Chondrogenic Properties of Primary Human Chondrocytes Culture in Hyaluronic Acid Treated Gelatin Scaffold

Dumnoensun Pruksakorn MD*, Nuengruethai Khamwaen MSc**,
Peraphan Pothacharoen PhD**, Olarn Arpornchayanon MD*,
Sattaya Rojanasthien MD*, Prachya Kongtawelert PhD**

** Department of Orthopedics, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
*** Thailand Excellence Centre for Tissue Engineering, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*

Objective: *To study the possibility of primary human chondrocytes culture in gelatin scaffold and the effects of exogenous HA on chondrocyte differentiation and synthesis of the hyaline-like extracellular matrix.*
Material and Method: *Cartilage tissue was engineered by using primary human chondrocytes with HA-treated gelatin scaffolds and gelatin scaffolds. The chondrogenic properties were monitored for chondrocyte proliferation, adhesion, and hyaline-like extracellular matrix production in both groups. The results were compared to each other.*
Results: *Chondrocyte proliferation, adhesive activity, and new HA production were significantly increased in HA-treated gelatin scaffold ($p < 0.05$). Immuno histochemistry for WF6 epitope demonstrated the higher quality of hyaline-like extracellular matrix production. Moreover, the scanning electron micrograph showed a higher filling of extracellular matrix in the pore of scaffold of HA-treated gelatin scaffold than that in non-HA treated scaffold.*
Conclusion: *The present study demonstrated the possible role of commercial gelatin-based scaffold in cartilage tissue engineering. It also demonstrated that exogenous HA-treated scaffold provides positive effects for chondrocytes.*

Keywords: *Cartilage, Chondrocytes, Hyaluronic acid, Tissue engineering*

J Med Assoc Thai 2009; 92 (4): 483-90
Full text. e-Journal: *http://www.mat.or.th/journal*

Articular cartilage defect has long been known as an untreatable condition because articular cartilage has limited self-repair. Autologous chondrocyte implantation (ACI), which was first introduced in 1987, has proven to be clinically effective in treating full-thickness cartilage defects[1]. This technique not only provides excellent clinical outcome but demonstrates hyaline-like cartilage at the repair site as well. However, use of this ACI technique is limited because the surgery is complex and periosteal hypertrophy occurs frequently[2]. Cartilage tissue engineering transplantation is a promising procedure that provides more advantages than ACI, including (1) less invasive surgical procedure, (2) technical attraction, (3) predictable hyaline-like cartilage production, and (4) good clinical result[3,4].

For cartilage tissue engineering technology, there are several factors affecting the quality of cartilage tissue, including scaffold, chondrocyte, and extracellular matrix molecules. In normal human hyaline cartilage, hyaluronic acid (HA), which is the major component of the extracellular matrix, plays an important role in the skeletal network. HA correlated with cell proliferation, migration, and control of the suitable surrounding cellular environment for cell growth in the reparation process[5]. Even though

Correspondence to: Kongtawelert P, Thailand Excellence Center for Tissue Engineering, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Phone: 053-894-188, 053-945-322, Fax: 053-894-188, 053-217-144, 053-893-031, E-mail: pkongtaw @mail.med.cmu.ac.th

HA-based scaffold has already been used for cartilage tissue engineering, the impact of exogenous HA on the chondrocytes is of interest for in vitro control cartilage engineering.

In the present study, the authors evaluated the effects of exogenous HA on the chondrocyte morphology and whether there is extracellular matrix production in 3-dimensional gelatin scaffold model.

Material and Method

Chondrocyte isolation, expansion and passaging

Human non-osteoarthritis articular cartilage from 18-45 year old patients who were reconstructed by ACL was harvested during notchplasty operation. All patients gave consent and all procedures of the present study were approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University.

Tissue was carefully cut away from the underlying bone and the normal chondrocytes were isolated by a sequential trypsin/collagenases digestion and then grown as monolayer in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. The explants were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere. The culture was passed every three days before confluence was reached and the media were exchanged on day 3 of the culture.

When the cells had reached confluence the media was removed and the cells were washed in 10 ml Hanks balanced salt solution (HBSS, Gibco) to remove any trace of FCS. After removal from HBSS, the cells were trypsinized with 3 ml of Trypsin-EDTA (Gibco). After examining the cells with an inverted microscope to ensure that all cells were detached and floated, 7 ml of fresh complete media was added.

The media plus trypsinized cells were divided into the appropriate number of flasks (depending on the desired splitting ratio) and the volume in each flask was increased to 10 ml with fresh complete media.

Scaffold preparation

The gelatin scaffold (Spongostan standard, Johnson & Johnson) was cut to 1 x 1 cm^2, sterilized with 70% ethanol for 15 minutes, and then washed with sterile PBS three times. HA treated gelatin was prepared by treatment of HA0.07% (v/v) overnight. The cells were harvested at confluence with 0.25% trypsin/2.5 mM EDTA. The cells were seeded and cultivated within the gelatin scaffold with and without HA0.07% presterilized with 70% (v/v) ethanol aqueous solution. A 1,500 μl cell suspension containing 3 x 10^5 cells was loaded onto an upper side of each pre-wetted scaffold and allowed to penetrate into the scaffold. Each sample was transferred to a 24-well tissue culture plate and then incubated at 37°C under 5% CO_2 condition for 4 hours. One ml of culture media was added to each well. Culture media were renewed every 3 days. At an indicated time interval, cells/scaffold constructs were collected, rinsed with PBS, and stored at -80°C until assayed. Each experiment was performed in triplicate.

Analytical methods

Cell proliferation

Cell viability and proliferation were assessed by AlamarBlue assay following the vendor's instructions. AlamarBlue is a non-toxic aqueous fluorescent dye that does not affect phenotype, viability, or proliferation of the cell. The AlamarBlue assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indication of both fluorescence and changes of color in response to chemical reduction of growth medium resulting from cell growth. The gelatin scaffolds were seeded with chondrocytes that were incubated in medium supplemented with 10% (v/v) AlamarBlue fluorescent dye for 4 h at 37°C. One hundred microliters of medium from each sample was read at absorbances of 540 and 630 nm in the Titertek Multiskan M340 multiplate reader[6].

Cell adhesion

The gelatin scaffold was cut into 1 x 1 cm^2 and then 0.5 ml of chondrocyte suspension (0.5 x 10^6 cells) was loaded directly onto the gelatin scaffold at room temperature. The cells were allowed to adhere in a humidified incubator (37°C, 5% CO_2) for 4 hours. Each scaffold was gently rinsed with 1 ml of 1M phosphate buffer saline (PBS), and the number of unattached cells in the rinsed solution was quantified by microscopic observation using a hemocytometer[7].

Histological and immunohistochemistry analysis

The gelatin scaffolds were washed in PBS, fixed in 4% paraformaldehyde at 4°C overnight, dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned at a thickness of 5 μm. For histological analysis, sections were then deparaffinized in xylene two times, rehydrated using a

graded series of ethanol (100%, 90%, 80%, 70% to 50%) and stained with Hematoxylin and Eosin (H&E) and Safranin-O for sulfated glycoaminoglycan (sGAG)[8].

Dye binding assay

The sGAG concentrations were determined using a colorimetric dye binding assay modified by Farndale et al. The assay is based on a metachromatic shift in absorption maximal from 690 nm to 535 nm as a complex compound is formed in a mixture of 1,9-dimethylmethylene blue (DMMB) and the sGAG in the sample and standard. The dye solution was made by adding 16 mg of 1,9-dimethylmethylene blue to 5 ml ethanol to 2 g of sodium formate and 2 ml of formic acid in a total volume of 1 liter at pH 3.5. The maximum absorbance of the dye solution was at 620 nm. This solution was stored at 4°C in a dark bottle. Chondroitin 6-sulfate (C-6-S) standards (0-40 mg/ml: 50 µl) or samples (50 µl) were transferred to a microtiter plate. The dye solution (200 µl) was added immediately to each well and absorbance was measured at 620 nm; a precipitate might form on standing. A standard curve of C-6-S concentration and absorbance 620 nm was plotted. The concentrations of C-6-S in the samples were calculated from the standard curve[9].

Enzyme-linked immunosorbent assay for HA

Microtiter plates (Maxisorp, Nunc) at 4°C overnight were coated with umbilical cord HA (100 µl/well) in the coating buffer. The uncoated area was then blocked with 150 µl/well of 1% (w/v) BSA in the incubating buffer for 60 minutes at 25°C. After washing, 100 µl of the mixture, either sample or standard competitor (HA Healon: range 39.06-10,000 ng/ml) in B-HABPs (1:100), was added. After incubation for 60 minutes at 25°C, plates were washed and then peroxidase-mouse monoclonal anti-biotin (100 µl/well; 1:4,000) was added and incubated for 60 minutes at 25°C. The plates were washed again and then the peroxidase substrate (100 µl/well) was added and incubated at 37°C for 20 minutes to allow the color to develop. The reaction was stopped by the addition of 50 µl of 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader[10].

Immunohistological analysis

The gelatin scaffolds were harvested following 21 days of culture, fixed in 4% paraformaldehyde overnight, washed three times with PBS dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned at a thickness of 5 µm. Sections were then deparaffinized in xylene two times, rehydrated using a graded series of ethanol (100%, 90%, 80%, 70% to 50%). Immunohistochemical analysis was examined by WF6, monoclonal antibody specific with C-6-S, which is part of aggrecan. WF6 was developed in Cell & Tissue Engineering Laboratory, Biochemistry Department of Chiang Mai University. The sections were pre-digested with chondroitinase ABC (1 unit/ml) in sodium acetate buffer pH 7.4 at 37°C, 90 minutes, and then washed with PBS three times. The sections were blocked with 3% BSA at RT for 15 minutes before incubation in 1:500 of WF6 at 37°C for 90 minutes, then washed with PBS three times. Then the sections were incubated in 1:100 of per-conjugated anti-IgM at 37°C for 90 minutes, then washed with PBS three times. After the substrate diaminobenzidine (DAB) was added in DAB buffer pH 7.4 and 35% H_2O_2, reaction was stopped with distilled water (DW) and washed again with DW. All sections were covered with cover slips and observed under a light microscope[11].

Scanning electron microscopy (SEM) examination

The specimens were washed twice by PBS and immersed in PBS three times. They were fixed in 2.5% glutaraldehyde (pH 7.4) overnight at 4°C. They were then dehydrated in increasing concentrations of ethanol (from 50%, 75%, 90% to 100%) followed by vacuum drying. Dry scaffolds were sputter-coated with gold at 40 mA prior to observing under SEM[12].

Statistical method

The percentages of cell proliferation and adhesion were calculated by the difference in reduction between treated and control cells in proliferation assays. The significance of the differences between groups of data was tested using non-parametric Wilcoxan rank-sum test. Statistical significance was considered when $p < 0.05$.

Results

The metabolic activity of chondrocyte in HA-treated scaffold showed a significant higher at the first day ($p < 0.05$); however the proliferation rate maintained in the following two weeks and increased in the fourth week in the HA-treated scaffold, whereas it decreased in the non-HA-treated scaffold ($p < 0.05$) (Fig. 1A, B). HA-treated gelatin scaffold also showed significantly increased adhesive activity of chondrocyte to the scaffold ($p < 0.05$) (Fig. 2). Additionally, histological examination showed a correspondingly large

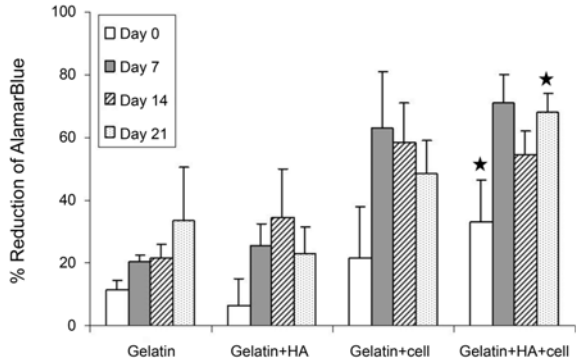

Fig. 1A The graphs demonstrated the proliferation of chondrocytes in gelatin with and without HA0.07% (v/v) treated scaffold and controls. Results are expressed as percent reduction of 10% AlamarBlue. Values represent means and standard deviations (n = 3)

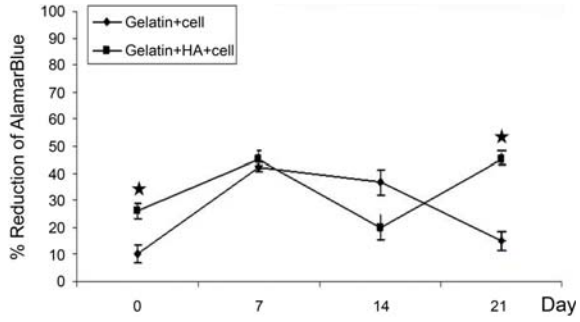

Fig. 1B Percentage of reducing AlamarBlue were compared with two groups, gelatin added cells and gelatin treated with HA0.07% (v/v) added cells. Normalized with each control group, gelatin and gelatin treated HA0.07% (v/v) without cells respectively. Error bars of means and standard deviations (n = 3) are shown

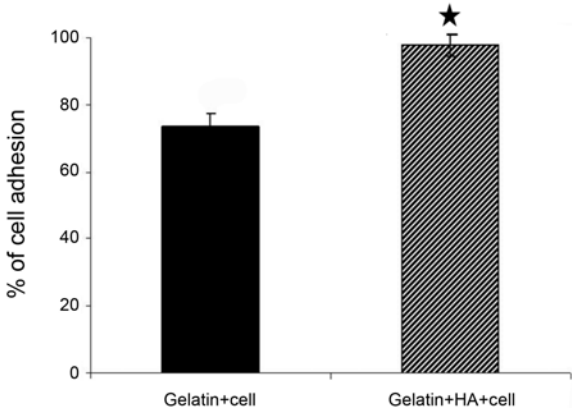

Fig. 2 The graph demonstrates the percentage of cell adhesion result with seeding chondrocytes 0.5 x 10^6 cell/ml each group after incubation for 6 hours

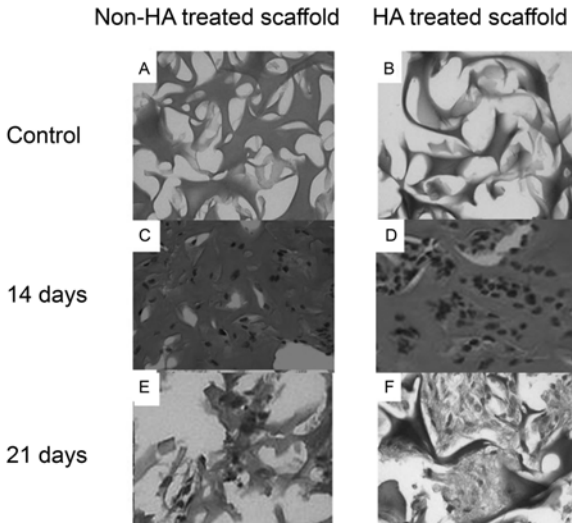

Fig. 3 Sections from the three-dimensional constructs were stained with H&E (X100). A, B demonstrates typical histology of the gelatin scaffolds without cell transplantation (control). C, D - Chondrocytes (black color) culture maintained in a chondrogenic medium supplemented with and without HA0.07% for 14 days, and E, F at 21 days

amount of cellular appearance in the HA-treated gelatin group at both 14 and 21 days (Fig. 3).

The chondrogenic activity of the chondrocyte embedded scaffold was monitored in the role of producing a hyaline-like extracellular matrix such as sGAG, C-6-S, and HA. sGAG production was presented in Safranin-O staining section of both HA-treated, non-HA(data not shown) treated gelatin scaffold (Fig. 4), and progressively increased from week to week in both kinds of scaffold in normalized dye binding assay analysis (Fig. 5). Immunohistochemically stained chondroitin sulfate WF6 epitope showed that HA-treated gelatin demonstrated more C-6-S production than the non-treated gelatin scaffold as well as a progressive increase in C-6-S accumulation from the first to the 21st day (Fig. 6). Moreover, HA-treated gelatin scaffold showed significantly higher quantities of the newly synthesized HA($p < 0.05$), which is

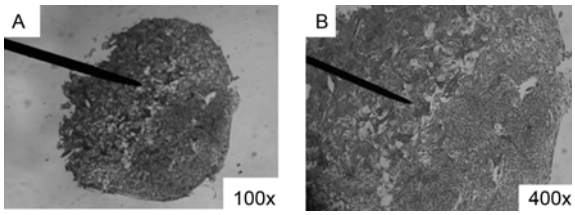

Fig. 4 HA-treated gelatin scaffold seeded with primary human chondrocytes after 21 days of cultivation. (A) (x100), (B) (x400) section demonstrates the deep brown color of extracellular matrix (pointer) which was positive staining with Safranin-O for sGAG

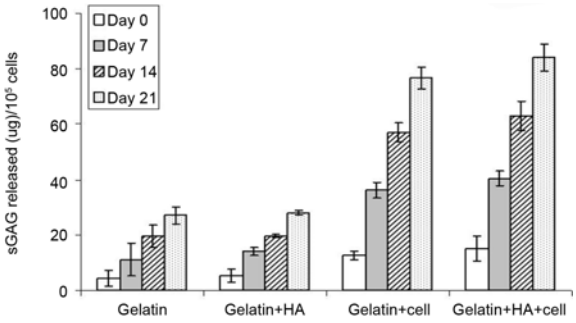

Fig. 5A The sGAG released on gelatin scaffold treated with and without HA0.07% cultures analyzed by DMMB assay. Error bars of means and standard deviations (n = 3) are shown

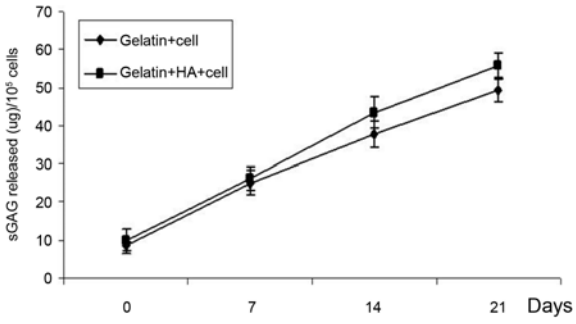

Fig. 5B Accumulated sGAG from chondrocytes cultivated which were compared between two groups. Gelatin added cells and gelatin treated with HA0.07% (v/v) added cells. Error bars of means and standard deviations (n = 3) are shown

the crucial substance for maintaining chondrocyte morphology and activity in cartilage tissue engineering, than of non-HA treated gelatin scaffold. The

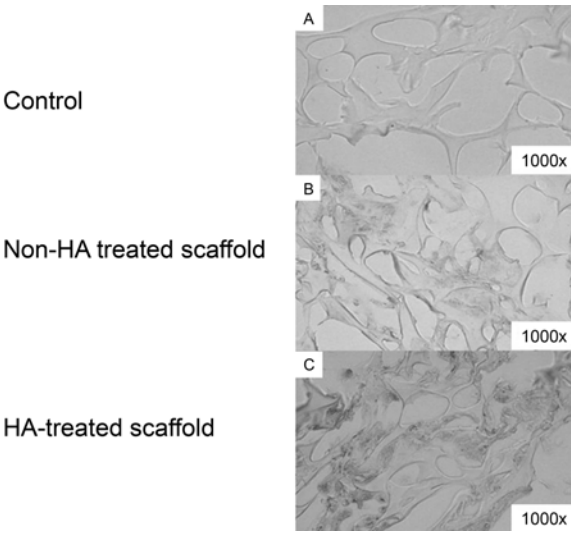

Fig. 6 Immunohistochemistry with WF6 epitope (x1000): positive staining was detected by the dark grey precipitate in the vicinity of the dark grey (stained with Hematoxylin). Gelatin scaffold without cells (A) compared with gelatin without (B) and with (C) HA 0.07% (v/v) for 21 days in growth factor medium

electron microscope confirmed a higher filling of extracellular matrix proteoglycans in the pore of scaffold at the 21st day in HA-treated gelatin scaffold than that in control.

Discussion

The present study demonstrated that chondrocytes could proliferate and express their specific extracellular matrix biomolecules, such as chondroitin sulfate and HA, in HA-treated gelatin scaffold better than that gelatin scaffold alone. Due to the viscosity which is a physiological property of HA, it can promote the initial adhesion property of chondrocytes. Moreover, it is implicated that the initial locomotive behavior triggers via the RHAMM (the receptor for HA-mediated motility) which is involved in the Ras and ERK signaling pathways associated with the cellular cytoskeleton[13,14]. HA provides an advantageous environment for the chondrocyte in 3-dimensional gelatin scaffold, including a hydrated zone around a cell, which facilitates cell mitosis and migration[13], as demonstrated by the proliferate maintaining of chondrocytes in the present results.

Exogenous HA promoted sGAG production gave a positive Safranin-O staining. Even though the

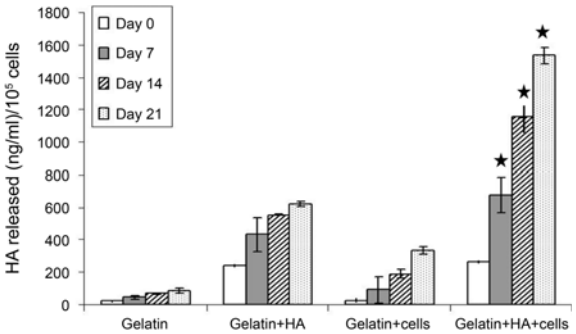

Fig. 7A The HA released of gelatin treated with and without HA0.07% (v/v) and control construct after culture. Values represent means and standard deviations (n = 3)

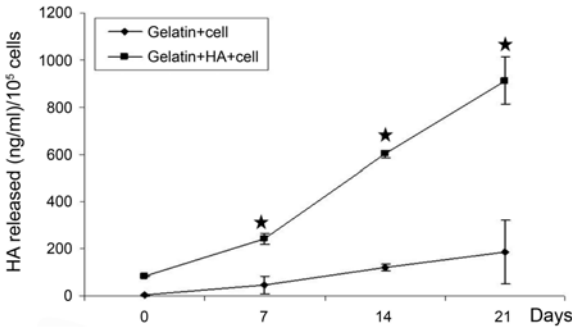

Fig. 7B HA released are compared between two groups, gelatin added cells and gelatin treated with HA0.07% (v/v) added cells. Normalized with each control group, gelatin and gelatin treated HA0.07% (v/v) without cells respectively

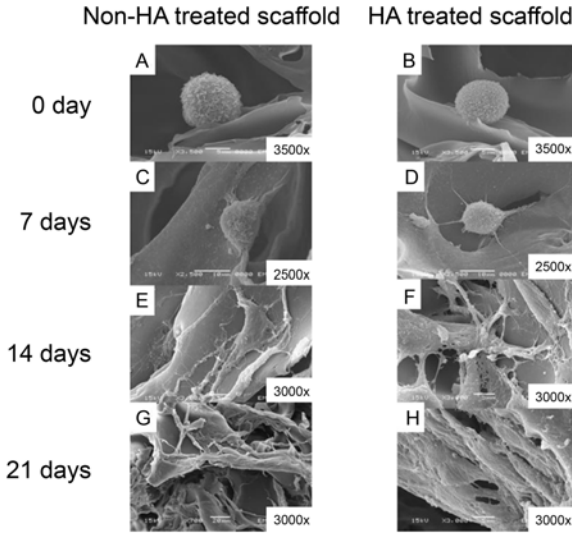

Fig. 8A A typical scanning electron micrograph of the primary human chondrocytes grow on the gelatin scaffold treated with and without HA0.07%. A, B demonstate the chondrocyte on the porous scaffold at 0 day. Chondrocytes change their morphology for adhering to the surface of scaffold at 7 days of incubation time (C, D). Progressive extracellular matrix production were observed on the 14 (E, F), and 21 (H, G) days of incubation times (x3000)

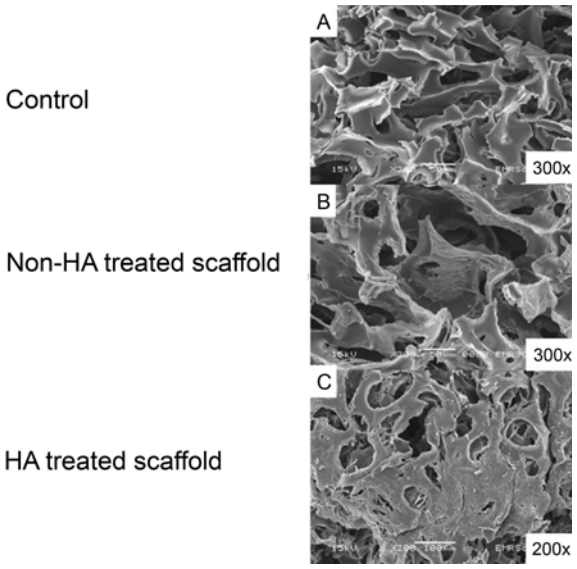

Fig. 8B A typical scanning electron micrograph of the primary human chondrocytes grown on the gelatin scaffold treated with and without HA0.07% (B, C) at 21 days incubation time compared with isolated gelatin scaffold without cell (A). Picture C demonstrate a large amount of extracullular matrix fill in the porous scaffold compared to the picture A and B (x200)

productive capacity of the two groups demonstrated equally, the hyaline-like mimicking properties in HA-treated scaffold was much higher than in the non-treated one that demonstrated in WF6 epitope antibody staining section. Furthermore, exogenous HA induced the new synthesis of HA that will further benefit embedded chondrocytes in maintaining their chondrogenic metabolism and morphology.

Scaffold is the most important factor and should contain several characteristics, such as being

fully biocompatible and degrading gradually as the cells start secreting their own extracellular matrix, allowing the optimal integration between the newly formed and existing tissue, promoting chondrocyte attachment and proliferation, and maintaining a cell-differentiated phenotype[15]. An absorbable gelatin sponge (Spongostan standard, Johnson & Johnson) composed of gelatin derived from porcine skin has the potential to be used as a cell delivery system. Gelatin-bases scaffold is denatured collagen, which presumably retains the Arg-Gly-Asp informational signals[16] and the uniform pore size from manufacturing process. Additionally, HA, the polysaccharide component molecules responsible for its function in tissue reparation, plays an important role for demonstrating and maintaining the phenotype of chondrocyte in cartilage tissue engineering[5].

The present study demonstrated not only that the commercial gelatin-based scaffold can be used in cartilage tissue engineering, but also that exogenous HA provides advantage for chondrocytes. These results confirmed the positive effect of exogenous HA in cartilage tissue engineering with biodegradable tissue. Further studies will consider natural products that have suitable properties when combined with exogenous HA to use in cartilage tissue engineering, and the extensive exploration of the molecular mechanism of HA to the chondrocytes.

Acknowledgements

The authors wish to thank Sompant Padungkert, MD; Naris Thiranont, MD; Taninnit Leerapun, MD; Prasit Wontreratanachai, MD; and Nantawit Sugandhavesa, MD from the orthopedic department for their assistance in harvesting cartilage tissue. This work was partially supported by the Faculty of Medicine Endowment Fund, Faculty of Medicine, Chiang Mai University (CMU), Center of Excellence Research Fund from CMU (to PK), and the National Research Council of Thailand (to PK).

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การศึกษาสมบัติเซลล์กระดูกอ่อนผิวข้อมนุษย์ที่เพาะเลี้ยงในโครงร่างของเจลลาตินเคลือบด้วยกรดไฮยาลูโรนิค

ดำเนินสันต์ พฤกษากร, หนึ่งฤทัย คำแหวน, พีรพรรณ โพธาเจริญ, โอฬาร อาภรณ์ชยานนท์, สัตยา โรจนเสถียร, ปรัชญา คงทวีเลิศ

วัตถุประสงค์*: ศึกษาความเป็นไปได้ในการสร้างเนื้อเยื่อกระดูกอ่อนผิวข้อจากโครงร่างเจลลาติน และอิทธิพลของกรดไฮยาลูโรนิคต่อการเจริญเติบโต และการสร้างสารเมทริกส์ของเซลล์กระดูกอ่อนผิวข้อ*

วัสดุและวิธีการ*: ปลูกถ่ายเซลล์กระดูกอ่อนผิวข้อของมนุษย์บนโครงร่างเจลลาตินที่เคลือบและไม่ได้เคลือบด้วยกรดไฮยาลูโรนิค ทำการวัดอัตราการเจริญของเซลล์กระดูกอ่อนผิวข้อ ความสามารถในการยึดเกาะต่อผิวโครงร่าง และความสามารถในการสร้างสารเมทริกส์*

ผลการศึกษา*: โครงร่างเจลลาตินที่เคลือบด้วยกรดไฮยาลูโรนิค ให้ผลเพิ่มจำนวนเซลล์กระดูกอ่อนผิวข้อ ความสามารถในการยึดเกาะ กระตุ้นการสร้างสารคอนดรอยตินซัลเฟต และกรดไฮยาลูโรนิค อย่างมีนัยสำคัญเมื่อเทียบกับโครงร่างที่ไม่มีการเคลือบกรดไฮยาลูโรนิค ภาพแสดงจากกล้องจุลทรรศน์อิเล็กตรอนพบการสร้างสารเมทริกส์เติมเต็มในช่องของโครงร่างเจลลาตินที่เคลือบด้วยกรดไฮยาลูโรนิคมากกว่าโครงร่างที่ไม่ได้ทำการเคลือบ*

สรุป*: สามารถใช้โครงร่างเจลลาตินในการสร้างเนื้อเยื่อกระดูกอ่อนผิวข้อได้ และการเคลือบโครงร่างด้วยกรดไฮยาลูโรนิคให้ผลดีต่อคุณภาพของเนื้อเยื่อกระดูกอ่อนผิวข้อที่สร้างขึ้น มากกว่าโครงร่างเจลลาตินธรรมดา*