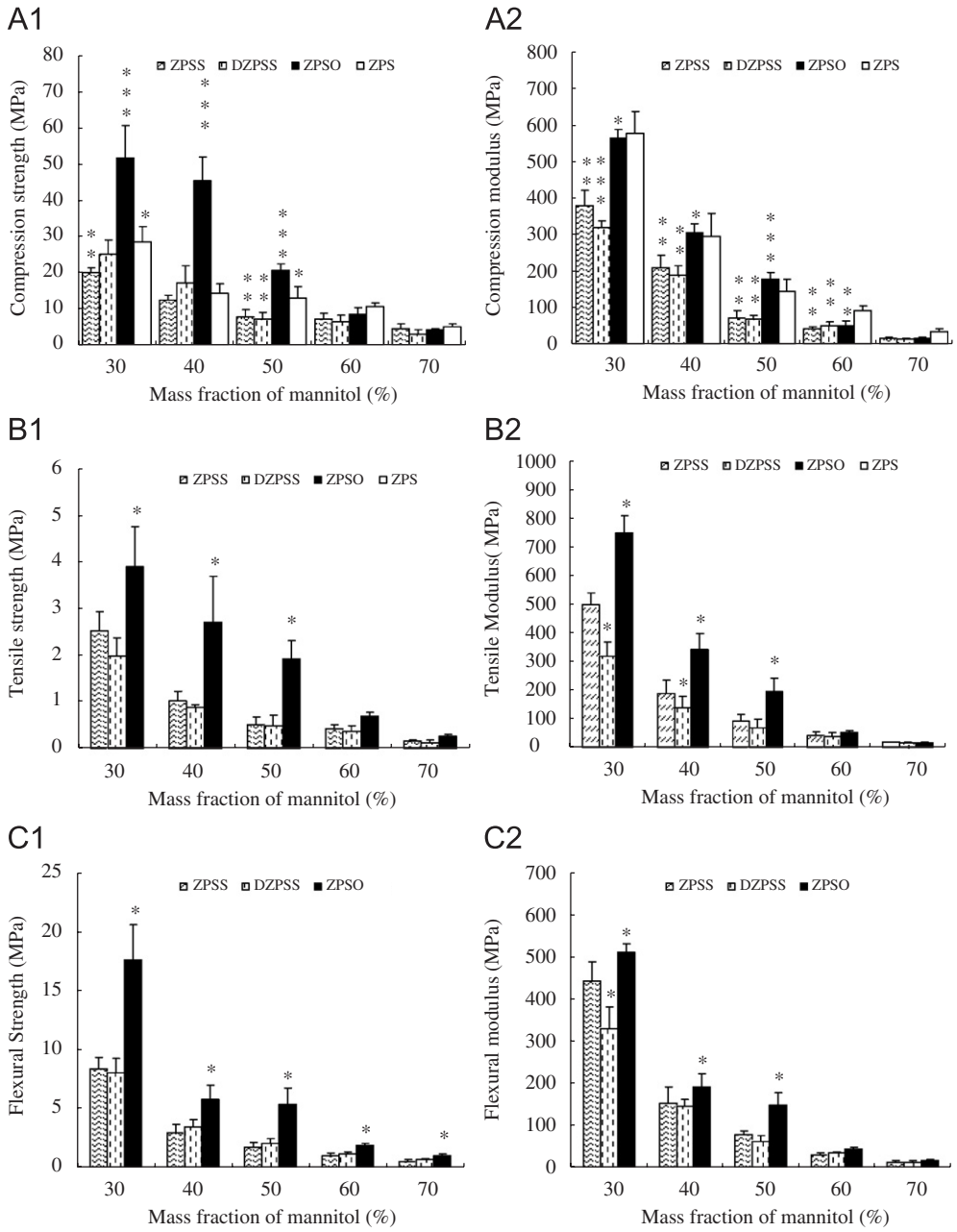

Fig. 7. Effects of adding 15% stearic acid (ZPSS) during the fabrication, and after further degreasing process (DZPSS), or 20% oleic acid (ZPSO) to the scaffolds on the mechanical properties of zein porous scaffold (ZPS) under the conditions of different ratios of mannitol to zein (w/w), including compression strength (A_1) and modulus (A_2), tensile strength (B_1) and modulus (B_2), flexural strength (C_1) and modulus (C_2). *Means significant difference compared with ZPSS; **means significant difference compared with ZPS; ***means significant difference compared with both.

modulus could not be measured as it was so brittle, although the compression properties were sufficient.

Fatty acids have been widely used as plasticizers, and the type and amount of fatty acids will affect the mechanical properties of the materials. According to the report of Budi Santona et al., the tensile properties and tractility of zein film could be enhanced by adding fatty acids [30]. The plasticization of zein with OA involved dispersion of rod-shaped zein molecules in aqueous alcohol and adsorption of fatty acids to hydrophilic regions of the zein surface [31]. To elucidate the possible interaction of fatty acid with the zein protein, FTIR spectra of the mixed powder of the scaffolds and KBr pellet in the presence of OA (ZPSO), stearic acid (ZPSS) and degreasing treatment of ZPSS (DZPSS) were performed after lyophilization. As shown in Fig. 8, the bands at 3600–3100 cm^{-1}, 1750–1600 cm^{-1}, and 1500–1400 cm^{-1} are clearly separated and can be assigned to the bands of amide A, amide I and amide II, which are the typical protein absorption bands [32]. These bands were also observed in the FTIR spectra of ZPSS, DZPSS and ZPSO. In particular, the DZPSS spectrum was very similar to the ZPS spectrum. However, the presence of CH and

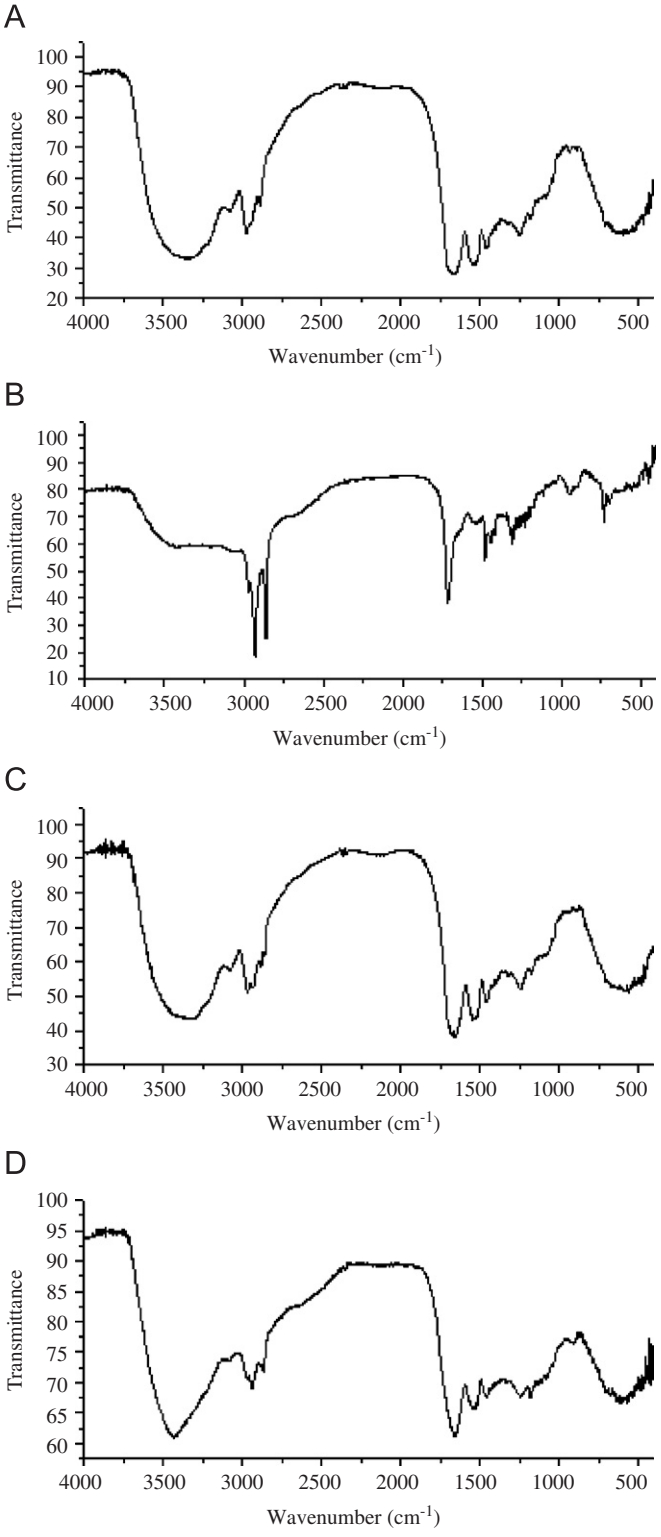

Fig. 8. FTIR spectra of 3-D biodegradable zein porous scaffolds in the presence of stearic acid (ZPSS) or oleic acid (ZPSO), and in the absence of stearic acid with (DZPSS) or without (ZPS) degreasing process. The mass fraction of porogen was 50%. (A) ZPS; (B) ZPSS; (C) DZPSS; (D) ZPSO.

carbonyl groups of the free fatty acids resulted in the differences in the intensity of their signals from 2950 to 2850 cm^{-1} and a shoulder at 1750 cm^{-1} in the ZPSS, ZPSO and DZPSS spectra, compared to that of ZPS reported by Forato et al. [32]. The peak between 3500 and 3200 cm^{-1} disappeared in the ZPSS spectrum, which was attributed to the formation of an electrovalent bond between the carboxyl of stearic acid and the free amino group of zein. This was proved by the reappearance of the peak after the degreasing process. As for the ZPSO spectrum, we considered that the peak at 3417 cm^{-1} was from CH = CH. Wang et al. proved by surface plasmon resonance that zein has a higher affinity for polar vs. non-polar surfaces [33], and Forato et al. also gave evidence about the polar interaction between C1 of oleic acid and the protein by ^{13}C NMR [34].

3.4. Cytotoxic test of porous scaffolds

As described above, the addition of fatty acid could improve the mechanical properties of zein porous scaffold, while these changes might cause cytotoxic effects and reduce biocompatibility [26]. MSCs are multipotent cells that can replicate as undifferentiated cells with the potential to differentiate and produce mesenchymal tissue [35]. MSC-based bone tissue engineering has been studied as a therapy for bone defects, and MSCs must differentiate into osteoblasts for the formation of bone tissue [36].

Therefore, we seeded MSCs onto the scaffolds and examined the cellular response to the scaffolds by adding 15% SA (ZPSS) or 20% OA (ZPSO); ZPS and DZPSS were chosen as the controls. Fig. 9A shows MTT values of MSCs seeded on zein porous scaffolds analyzed spectrophotometrically at 490 nm.There was no significant difference among the four groups of scaffolds within 4 days of culture, and after 8 days of culture, MSCs showed greater proliferation activity on the scaffolds with added stearic acid than the ZPS control ($n = 3$, $p < 0.05$). The presence of oleic acid in the scaffold slowed the cellular proliferation to some extent, while no significant difference could be observed ($n = 3$, $p > 0.05$).

In addition, dexamethasone has been shown to direct osteoblastic differentiation of bone MSCs *in vitro* [37]. Thus, the cell-seed scaffolds were also cultured in osteogenic media by adding dexamethasone, vitamin C and β-glycerolphosphate. As for the MTT analysis, the cellular proliferation on the scaffolds with added stearic acid was enhanced up to 1.89 times that of the ZPS control after 8 days, and 1.44 times after 12 days of culture (Fig. 9B). At the same time, in each group the cells showed significant osteoblastic differentiation activity according to the results of ALP activity analysis, which is a transient early marker after the appearance of onset genes (e.g., *fos-jun*) and type I collagen, and followed later by a series of bone-specific proteins (e.g., osteopontin and osteocalcin) during the osteoblastic differentiation of MSCs; it is also a good indicator of differentiation [38]. The slight change of ALP analysis values of MSCs not treated with osteoblastic media indicated a slow osteoblastic differentiation. ALP activity was significantly reduced by the addition of oleic

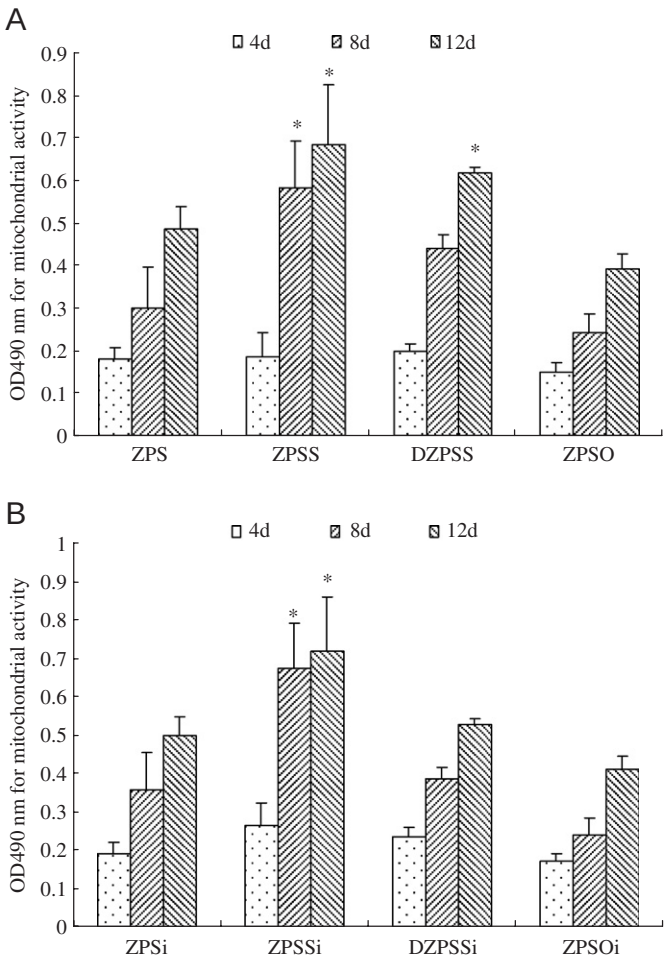

Fig. 9. MTT analysis of proliferation of MSCs (A) and induced MSCs (B) seeded on porous zein scaffolds analyzed spectrophotometrically at 490 nm. *Means significant difference compared with the control ($p<0.05$, $n=3$).

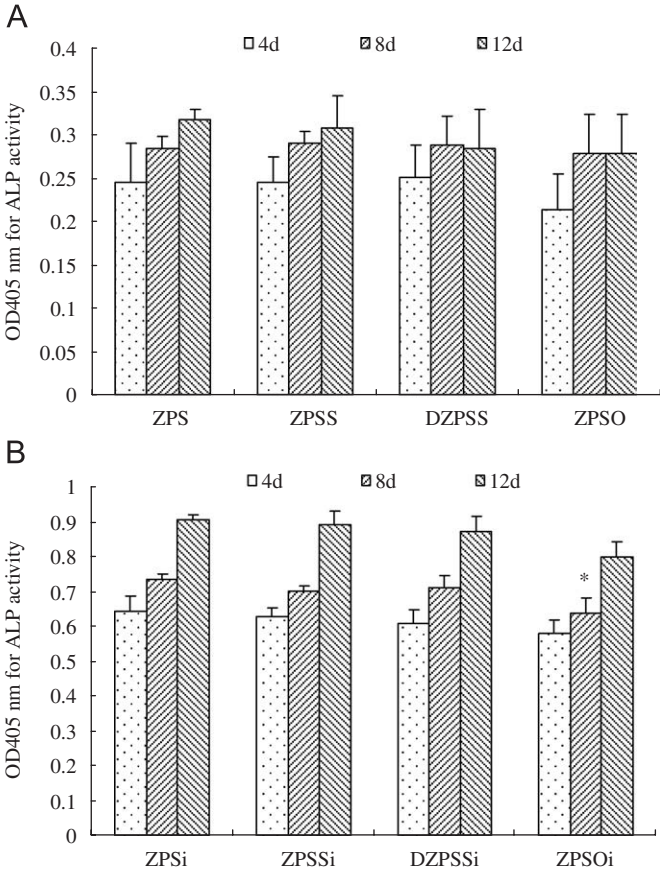

Fig. 10. The absorption at 405 nm for relative activities of alkaline phosphatase of MSCs (A) and induced MSCs (B) during 12 days of culture on porous scaffolds. *Means significant difference compared with the control ($p<0.05$, $n=3$).

acid ($n=3$, $p<0.05$), and 0.88 times that of ZPS control after 8 days of culture (Fig. 10).

4. Conclusions

On the basis of our observations, we concluded that zein porous scaffolds had good tissue compatibility, blood vessels could form in the scaffold, and the degradation could be completed within 8 months. Mechanical properties of the scaffolds were improved significantly by adding fatty acids. In particular, oleic acid addition (ZPSO) achieved a more than 1.5 times higher average tensile modulus value, 2.2 times higher average tensile strength value, 1.4 times higher average flexural modulus value and 2.1 times higher average flexural strength value than those achieved with stearic acid addition. The suitable pore size (100–380 μm), porosity (>60%) and interconnection of pores could also be obtained by modulating the size of club-shaped mannitol and its ratio to zein. Moreover, the degreasing process had no significant effect on the mechanical properties. Cytotoxic testing indicated that the MSCs could adhere, proliferate, and differentiate on all the porous scaffolds, and no signs of toxicity could be detected.

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