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Chitosan-alginate Gel Modified Poly (L-Lactic-co-ε-Caprolactone) (PLCL) as a Scaffold for Cartilage Tissue Engineering

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Abstract : This study was designed in the fabricated poly (L-Lactic-co-ε-Caprolactone) (PLCL) scaffold using chitosan-alginate hydrogel, which would be more suitable to maintain the biological and physiological functions continuing three dimensional spatial organizations for chondrocytes. As a scaffold, hydrogels alone is weak at endure complex loading within the body. In this study, we made cell hybrid scaffold constructs with poly (L-Lactic-co-ε-Caprolactone) (PLCL) scaffold and hydrogels to make a three-dimensional composition of cells and extracellular matrix, which would be a mimic of a native cartilage. Using a particle leaching technique with NaCl, we fabricated a highly-elastic scaffold from PLCL with 85% porosity and 300-500 μm pore size. A mixture of bovine chondrocytes and chitosan-alginate gel was seeded and compared with alginate as a control on the PLCL scaffold. The cell maturation, proliferation, extracellular matrix synthesis, glycosaminoglycans (sGAG) production and collagen type-II expressions were better in chondrocytes seeded in chitosan-alginate hydrogel than in alginate only. These results indicate that chondrocytes with chitosan-alginate gel on PLCL scaffolds provide an appropriate biomimetic environment for cell proliferation and matrix synthesis, which could successfully be used for cartilage repair and regeneration.

Key words **:** PLCL, bovine chondrocytes, cartilage repair.

Introduction

Articular cartilage has a poor intrinsic capacity to heal under normal physiological circumstances; even minor injuries have the capacity to become severe cartilage defects, which may eventually lead to further degeneration and functional impairment (44). Untreated lesions can lead to devastate joint pain and degenerative arthritis. Hence, the management of articular cartilage defects continues to be one of the most challenging clinical problems for orthopaedic surgeons (25, 37). Although there are a lot of techniques such as autologous chondrocyte implantation or marrow-stimulation techniques have widely been used at the clinic, but formation of fibrocartilage, decreased mechanical strength of the newly formed tissues were common, which resulted in a limited repair capacity (5,42). Therefore, to overcome these limitations, the delivery of tissue engineered cartilaginous implant to the injured site is one of the important strategies to promote tissue integration and tissue regeneration (40).

In the recent years, tissue-engineered cartilage using scaffolds and cells has been considered for improved treatment of cartilage defects (16,31). Scaffolds can play a crucial role for cartilage tissue engineering by maintaining the mechanical integrity, withstanding stress loads within the body and thus providing a sufficient mechanical environment to cells. Mechanical environment is a vital point to cell fate (16); especially, the maintenance of chondrocyte phenotype and synthesis of the extracellular matrix (31). Additionally, chondrocytes tend to lose their phenotype and become fibroblast-like in monolayer culture but can redifferentiate when returning to a 3-dimensional environment (7).

Hydrogels have structural similarity to the macromolecular based components within the body and are considered as a useful biomaterial. These are crosslinked polymeric networks, which may absorb from 10-20% (an arbitrary lower limit) up to thousands times of their dry weight in water. Hydrogels may be chemically stable or may degrade and eventually disintegrate and dissolve. Both natural and synthetic polymers of hydrogels are commonly used for encapsulation of cells (22,35) and also attractive to the new field of 'tissue engineering' as matrices for repairing and regenerating a wide variety of tissues and organs (14,29). They have been utilized as an effective carrier for injectable cartilage in the clinical treatment option and in the preoperative molding of simple facial implants. On the other hand, neither cell suspension nor the use of hydrogels alone helps to make and keep shape as an entire structure of a joint or ear. Instead, a solid porous polymer scaffold can afford the accurate dimensions for materials used for such construction (9). Therefore, the combination of a biodegradable polymer scaffold and hydrogels could enhance cellular proliferation and matrix synthesis for three-dimensional tissue regeneration, which would be applicable to make any desirable shape as our requirements. Recently, it was found out that chitosan and many of its derivatives were potentially favorable materials for the growth of many cells with supporting the attachment, morphology, and proliferation of various kinds of cells, including chondrocytes (13,24). However, *in vivo* applications of

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alginate matrices induce severe foreign body giant cell reactions and immunological responses when implanted within full- thickness defects in experimental animals (34). Therefore, the objectives of this study were to investigate the use of chitosan-alginate mixed hydrogel that might be useful to enhance the chondrocytes function in creating cartilage tissue onto the fabricated poly (L-Lactic-co-ε-Caprolactone) (PLCL) scaffold for advanced use for the repair of damaged cartilage. The cell maturity, extracellular matrix production and collagen type-II gene expression were investigated up to 28 days *in vitro*.

Materials and Methods

Fabrication of the PLCL Scaffold

PLCL (LA/CL = 7:3; Mw 23000) was kindly provided by Professor Zigang Ge, Peking University, China, was dissolved in 1,4-dioxane (10%, w/v) and mixed with a proportional amount of NaCl (200-500 μm, 85% w/w). After agitation, the NaCl/PLCL mixtures were cast in glass molds and then frozen at −20°C for 24 h. The frozen mixtures were then immersed in 70% ethanol aqueous solution for 72 h that was pre-cooled to −20°C. Then the parts of NaCl were substituted with ethanol aqueous solution, and the scaffolds were removed from the template and rinsed with distilled water for 24 h. Porous scaffolds were then dried in hood and sterilized with ethylene oxide gas and finally stored in a refrigerator in dry condition until use. The overall fabrication procedures are summarized in Fig 1.

Isolation and culture of bovine chondrocyte

The chondrocytes were isolated from a six month-old Holstein calf, which died accidentally without suffering any osteoarthritic disease. Full thickness of articular cartilage was aseptically collected from the distal condyle of a femur and digested with collagenase (Welgene, Daegu, South Korea) for 18 h at 37°C in a shaking water bath. Isolated chondrocytes were then cultured with high glucose DMEM containing 10% fetal bovine serum at 37°C in 95% air with 5% CO_2 humidified atmosphere. The culture medium was changed twice a week. At 100% confluence, cells were harvested with 0.25% trypsin-EDTA treatment. Primary chondrocytes were used for this experiment. Cell number and viability were assessed with a hemocytometer by Trypan blue exclusion assay. More than 95% of the cells were usually viable after isolation.

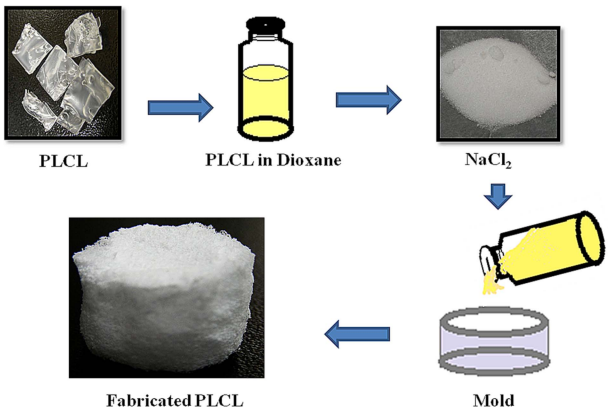

Fig 1. Schematic diagram to illustrate the fabrication process of PLCL scaffold. PLCL scaffold fabricated by the particulate-leaching method.

Preparation and culture of chondrocyte-chitosan-alginate and chondrocyte-alginate construct

Chitosan and alginate solutions were prepared separately (2,43) as described elsewhere, with some modifications. Briefly, 0.5 g of chitosan (degree of deacetylation 85%, Sigma-Aldrich, St Louis, MO, USA, Cat. # 448877) was dissolved in 40 ml 5% (v/v) lactic acid solution and then 160 ml methanol was added to dilute the solution. One and half gram of succinic anhydride (Sigma-Aldrich, St Louis, MO, USA) was added to this solution with stirring at room temperature. The succinyl modified chitosan was precipitated by adjusting the solution pH to 6~7 after 24 h. The precipitate was filtered, re-dissolved in distilled water, and dialyzed for three days. The purified product was freeze-dried and dissolved in PBS at a concentration of 24 mg/ml until further use. Alginate (Sigma-Aldrich, St Louis, MO, USA, Cat. # A0682) solution was prepared by dissolving 0.96 g sodium alginate (low viscosity) in 40 ml 0.15 M NaCl solution. Chondrocytes were added to 8×10^5 cells/20 μL of volume after chitosan and alginate solutions were mixed at ratio 1:1. Scaffolds were then placed individually in the wells of 6-well-plate. Half of the cell suspension (10 μL) was slowly applied on the surface of each scaffold using a pipette. Then the scaffolds were turned upside down, and the reminder of the suspension was slowly applied to the other surface. Same cell concentration and procedure were used for 1.2% alginate suspension in another group as a control. The scaffolds containing cell suspension were then dropped into 102 mM $CaCl_2$ solution to make suspension to gel, which were cultured with high glucose DMEM containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed at three-day intervals, and scaffolds were harvested at the different time points up to 28 days.

Histological and Immunohistological Assessment

For histological analysis, the constructs were fixed in 10% (v/v) buffered formalin for 24 h and were dehydrated using a graded ethanol series and dried. Specimens were then embedded in paraffin blocks and prepared for sectioning. Transverse sections were then cut at 8 μm and routinely stained using Hematoxylin and Eosin (H&E) for nucleus and cytoplasm and Alcian blue (pH-1.0) for sGAG synthesis. Cell numbers were counted and expressed statistically as the average number from 10 fields in each group at a magnification of × 400.

For immunohistochemical analyses, rabbit polyclonal antibody (Rabbit Antibovine collagen type-II, Chemicon International, USA) was used as the primary antibody for indirect immunostaining. Deparaffinized sections of the scaffolds were incubated with 3% H_2O_2 for 30 min to block the endogenous peroxidase activity before 10 min proteinase-k treatment. Nonspecific antibody binding sites were blocked by incubation with normal goat serum in Tris buffer for 60 min. The sections were then incubated for overnight in primary anti-

body diluted 1/100 in Tris buffer at 4ºC. Control sections did not receive the primary antibody, otherwise they were treated identically. The sections were then washed and incubated with a secondary biotinylated goat anti-mouse antibody, and peroxidase labeled streptavidin (Chemicon, Millipore). After color development using 3, 3-diaminobenzidine tetrahydrochloride, the sections were dehydrated through an ethanol-xylene series and mounted permanently in canada balsam by the usual methods for long-term storage. Photographs were taken using a DCM-130 1.3-megapixel digital video eye-piece camera with ScopePhoto 2.0 image software (TrueVision Microscopes, Incorporated, St. Louis, MO).

Statistical analysis

All data were evaluated as the mean ± standard deviation. The statistical significance in the differences between the mean values among the groups was analyzed by one-way analysis of variance (ANOVA) with Tukey's test using the SPSS 11.5 statistical software (SPSS Inc., Chicago, USA). P value < 0.05 was considered statistically significant.

Results

Scaffold fabrication and morphology

PLCL scaffolds with 85% porosity and a 200-500 μm pore sizes were fabricated using a particle leaching technique. The scaffolds have homogeneously interrelated and open three-dimensional porous structures without a skin layer, and possess a completely rubberlike elasticity. The gross and cross sectional images of PLCL scaffolds are shown in Fig 2. The porous surfaces were almost smooth and fiber diameter was found to be uniform in scaffolds. Individual fibers are found crossed and/or fused together with neighboring fibers. Mechanical property was improved on a forceps pressure during the culture time.

Histological evaluation

The distribution and morphological changes of the cells were examined by histological staining. Hematoxylin-Eosin staining revealed that homogenously distributed cells with high viability were observed throughout the scaffolds during the culture period. The chondrocytes maintained a typically round and plumb form, characteristics of chondrocytes, throughout the culture time. The cells gradually formed lacunae within the scaffolds after 7 days of culture (Fig 3). After

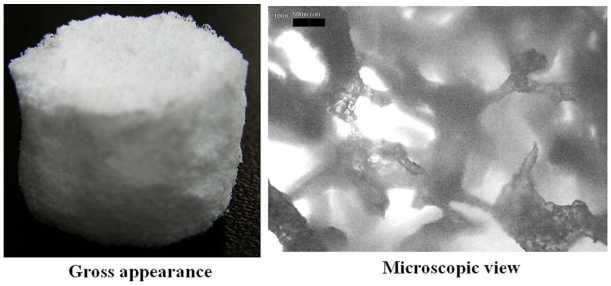

Fig 2. The gross and microscopic views of fabricated PLCL. The microscopic view of the PLCL scaffold was taken using a light microscope connected with a digital video eye-piece camera with ScopePhoto image software (scale bar = 100 μm).

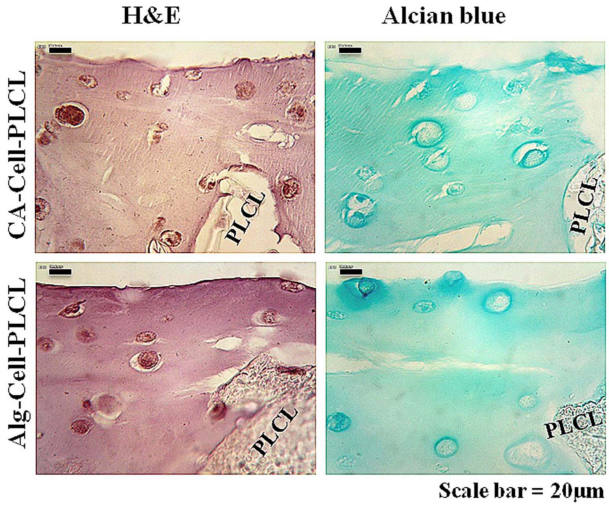

Fig 3. Histological studies of bovine chondrocytes cultured in chitosan-alginate (CA) or alginate (Alg) gel modified PLCL scaffold. The sections were stained at one week with H&E and Alcian blue staining methods. Images were taken at × 400 magnification and are representative of two experiments.

7 and 28 days of culture, the average cell numbers of different scaffolds were 134 ± 4.16 (chitosan-alginate-PLCL), 108.33 ± 9.01 (alginate-PLCL) and 265 ± 6.08 (chitosan-alginate-PLCL), 218.33 ± 11.23 (alginate-PLCL) respectively.

The alcian blue-positive stain indicated that glycosaminoglycans were abundant and homogenously distributed around the cells. Chondrocytes cultured in PLCL scaffolds showed the increasing patterns sGAG in the chitosan-alginate group during the culture time in alcian blue staining (Fig 4).

Immunohistochemistry

Collagen type II expression of chondrocytes was measured in Immunohistochemistry. Chondrocytes expressed better in chitosan-alginate-PLCL compared with the alginate-PLCL (Fig 4). No staining was detected in negative control.

Discussion

Our previously published article demonstrated that the bovine chondrocytes enhanced the cell proliferation and sGAG synthesis in chitosan-alginate gel with maintenance collagen type II expressions (41). In this experiment, we studied the hard scaffold PLCL to find the chondrocytes nature with production of the extracellular matrix that more mimics to use *in vivo* condition. The results obtained showed that chitosan-alginate is effectively helpful media for cell proliferation and matrix production in PLCL scaffold, which is a new window for cartilage tissue engineering. Chitosan is a naturally-occurring polysaccharide and has been widely applied to tissue engineering due to its relatively good biocompatibility and ease of usage. It can be considered as a biochemical counterpart of glycosaminoglycans (sGAG, main components of proteoglycan) due to its similar hexose consisting of water-soluble structural units. On the other hand, PLCL is a highly elastic synthetic macromolecular compound which simulated an elastic collagen fiber network in native cartilage with promoting good cellular interaction and

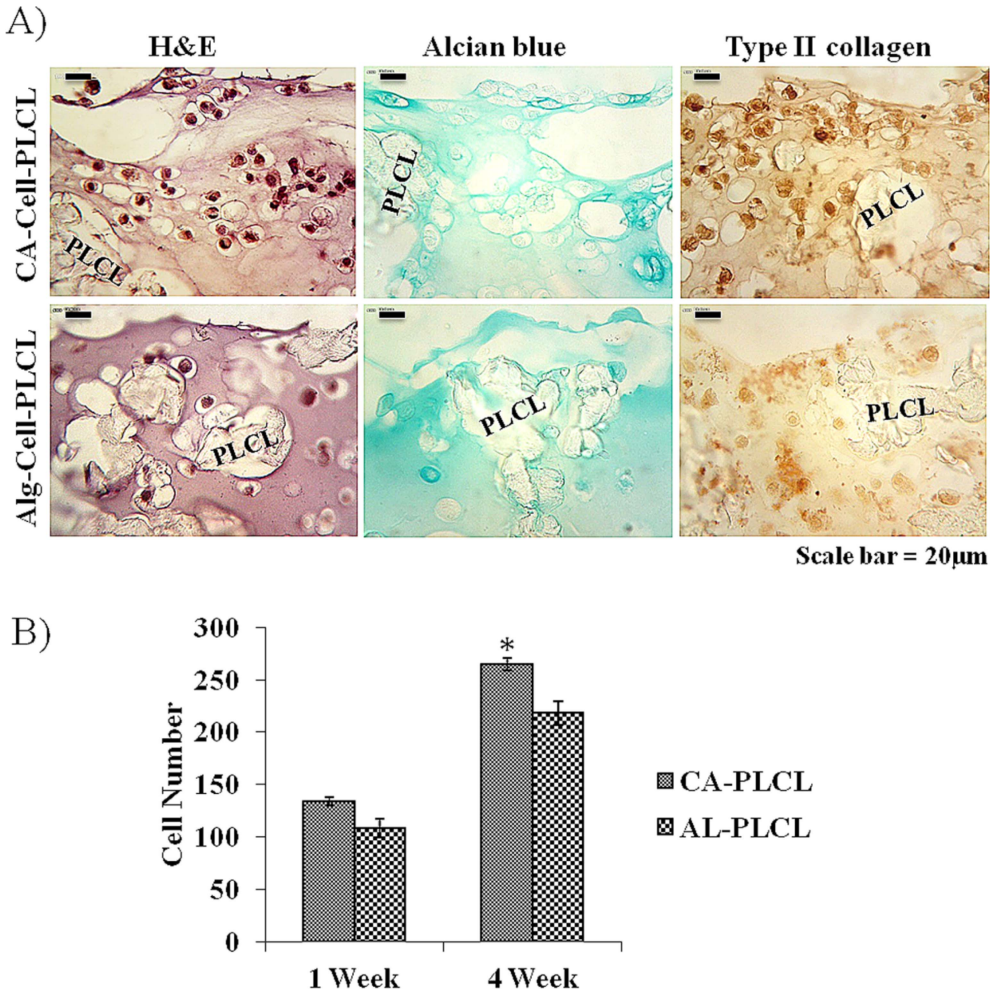

Fig 4. Histological studies of bovine chondrocytes cultured in chitosan-alginate (CA) or alginate (Alg) gel modified PLCL scaffold at 4 weeks. A) The sections were stained with H& E, Alcian blue and type II collagen antibody. Images were taken at × 400 magnification and are representative of two experiments. B) Proliferation of chondrocytes on chitosan-alginate and alginate modified PLCL scaffold at week 1 and 4 was detected by image analysis. The cell nuclei were counted and analyzed as the mean ± standard deviation. P value < 0.05 was considered statistically significant.

degrades in a set time period without toxicity (21,45). It was chosen in place of the broadly adopted PLA and PLGA, mainly due to its intrinsic elasticity. Furthermore, when PLCL degrades into relatively mild acidic products compared to PLGA, chitosan could neutralize the acidic products from the degradation of PLCL (12). In the current study, chitosan-alginate gel was immobilized onto the PLCL scaffold by $CaCl_2$ to maintain the 3D environment for chondrocyte for proliferation and matrix synthesis. During *in vitro* culture, the consistency was well maintained for both scaffold types. Scaffold degradation was negligible and mechanical properties were improved after four weeks of culture, probably due to the deposition of matrix materials by chondrocytes. Several *in vitro* studies prove that the chitosan has a potential value as a matrix to facilitate articular cartilage repair, which efficiently supports not only chondrogenic activities (1,32), but also the *in vitro* expression of cartilage extracellular matrix proteins by human chondrocytes (24). It can also serve as a carrier for growth factors (17). Cell attachment to the surface of chitosan involves the binding of negative groups in the cell surface to the positive charges on the chitosan (15,27) and the number of attached cells on a chitosan matrix increased in the order of chondrocyte > fibroblast > keratinocytes (11). They assumed that the negative charge density in the surface of chondrocyte > fibroblast > keratinocytes, because a negative surface charge of the cells preferred to attach on the positive charge surface on the chitosan matrix. Although, the morphology of attached cells is dependent on deacetylation procedure of chitosan (3). Other reports observed that chitosan films supported the proliferation of chondrocyte (11,19). On the other hand, alginate is a natural polymer used as a biomaterial for cell encapsulation, which can readily redifferentiate from dedifferentiation of chondrocytes (28). Ionic gelation method is an important method to form complex between two different ionic classes. Coacervation between chitosan and alginate (1:1) occurred by electrostatic attraction between the two oppositely charged polymers. Therefore, in this study, we mixed the chondrocytes with chitosan-alginate gel to seed cells into the porous PLCL scaffold. When chitosan-alginate was cross-linked, it formed gel, and chondrocytes were suspended in gel occupying in porous cavity of PLCL. Advantage of this technique is

easy to seed cells into the porous scaffold and accomplish high initial cell concentration. Cell number was noticeably greater in PLCL containing chitosan-alginate gel than alginate gel only, which may results from a synergistic effect of chitosan-alginate combination in terms of cell in-growth and proliferation. The another report also demonstrated that chitosan solution injected into the knee articular cavity of rats caused a significant increase in the density of chondrocyte cartilage (26). The physical property of PLCL scaffolds was also important for proper environments of cell adhesion and in-growth, which also maintained mechanical integrity and deliver mechanical signals to adherent cells (20). Surface topography of the scaffold also directly influenced the tissue response by affecting protein adsorption and by controlling different cellular activities with proliferation and differentiation (8,36). In our study, we used chitosan-alginate and alginate only gel in PLCL scaffold, which increased the adhesive nature on the surface with hydrophilicity of the PLCL scaffold, without altering the scaffold porosity, and the elastic nature. The enhanced wettability and permeability of the gel modified PLCL scaffold promoted homogenous distribution of the seeded chondrocytes throughout the scaffold. Recent studies also support that small changes in hydrophobicity through modification of surface chemistry can dramatically alter cell-matrix interaction, thus making a profound impact on cellular behaviors such as adhesion, morphology, motility, cytoskeletal organization, and differentiation (4,30). Alcian blue staining showed that sGAG production was started as the same manner in both types of gel containing PLCL scaffold, but it was continued and increased in chitosan containing PLCL scaffold up to 28 days of culture due to the ionic nature of chitosan. Several studies have examined that chitosan has a cationic nature, which is primarily responsible for electrostatic interactions with anionic sGAG and allows the formation of insoluble ionic complexes. This property allows chitosan to retain and concentrate bio-molecules in chitosan based scaffolds (23,38). However, alginate has a limited ability to retain the sGAG (41). The results from our study clearly indicate that the chondrocytes seeded in chitosan-alginate gel showed the increased expression of collagen type-II. Studies showed that chondrocyte cells retain a round shape on a chitosan-coated surface (10,33) and produce their characteristic ECM components such as collagen type II and proteoglycan (18,24). Chondrocytes cultured in alginate also support the synthesis of collagen type II (6). However, collagen type-II expression in chitosan mixed alginate is unknown. Spherical shaped morphology of chondrocytes might induce the synthesis and secretion of type II collagen by chondrocytes because they did not undergo phenotypical change (dedifferentiation) (39). Histological findings generally concurred with sGAG and immunihistochemical findings. Seeded chondrocytes effectively penetrated scaffold centers, proliferated with extracellular matrix demonstrates the affinity of chitosan-alginate modified PLCL scaffold for cartilage tissue engineering.

In cartilage tissue engineering, it is essential to maintain the phenotype of seeded chondrocytes and to induce cartilage regeneration. Chitosan and alginate are the most common polysaccharides for cartilage tissue engineering. Both have advantages for forming engineered cartilage due to their biological functions. Although, the weak mechanical properties with rapid degradation of hydrogels have posed problems in their application for articular cartilage defects. In our study, we evaluated the potentiality of cell-hydrogel modified scaffold constructs as an engineered cartilage for the treatment of articular cartilage defects. The three-dimensional environment for seeded chondrocytes was maintained, and the cell seeding efficiency was enhanced in the chitosan-alginate gel with PLCL scaffold. In addition, histological analysis showed that cells onto chitosan-alginate gel in PLCL scaffolds formed mature and well-developed cartilaginous tissue, as evidenced by chondrocytes within lacunae similar to that of native articular cartilage. In summary, the combination of hydrogels in PLCL scaffold for three-dimensional culture of cells would provide a biomimetic environment where cartilage tissue growth is enhanced and facilitated. It can also promote chondrocytes to preserve their phenotypes, enhance cartilaginous extracellular matrix production and, consequently, helps to form the improved quality of cartilaginous tissue.

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변형된 키토산 알지네이트 겔 poly (L-Lactic-co-ε-Caprolactone) 지지체의 연골 조직 재생 평가

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요 약 : 본 연구는 키토산 알지네이트 수화겔을 사용하여 제작된 연골세포의 3차원 구조를 유지하며 생물학적, 생리학적인 기능을 유지하는데 적합한 poly (L-Lactic-co-ε-Caprolactone) (PLCL) 지지체의 효과에 대한 연구이다. 체내에서 수화겔은 단독으로 지지체 역할을 하기에는 부하를 견디기에 약하다. 이에 본 연구에서는 연골세포와 유사한 세포, 세포외 기질의 3차원적 구성을 만들기 위해 PLCL 지지체와 수화겔을 사용하여 합성 지지체를 제작하였다. 염화나트륨을 사용한 입자 침출 기법으로 85%의 다공성, 300-500 μm 크기의 구멍을 가진 탄성력 높은 지지체를 제작하였다. 소의 연골세포와 키토산 알지네이트 겔 혼합물이 PLCL 지지체에 적용되었고 대조군의 알지네이트와 비교 연구하였다. 키토산 알지네이트 수화겔과 연골세포가 혼합된 경우에 알지네이트 단독 사용에 비해 세포 성숙, 증식, 세포외 기질의 합성, sGAG 생성과 II 형 콜라겐의 발현 등의 효과가 좋은 것으로 확인되었다. 본 연구 결과를 통해 PLCL 지지체에 연골세포와 키토산 알지네이트 겔 혼합물을 적용할 경우 세포 증식과 기질의 합성에 적합한 환경을 만들 수 있으며 연골의 복구와 재생에 효과적으로 사용될 수 있을 것으로 기대된다.

주요어 : PLCL, 소 연골세포, 연골 수복