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Growth and osteogenic differentiation of adipose-derived and bone marrow-derived stem cells on chitosan and chitooligosaccharide films

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ABSTRACT

Very low molecular weight chitooligosaccharide (COS, 1.4 kDa) and high molecular weight chitosan (1000 kDa) were comparatively studied in terms of physical and biological characteristics. Thin films of COS, chitosan and gelatin were prepared and crosslinked by dehydrothermal treatment at 140 °C for 24 h. COS film presented more hydrophilic property than chitosan film. Behaviors of rat adipose-derived stem cells (ASCs) and bone marrow-derived stem cells (MSCs) were investigated on COS and chitosan films, comparing to those on gelatin film. The results on cell spreading suggested that both ASCs and MSCs preferred to attach on COS film than chitosan film with 6–7 times larger cell areas. Numbers of both stem cells proliferated on COS film were approximately 3-fold higher than those on chitosan film. In addition, COS film enhanced osteogenic differentiating potential of MSCs, as observed from the alkaline phosphatase activity and calcium deposition. Therefore, in this work, COS was shown to be a more favorable material for the growth and osteogenic differentiation of both ASCs and MSCs, compared to high molecular weight chitosan.

Detailed report on the effects of blended materials on MSCs were under publishing in Journal of Biomaterials 29 Science, Polymer Edition (2009)

1. Introduction

Bone tissue engineering has been developed for the treatment of bone defect using osteoconductive materials, osteogenic cells and osteoinductive molecules (Mistry & Mikos, 2005). Osteoconductive material provides mechanical support, sites for cell attachment and vascular ingrowth, and serves as a delivery vehicle for implanted growth factors and cells. Proteins, such as collagen and gelatin, are widely used materials due to their appropriate biological characteristics. Gelatin is the denatured product of collagen in which its structure consists of amino acids mainly glycine, proline and hydroxyproline. It is biocompatible, biodegradable, non-immunogenic and non-antigenic. Gelatin molecule also contains Arg–Gly–Asp (RGD)-like sequence that promotes cell adhesion and migration (Takahashi, Yamamoto, & Tabata, 2005). Recently, polysaccharides like chitosan and its derivative have become of increasing interest in the field of tissue engineering (Lee et al., 2002; Muzzarelli, 2009; Seol et al., 2004). Chitosan, the deacetylated derivative of chitin, is an amino polysaccharide consisting of β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-2-amino-2-deoxy-D-glucose units. It has attractive biological properties such as antimicrobial, antitumor, and haemostatic (Liu, Du, Yang, & Zhu, 2004). In addition, chitosan is reported to accelerate wound healing and enhance bone formation (Joon et al., 2005). Depending on source and preparation process, the molecular weight of chitosan can be varied in a wide range. Our previous study has shown that low molecular weight chitosan was more effective to promote the proliferation of mouse fibroblasts than high molecular weight chitosan (Tangsadthakun et al., 2007). Chitooligosaccharide (COS) is the low molecular weight water-soluble chitosan that showed better biocompatibility and solubility than chitosan (Tian, Liu, Hu, & Zhao, 2003) while preserves chitosan characteristics like anti-diabetic (Lee, Park, Choi, Yi, & Shin, 2003), antimicrobial (Tsai, Wu, & Su, 2000), and antitumor activities (HarishPrashanth & Tharanathan, 2005).

To explore the potentials of materials for tissue engineering application, mesenchymal stem cells are widely employed since they exhibit proliferation, self-renewal and multipotent differentiation capacities. They are able to differentiate into various cell types of mesodermal origin, such as osteoblasts, chondrocytes, adipocytes and muscle cells. Mesenchymal stem cells have been initially identified in bone marrow as non-hematopoietic stem cells, called bone marrow-derived stem cells (MSCs). The osteogenic differentiation potential of MSCs was described many years ago

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(Maniatopoulos et al., 1988). However, MSCs are rare in bone marrow (1 cell/10^5 nucleated cells) and their quantity decreases with age (Pittenger et al., 1999). Also, difficulty of cell isolation limits their uses. Adipose tissue, which is often removed from plastic surgery, has become another source of mesenchymal stem cells. Adipose tissue has several advantages over bone marrow, such as minimal morbidity upon harvest. High number of adipose-derived stem cells (ASCs) can be extracted from adipose tissue isolates, potentially eliminating the need for *in vitro* expansion. ASCs show higher proliferation rates than MSCs while perform potential of multiple differentiation similar to MSCs (Zhang, Gai, & Liu, 2008). Several works have showed the effective multipotential differentiation of ASCs (Strem et al., 2005; Wu et al., 2007). Comparing on multipotential differentiation between ASCs and MSCs, there are many controversial reports. Mauney et al. (2007) studied *in vitro* and *in vivo* adipogenic differentiation utilizing human ASCs and MSCs cultured on silk fibroin scaffolds. On such system, the difference of adipogenesis between hMSCs and hASCs could not be noticed. Noël et al. (2008) also reported that both stem cells underwent adipogenic differentiation with a similar extent when cultured on tissue culture plates under adipogenic differentiation. However, some studies found that MSCs tended to have higher potentials of osteogenic and chondrogenic differentiation while ASCs preferred to differentiate into adipocytes (Im, Shin, & Lee, 2005; Liu et al., 2007). Nevertheless, those previous reports did not take into account the effects of material on the differentiation potentials of both stem cells. Base on our best knowledge, we are here the first to investigate and compare the biological characteristics of low molecular weight chitooligosaccharide and high molecular weight chitosan using two types of stem cells, ASCs and MSCs. In addition to both polysaccharides, gelatin is employed in this study as a control material.

2. Materials and methods

2.1. Materials

Squid pen chitin and chitosan were obtained from Taming. Enterprises Co., Thailand. Gelatin with an isoelectric point of 9.0 was purchased from Nitta Gelatin Co., Osaka, Japan. Other chemicals used were analytical and cell culture tested grades.

2.2. Preparation of chitooligosaccharide and chitosan films

Both chitosan and chitooligosaccharide were prepared from squid pen chitin. High molecular weight chitosan was obtained from deacetylation process with concentrated NaOH (50% w/w) at room temperature for 4 days. Chitooligosaccharide (COS) was produced from chitosan (MW 500–800 kDa) by free radical degradation process using hydrogen peroxide (H_2O_2) as an oxidizing agent (Chang, Tai, & Cheng, 2001). Briefly, Chitosan in acetic acid solution is hydrolyzed by H_2O_2 at 50 °C. The solution is then dialyzed with cellulose dialysis membrane (MWCO 500 Da) and freeze dried to obtain COS. Molecular weight (MW) of COS and chitosan were characterized by Gel Permeation Chromatography (GPC 110, Shropshire SY6 6AX, UK) using pullulans (MW 5800–1,660,000 Da) as the standard samples. Deacetylation degree (%DD) of COS and chitosan were analyzed by Fourier Transform Infrared Spectroscopy (FT-IR, Perkin-Elmer Spectrum GX, USA). COS, chitosan and gelatin solutions (0.05% w/v in 1% v/v acetic acid) were cast on glass slips (15 mm in diameter) to prepare thin films. To avoid the solubility of COS and gelatin, all films were crosslinked by dehydrothermal treatment at 140 °C for 24 h in a vacuum oven (VD23, Binder, Germany).

2.3. Measurement of water contact angle

Water contact angle of COS, chitosan and gelatin films was evaluated using sessile drop method. The water contact angle was measured using video contact analyzer (Data Physics, OCA 15 Plus, Germany) and imaged using SCA 20 software. Deionized water was dropped onto the film using a gastight Hamilton precision syringe. Images were captured at 60 s after dropping. The baseline and the tangent were drawn using software and the contact angles were measured from three different points.

2.4. X-ray photoelectron spectroscopy

To obtain chemical information of surface, angle resolved X-ray photoelectron spectroscopy (XPS) analysis has been carried out on the films. The XPS spectra have been obtained using a PHI spectrometer equipped with an Mg/Al dual mode source and a small area analyzer with PSD detector. An achromatic X-ray (1253.6 eV) source was operated at 300 W. The vacuum pressure was 10^{-8} Torr during spectra acquisition. The N 1s, O 1s and C 1s regions have been recorded. Ratios of N/C and N/O were quantified from the percentage of atomic concentration.

2.5. Isolation and cultivation of rat adipose-derived and bone marrow-derived stem cells

The animal experiment was performed according to Chulalongkorn University Animal Care and Use Committee (CU-ACUC) and with ethics approval from the research ethical committee, Faculty of Medicine, Chulalongkorn University. Adipose-derived stem cells (ASCs) were isolated from subcutaneous adipose tissue of 3-week-old female Wistar rats by enzymatic digestion as described by Hong et al. (2007). After scarifying, adipose tissue was removed from subcutaneous skin and digested with 0.075% collagenase under agitation at 37 °C for 30 min. After complete digestion, adipose tissue was filtered through 200 μm Nylon mesh and the filtrate was then centrifuged to remove mature adipocytes. ASCs were collected and cultured in 199 medium (10% FBS, 100 U/ml penicillin/streptomycin) at 37 °C, 5% CO_2. The medium was changed on the 4th day after isolation and every 3 days thereafter. When the cells become subconfluent, the cells were trypsinized using 0.25% trypsin–EDTA. The cells of the second- and third-passages at subconfluence were used for all experiments.

Bone marrow-derived stem cells (MSCs) were isolated from the bone shaft of femurs of same rats according to the technique reported by Hosseinkhani, Hosseinkhani, Tian, Kobayashi, and Tabata (2006). After scarifying, both ends of rat femurs were cut away from epiphysis. Bone marrow was flushed out with 1 ml of medium. Then, the cell suspension was cultured in proliferating medium (α-MEM, 15% FBS, 100 U/ml penicillin/streptomycin) at 37 °C, 5% CO_2. Other details were the same as those of ASCs.

2.6. Adipogenic and osteogenic linages of rat adipose-derived and bone marrow-derived stem cells

To verify the differentiating potential of isolated ASCs and MSCs, cells were seeded on 6-well tissue culture plates (3×10^4 cells/cm^2) and induced to differentiate to adipocytes and osteoblasts by cultured in differentiating medium. To differentiate to adipocytes, both stem cells were cultured in adipogenic medium (ITT medium: DME/Ham's F12 medium containing 0.05 μM insulin, 0.2 nM 3,5,3′-triiodothyronine, 100 nM transferring, 17 μM calcium pantotenate, 33 μM biotin and 100 nM dexamethasone). Adipogenic differentiating markers including glycerol-3-phosphate dehydrogenase (GPDH) activities and lipid droplets formation of both stem cells after 4-week culture were assessed.

Table 1
Characteristics of COS and chitosan.

Sample	MW (kDa)	DD (%)	Water contact angle (°)	Atomic ratio	
				N/O	N/C
COS	1.4	80	81.97 ± 0.78[a]	0.128	0.069
Chitosan	1000	82	92.50 ± 0.44[a]	0.120	0.055

[a] Represented significant difference between COS and chitosan at $p < .05$.

For osteogenic differentiation, cells were cultured in osteogenic medium (α-MEM, 10% FBS, 10 mM β-glycerol phosphate, 50 μg/ml L-ascorbic acid and 10 nM dexamethasone). After 4-week culture, osteogenic differentiating markers including alkaline phosphatase (ALP) activity and calcium deposition were determined by *p*-nitrophenyl phosphate and von Kossa staining methods, respectively.

2.7. Cell immunostaining and spreading observation

ASCs and MSCs were seeded onto sterilized COS, chitosan and gelatin films (2×10^4 cells/film) and cultured in proliferating medium (α-MEM, 15% FBS, 100 U/ml penicillin/streptomycin) at 37 °C, 5% CO_2. On the 1st day after seeding, photographs of 100 cells attached on each film were taken at 10× magnification using phase-contrast microscope (IX70, Olympus Optical Co., Japan). Cell spreading area was quantified using Metamorph program (Universal Imaging Systems, Molecular Devices Inc., Canada). In addition, filamentous actin fiber of spreading cells was observed by immunostaining method (Inoue, Imamura, Umezawa, & Tabata, 2008). Briefly, attached cells were rinsed twice with PBS and fixed in 10% formaldehyde solution at 4 °C for 20 min. After washing with PBS, cells were treated with 2% bovine serum albumin in PBS at room temperature for 20 min. Next, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated phalloidin (10 μg/ml, Sigma–Aldrich Co., USA) at room temperature for 1 h. Following PBS washing, cells were mounted in FluorSave Reagent (Calbiochem Inc., USA) and viewed using a confocal fluorescence microscope (Fluoview FV300, Olympus Optical Co., Japan).

2.8. Attachment, proliferation and osteogenic differentiation of rat adipose-derived and bone marrow-derived stem cells on the films

ASCs and MSCs were seeded onto the films (2×10^4 cells/film) and cultured in proliferating medium (α-MEM, 15% FBS, 100 U/ml penicillin/streptomycin) at 37 °C, 5% CO_2. The numbers of cells attached on the 1st day and cells proliferated on the 3rd and 5th days after seeding were quantified by MTT assay (Mosmann, 1983). To assess osteogenic differentiation of ASCs and MSCs cultured on the films, cells were seeded on the films (2×10^4 cells/cm^2) and cultured in proliferating medium at 37 °C, 5% CO_2. One day after seeding, the medium was changed into osteogenic medium and changed every 2 days thereafter. After 7 days cultured in osteogenic medium, ALP activity and calcium deposition were analyzed as described above.

2.9. Statistical analysis

All statistical calculations were performed using MINITAB (Statistical software, version 13.2, PA, USA). The differences of data analyzed using 2 sample *t*-test were considered at $p < .05$ and $p < .01$.

3. Results and discussion

3.1. Characteristics of chitooligosaccharide and chitosan

Characteristics of COS and chitosan were first characterized and reported in Table 1. It was demonstrated that COS and chitosan had a great difference in molecular weight (1.4 and 1000 kDa, respectively) while the deacetylation degrees (DD) were similar. Water contact angle of the dehydrothermal crosslinked COS film (81.97°) was lower than that of crosslinked chitosan film (92.50°), representing more hydrophilic surface of COS film. This was due to the very short molecular chains of low molecular weight COS that allow water to easily penetrate and interact with. However, atomic ratios of N/O and N/C which represented the presence of NH_2 and OH on COS and chitosan film surfaces did not show any significant differences.

3.2. Adipogenic and osteogenic linages of rat adipose-derived and bone marrow-derived stem cells

The results on adipogenic differentiation showed that MSCs formed larger aggregates of lipid droplets than ASCs, as demonstrated in Fig. 1. Our results on GPDH activities of MSCs also corre-

Table 2
Spreading area of ASCs and MSCs attached on COS, chitosan and gelatin films measured on the 1st day after seeding.

Materials	Cell area (μm²)	
	ASCs	MSCs
COS	88.37 ± 17.90[a,b]	69.73 ± 15.95[a,b]
Chitosan	12.98 ± 3.20[a]	12.72 ± 3.84[a]
Gelatin	66.54 ± 13.75[b]	45.17 ± 8.66[b]

[a] Represented significant difference between COS and chitosan within same cell type at $p < .01$.
[b] Represented significant difference between ASCs and MSCs within same material at $p < .01$.

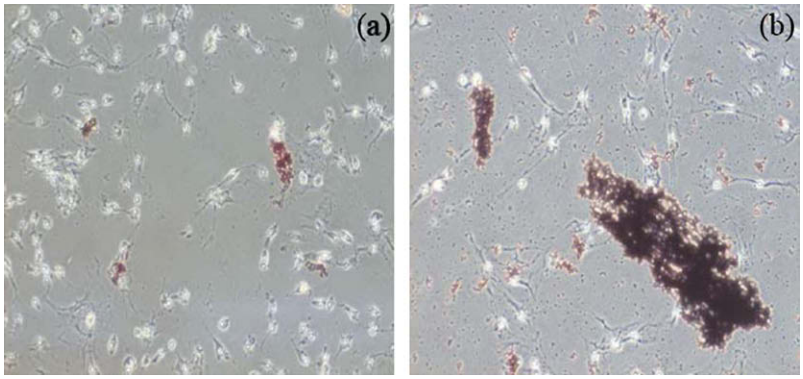

Fig. 1. Oil red O staining of (a) ASCs and (b) MSCs cultured on tissue culture plates for 4 weeks in adipogenic medium (ITT medium, seeding: 3×10^4 cells/cm^2).

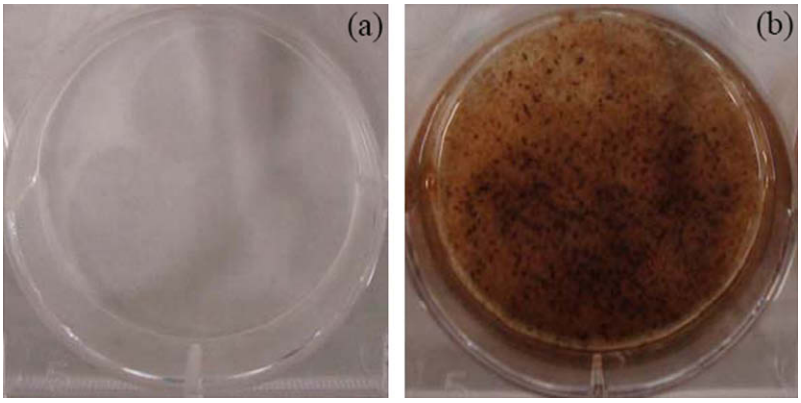

Fig. 2. Von Kossa staining of (a) ASCs and (b) MSCs cultured on tissue culture plates for 4 weeks in osteogenic medium (α-MEM supplemented with 10% FBS, 10 mM β-glycerol phosphate, 50 μg/ml L-ascorbic acid and 10 nM dexamethasone, seeding: 3×10^4 cells/cm^2).

sponded to the Oil red O staining pictures (data not shown here). From osteogenic differentiation, von Kossa staining obviously revealed that MSCs could deposit more calcium than ASCs, as seen from a dark staining in Fig. 2. From this comparative study, all results supported that mesenchymal stem cells derived from bone marrow (MSCs) of Wistar rats had a greater adipogenic and osteogenic differentiating capabilities than those derived from adipose tissue (ASCs) under differentiating conditions.

3.3. Cell spreading on chitooligosaccharide, chitosan and gelatin films

The results from cell spreading observation on the 1st day after seeding showed that ASCs and MSCs could spread well on COS film, followed by gelatin film (Table 2). In contrast, both stem cells could not spread on chitosan film. Spreading areas of cells attached on COS film were 6–7 times larger than those on chitosan film. The results were confirmed by cell immunostaining shown in Fig. 3 that filamentous actins of the cells cultured on COS film could be clearly observed, comparing to those on gelatin film. The round-shaped morphologies of both stem cells on chitosan film were observed as presented in Fig. 3(c), (f), (i) and (l). Our previous work has proved that dehydrothermal crosslinked chitosan film surface presented highly negative charged (Ratanavaraporn, Kanokpanont, Tabata, & Damrongsakkul, in press). The negatively charged surface of the film could prohibit adhesion of negatively charged cells through electrostatic repulsion (Kishida, Iwata, Tamada, & Ikada, 1991). Besides, comparing between ASCs and MSCs, it was noticed that spreading areas of ASCs on gelatin and COS films were statically larger than those of MSCs ($p < .01$).

3.4. Attachment and proliferation of rat adipose-derived and bone marrow-derived stem cells on chitooligosaccharide, chitosan and gelatin films

The results of cell number attached and proliferated on the films presented in Fig. 4 showed that both stem cells behaved sim-

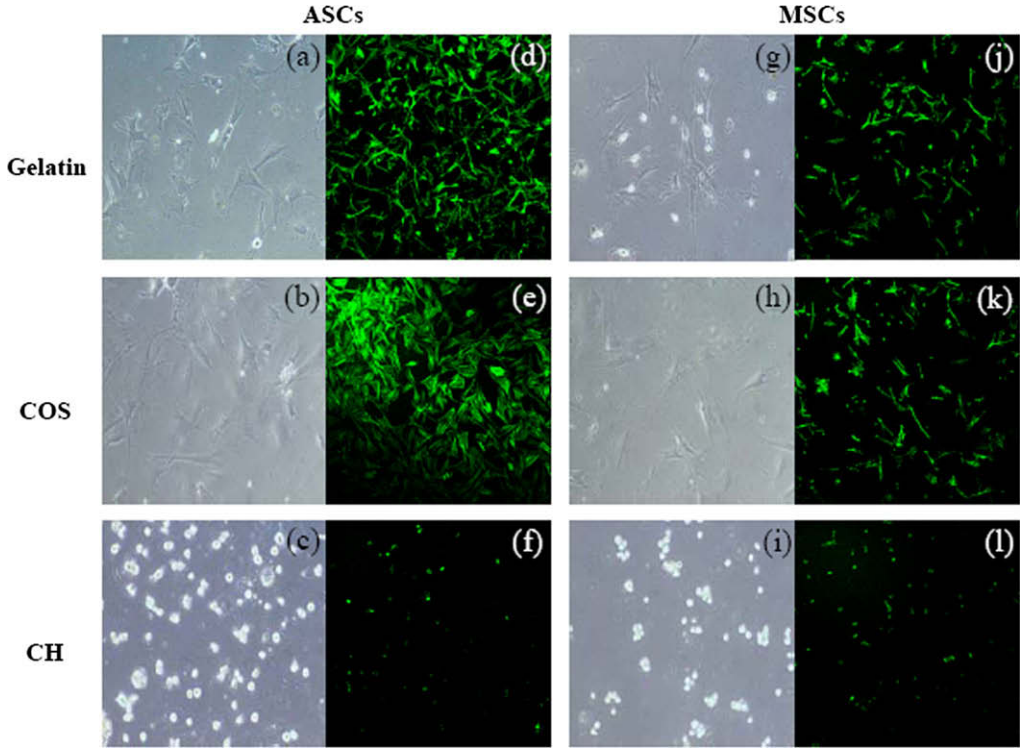

Fig. 3. Spreading morphology of ASCs and MSCs attached on gelatin (a, d, g, j), COS (b, e, h, k) and chitosan (CH) (c, f, i, l) films observed on the 1st day after seeding under a phase-contrast microscope (a–c, g–j) and a confocal fluorescence microscope using FITC-conjugated phalloidin immunostaining (d–f, j–l).

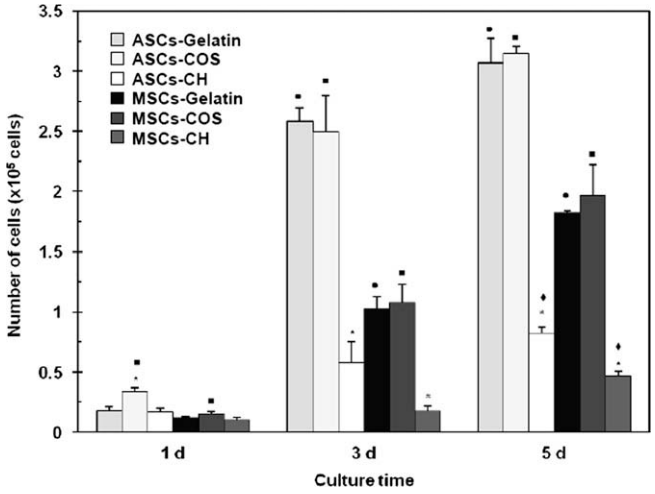

Fig. 4. Number of ASCs and MSCs attached and proliferated on gelatin, COS and chitosan (CH) films when cultured in proliferating medium (α-MEM supplemented with 15% FBS), determined by MTT assay (Seeding: 2×10^4 cells/film, * presented significant difference relative to gelatin within same cell type and culture period at $p < .05$, ●, ■, ♦ presented significant difference between ASCs and MSCs within same material and culture period at $p < .05$).

ilarly on the same material. Both stem cells could proliferate well on COS and gelatin films while very small increases of cells were observed on chitosan film. The numbers of both stem cells on COS and gelatin films were approximately 3-fold higher than those on chitosan film after 3-day culture. This trend of attachment and proliferative behavior of both stem cells on each material was correlated to the spreading behavior described previously. The results obviously indicated the less biocompatibility of high molecular weight chitosan compared to low molecular weight COS. The difference in biological activities affected by different molecular weight of chitosans corresponded to several works. Tangsadthakun et al. (2007) found that human skin fibroblasts could proliferate better on low molecular weight chitosan (180 kDa) than the high molecular weight ones (460 and 1450 kDa). Wang, Liu, Han, Yao, and Wei (2007) also reported a similar result using keratinocytes. Richardson, Kolbe, and Duncan (1999) reported that a variety of chitosans were cytotoxic and the extent was being dependent upon their molecular weight, degree of deacetylation and salt form.

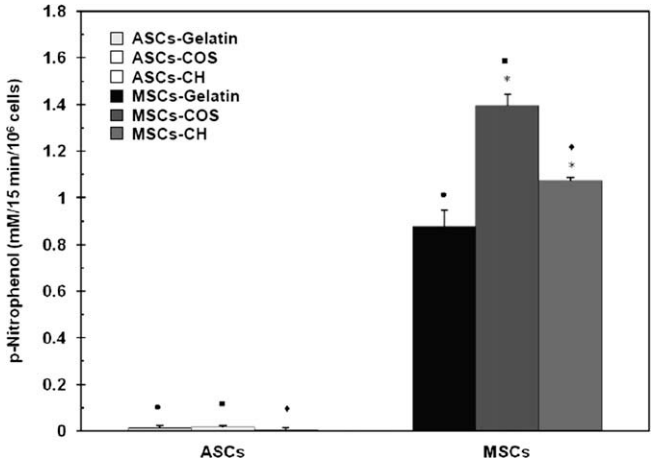

Fig. 5. ALP activity of ASCs and MSCs cultured on gelatin, COS and chitosan (CH) films for 7 days in osteogenic medium (α-MEM supplemented with 10% FBS, 10 mM β-glycerol phosphate, 50 μg/ml L-ascorbic acid and 10 nM dexamethasone), (Seeding: 2×10^4 cells/cm^2, * presented significant difference relative to gelatin within same cell type at $p < .05$, ●, ■, ♦ presented significant difference between ASCs and MSCs within same material at $p < .05$).

They showed that toxicity increased with increasing molecular weight. Subsequent studies using lower molecular weight chitosan showed that toxicity could be substantially decreased (Heller, Liu, Duncan, & Richardson, 1996). The cytotoxicity of chitosan and COS was also proved with MSCs using Annexin V/propidium iodide (PI) apoptosis test in our previous work (Ratanavaraporn et al., in press). We have confirmed that MSCs treated with high molecular weight chitosan were late apoptotic while those treated with COS remained viable. This could be explained that the toxicity of chitosan might be due to the imbalance charge between high molecular weight chitosan and cell surface.

Furthermore, molecular weight of water-soluble chitosan was also reported as a critical parameter of chitosan transport through the Caco-2 cell layer, revealed by Chae, Jang, and Nah (2005). Middle and low molecular weight (<22 kDa) water-soluble chitosan could penetrate through the Caco-2 cell layer, while high molecular weight (230 kDa) one could not. Schipper, Varum, and Artursson (1997) confirmed that chitosan with molecular weight higher than 30 kDa bound tightly to Caco-2 cell surface without cellular uptake. This could destabilize their structure and resulted in the rupture of cell membrane followed by cell death. In addition, it was shown by Porporatto, Bianco, Riera, and Correa (2003) that high molecular weight chitosan could be related to increased viscosity, reduced solubility or sterical hindrance that bring to a less efficient interaction of high molecular weight chitosan with the cells.

3.5. Osteogenic differentiation of rat adipose-derived and bone marrow-derived stem cells on chitooligosaccharide, chitosan and gelatin films

The results on osteogenic differentiation of ASCs and MSCs cultured on the films for 7 days in osteogenic medium presented that

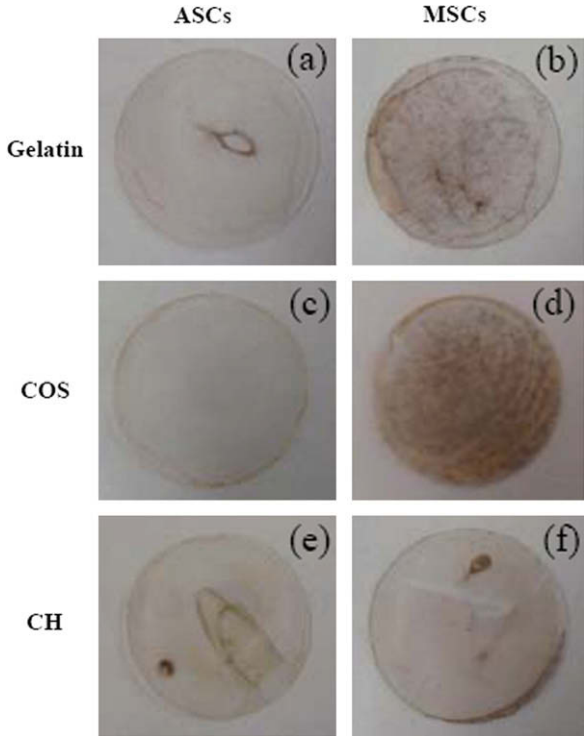

Fig. 6. Von Kossa staining of ASCs and MSCs cultured on gelatin, COS and chitosan (CH) films for 7 days in osteogenic medium (α-MEM supplemented with 10% FBS, 10 mM β-glycerol phosphate, 50 μg/ml L-ascorbic acid and 10 nM dexamethasone), (a) ASCs on gelatin, (b) MSCs on gelatin, (c) ASCs on COS, (d) MSCs on COS, (e) ASCs on CH and (f) MSCs on CH.

the ALP activities of MSCs were remarkably higher than those of ASCs in all materials (Fig. 5). Staining of calcium deposition supported the ALP results (Fig. 6). It could be concluded in this study that MSCs cultured on all films performed higher osteogenic differentiating potential than ASCs. Comparing among materials, COS films tended to promote ALP production and calcium deposition of MSCs than gelatin and chitosan films. The results endeavored osteoconductive property of very low molecular weight COS when compared to the high molecular weight chitosan and gelatin.

4. Conclusions

The study concerning on behaviors of ASCs and MSCs responded to chitooligosaccharide, chitosan and gelatin films clearly presented that COS film was the most preferable material for osteogenic differentiation of stem cells. COS film also supported the proliferation of both stem cells as good as gelatin film. In contrast, high molecular weight chitosan did not show appropriate biological characteristics with both stem cells. Additionally, we found that ASCs had larger spreading area and showed faster proliferative rate than MSCs when cultured on COS and gelatin films. However, MSCs performed remarkably higher osteogenic differentiating potential than ASCs when cultured on all materials under osteogenic induction.

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