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Coculture of Bovine Chondrocytes with Demineralized Bone Matrix in Alginate Bead and Pellet Cultures

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Abstract : Bio-integration of cartilage grafts with subchondral bone is a significant clinical challenge. To date, the use of demineralized bone matrix (DBM) has been one of the most effective strategies for bone cell proliferation *in vivo*. Here, we investigated whether coculture of chondrocytes and DBM could serve as a single-platform system containing all the essential elements for purposive bone and cartilage induction. The aim of this study was to evaluate and compare the phenotype and proliferation of bovine chondrocytes cocultured with DBM in two different culture systems, pellet and alginate bead culture. In alginate bead culture, we observed an increase in chondrocyte number and formation of cell clusters. Typical chondrocytic phenotype was maintained for entire eight weeks. Histological analysis showed that chondrocytes maintained a typical round, plump morphology and there was a gradual increase in lacunae. Both coculture systems yielded an expanded cell population as compared to the controls (chondrocytes alone). The production of glycosaminoglycans was also increased in the coculture systems as compared to controls.

Key words **:** coculture, bovine chondrocytes, demineralized bone matrix.

Introduction

Cartilage defects and injury present a challenge for surgical or clinical treatment because cartilage has a limited intrinsic capacity for repair (20). In advanced osteo-arthritis, joint space and osteophyte number are decreased due to the destruction of articular cartilage and growth of bone deposits at the margins of the joints. In experimental animals, repair and healing of full-thickness cartilage defects extending into the subchondral bone involves the formation of fibrocartilaginous repair tissue (14). Such repair tissue differs from normal cartilage in that it contains relatively low levels of type II collagen and aggrecan, which are characteristic markers of chondrocytes (20), and a relatively high amount of type I collagen, a fibrillar molecule that is not present in measurable amounts in normal adult articular cartilage (14). Because of the ineffectiveness of intrinsic repair, the development of artificial methods for stimulating cartilage repair is an active area of investigation.

Therapeutic strategies for stimulating cartilage repair include inhibition of matrix degradation and/or stimulation of cartilage anabolism in the diseased joint. A number of studies have demonstrated the beneficial effects of administration of anti-catabolic or anti-inflammatory cytokines such as interleukin (IL)-1Ra, IL-4, IL-10, or IL-11 to the affected cartilage. However, the clinical utility of this approach is limited by the enormous costs associated with these proteins and their short half-life *in vivo* (6). Implantation of an engineered construct containing autologous chondrocytes is currently the most promising approach (20). However, the applicability of autografts is also somewhat problematic due to their limited availability, size, risk of donor site morbidity, infection and rapid de-differentiation of chondrocytes during *in vitro* subculture (7,20). Recent studies have focused on identifying the mechanisms of cartilage destruction, as well as the prevention and gene therapy of osteoarthritis. Currently, modalities such as bone marrow tissue, composite bone marrow scaffolds, demineral-ized bone matrix (DBM) and bioglass have been used with variable success to stimulate the repair of bone and cartilage *in vivo* (7,36).

Despite these advances, purposive induction of local hard tissue development remains problematic. Recent approaches that incorporate tissue engineering appear to be effective in stimulating bone regeneration, and tissue-engineered bone substitutes represent a potential alternative to autologous bone transplantation (17,37). DBM is a promising candidate for such bone tissue-engineering scaffolds due to its close structural and functional similarity to autologous bone. Derived from native osseous tissue, DBM contains bone morphogenic proteins, which are potent osteoinductive glycoproteins, and matrix proteins, such as collagens, that serve as an osteoconductive matrix (19). In addition, DBM is slowly biodegradable and nonimmunogenic (30).

Coculture of various cells is used as a way to improve the

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rate of success of extracorporeal tissue engineering (23,35). It allows direct physical contact between plasma receptors of different cells, which may lead to more efficient transduction of molecular signals (16,20). We hypothesized that coculture of chondrocytes with DBM would provide all of the necessary conductive features of a carrier while at the same time serving as a natural source of inductive osteogenic and chondrogenic factors (7). Our goal is to create a self-sufficient unit that contains all of the essential elements for local formation of hard tissue in orthotopic or ectopic sites.

To date, systematic analysis of the effect of coculture with DBM on the proliferation of chondrocytes has yet to be reported. Here, we investigated the effects of DBM on chondrocyte proliferation and morphology in two well-established cell culture systems, pellet culture and alginate bead culture (36). The aim of this study was to determine whether the pellet or alginate bead culture system, or both, would support DBM cocultures as a new tissue engineering scaffold.

Materials and Methods

Materials

The 75 cm^2 cell culture flasks were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Collagenase, Dulbecco's modified Eagle's medium containing high glucose (DMEM-HG (4.5 g/L D-glucose, L-glutamine, pyridoxine hydrochloride and 110 mg/L sodium pyruvate), fetal bovine serum (FBS), trypsin-EDTA, trypan blue, penicillin, and streptomycin were obtained from Welgene Bioscience (Dalseogu, Daegu, Korea). Microcentrifuge tubes were from Axygen Biosciences (Union City, USA); low viscosity alginate, diethylether, 10% formalin, Alcian blue, Harris hematoxylin and eosin, and calcium chloride were from Sigma Chemicals Co. (St. Louis, MO, USA).

Isolation and culture of bovine chondrocytes (BCC)

Bovine cartilage was obtained from a 6 month-old Holstein calf that died accidentally, without suffering from any osteoarthritic disease. Full thickness articular cartilage was aseptically collected from the distal condyle of the femur. The cartilage was washed with sterile physiological saline and cut into small pieces. The pieces were digested with 1 mg/ml collagenase in DMEM containing 63.5 μg/ml penicillin and 100 μg/ml streptomycin for 18 hours at 37°C in a shaking water bath. Isolated chondrocytes were collected by filtration with sterilized gauze followed by centrifugation at 200 × *g* for 5 minutes. The cell pellets were resuspended and plated at a density of 1×10^6 cells/dish in DMEM containing 10% FBS in 75 cm^2 cell culture flasks. Cultures were maintained at 37°C in a 95% air/5% CO_2 humidified atmosphere. The culture medium was changed twice a week. When the cells reached 100% confluence, they were harvested by 0.25% trypsin/EDTA treatment. Second passage chondrocytes were used for all experiments. Cell number and viability were assessed by cell counts using trypan blue exclusion and a hemocytometer. Generally, cells were 95% viable after isolation.

Preparation of DBM

Demineralized bone matrix was prepared from the same calf that served as the source of chondrocytes. The cancellous part of the femur was collected aseptically into PBS and then cut into small pieces with Rongeurs. After washing several times with PBS, the bone particles were thawed in ethanol and air dried under a laminar flow hood. The particles were pulverized in a mortar with liquid nitrogen and then sieved. Pieces were decalcified in 0.6 mol/L HCl at 4°C for 24 hours under constant agitation. Fresh acid was added every eight hours. After demineralization, the bone particles were rinsed in deionized water and placed in PBS overnight. The bone particles were rinsed and the pH was adjusted to 7.3, after which they were placed in ethanol and allowed to evaporate overnight. The resultant DBM was packaged and stored at -20°C after ethylene oxide sterilization.

Coculture of chondrocytes with DBM in pellet culture

An aliquot containing 1×10^6 cells was placed into a 1.5 ml microfuge tube and subjected to centrifugation at 500 × *g* for 5 minutes. Cell pellets were evenly divided into two groups for culture with and without DBM. Cells were incubated in loosely capped tubes in DMEM-HG with 10% FBS for 8 weeks in a humidified atmosphere at 37°C with 5% CO_2 and 95% air. The medium was changed every 3-4 days. To monitor and compare cocultures to the controls (chondrocytes alone), samples were harvested at 2, 4, 6 and 8 weeks for histology.

Coculture of chondrocytes with DBM in alginate bead culture

An aliquot of cells was subjected to centrifugation in a 50 ml conical tube and then resuspended in 1 ml. An equal volume of sodium alginate (2.4% in 0.15 M NaCl, filter-sterilized using a 0.2-μm filter) was thoroughly mixed with the cell suspension by gentle aspiration to achieve a final alginate concentration of 1.2%. Unless otherwise noted, we used 5×10^6 cells/ml. Half of the alginate-cell suspension was slowly dispensed in a drop-wise fashion through a modified yellow pipette tip into a gelation solution (102 mM $CaCl_2$, pH 7.4) containing approximately 30 μl/bead. DBM was mixed with the other half of the cell suspension and dispensed in the same manner. The beads were allowed to polymerize for 20 minutes at room temperature. Calcium chloride was removed, and the newly formed beads were washed three times with 0.15 M NaCl, followed by two washes with DMEM-HG. Alginate beads (approximately 3-4 mm thick) were cultured in a six-well tissue culture plate (5 beads/well) with 5 ml DMEM-HG containing 10% FBS. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 for up to 8 weeks, and the medium was replaced two or three times per week. Samples were harvested on days 14, 28, 42 and 56. Bead cultures were transferred to new six-well plates every other week to avoid the formation of a cell monolayer on the bottom of well by chondrocytes that migrated out of the beads. Beads remained intact during the entire culture period.

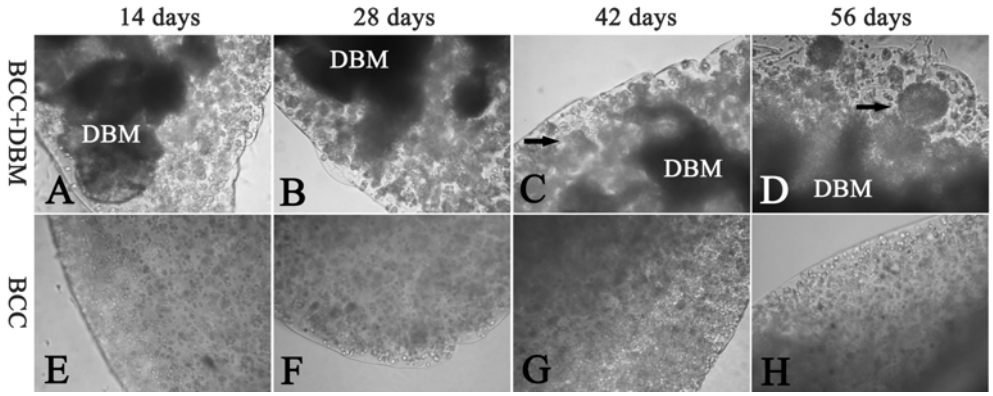

Fig 1. Photographs of alginate bead cultures viewed by phase contrast microscopy. Chondrocytes were encapsulated in alginate beads with or without DBM, and then cultured in high glucose medium for 8 weeks. Coculture of BCC with DBM (A-D) and BCC alone (E-H). Coculture with DBM enhanced chondrocyte proliferation, as demonstrated by the clusters (arrow) of cells in cocultures.

Evaluation

The gross appearance of the cultures was noted and stiffness was evaluated by forceps pressure. Histological evaluation of the engineered cartilage using hematoxylin-eosin (HE) and alcian blue staining of neo-cartilage was carried out during *in vitro* culture for 8 weeks. Harvested samples of alginate beads and pellet cultures were fixed in 10% neutral buffered formalin with 0.1 M calcium chloride for 12 hours at room temperature and then dehydrated using a conventional ethanol gradient dehydration procedure. Samples were cleaned with xylene, embedded in paraffin, and then cut into 5 μm thick sections for HE and alcian blue staining. Alcian blue staining at acidic pH was used as an indicator of highly sulfated glycosaminoglycans (GAGs). The paraffin sections were stained with alcian blue 8GX (pH 2.5) and then counterstained with Harris hematoxylin, according to standard protocols. The sections were viewed under a light microscope.

Results

Chondrocyte morphology

The phenotype of chondrocytes cultured with and without DBM in 1.2% alginate beads is shown in Fig 1. At the start of culture, isolated chondrocytes were homogenously distributed throughout the alginate beads and exhibited a round morphology when observed by phase contrast microscopy. At day 6 of culture, the cells appeared as small clumps scattered throughout the beads. Growth of the chondrocytes occurred gradually over the 8 weeks of culture, as evidenced by the formation of enlarged cell aggregates and numerous small clusters of densely packed round cells (Fig 1). These clusters were presumably the result of mitosis, with daughter cells being separated gradually from one another by deposition of the extracellular matrix. In the pellet culture system, all pellets exhibited a spherical or oval shape in the first few days after formation. Over time, we observed a marked increase in the size and solid three-dimensional tissue structure of the cocultures as compared to controls (chondrocytes alone) (data not shown).

Histology

Changes in cell morphology were investigated by histological staining. The persistence of BCC in the cocultures and controls was confirmed by chondrogenesis showed a round and plump morphology. In both culture systems, homogenously distributed cells with high viability were observed throughout the culture period. The cells maintained a round and plump morphology characteristic of chondrocytes, particularly in the cocultures, throughout the culture period. Cells gradually formed lacunae in both culture systems starting at day 14, but to a greater extent in the cocultures than in the controls (chondrocytes alone) (Fig 2 and 3).

Based on HE staining, the appearance and cellular distribution of neo-chondrocytes were better in the cocultures, in which most of the chondrocytes were mature and embedded in lacunas, as compared to the control cells (Fig 2). Coculture also resulted in an expanded cell population as compared to controls in both culture systems. Upon analysis of long term cultures, we observed more extensive cell growth and matrix elaboration in the cocultures as compared to the controls (Fig 2).

Alcian blue staining of cross-sections of alginate and pellet samples revealed characteristic peri-cellular deposition of GAGs. The amount of new GAG increased with prolonged culture and was greater in the coculture systems as compared to the controls (Fig 3). The number and roundness of chondrocytes were also greater in the cocultures as compared to chondrocytes alone.

Discussion

Articular cartilage is frequently damaged as a result of injury or disease, but has a limited capacity to regenerate. In chondral defects, whether or not a lesion is contained within the articular cartilage, there is no involvement of vasculature. Consequently, progenitor cells cannot enter the damaged region from the blood or marrow to influence or contribute to the repair process, which involves degenerative changes of cartilage and bone in the affected joint (11,27).

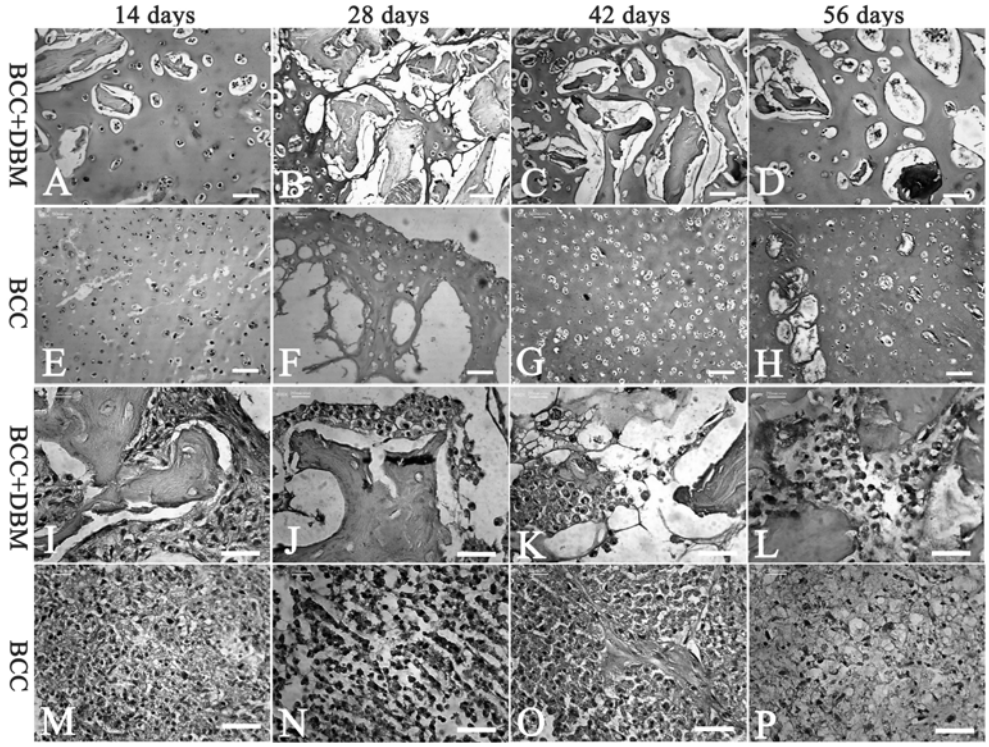

Fig 2. Histological analysis of chondrocyte morphology and distribution in alginate bead and pellet cultures during 8 weeks by HE staining (scale bar, 100 μm for A-H; scale bar, 40 μm for I-P). BCC alone (E-H) and with DBM (A-D) in alginate bead cultures. Bovine chondrocytes alone (M-P) and with DBM (I-L) in pellet cultures. Cocultures contained a greater number of healthy cells and lacunated chondrocytes surrounded by matrix as compared to the controls (chondrocytes alone).

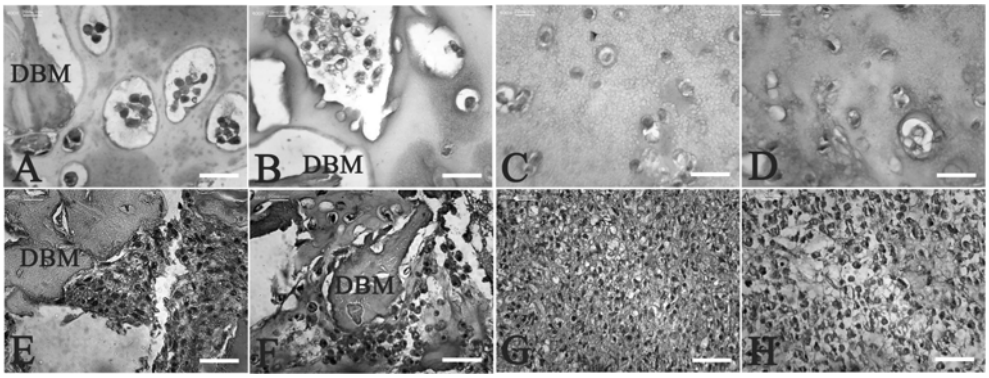

Fig 3. Histological analysis using Alcian blue staining of chondrocytes in alginate bead and pellet cultures during 8 weeks (scale bar, 40 μm) to detect newly synthesized GAG. BCC alone (C, D) and with DBM (A, B) in alginate bead cultures. BCC alone (G, H) and with DBM (E, F) in pellet cultures. Cell proliferation (clusters of cells) and the level of GAG were higher in cocultures as compared to the controls (chondrocytes alone).

Effective techniques for restoring defects in the articular surface are non-existent. Various surgical techniques have been reported in animals and in humans, including abrasion (13); microfracturing (24); slurry (28), periosteal (9), perichondral (10) and osteochondral grafting (8); and stem cell (3) or chondrocyte (33) transplantation. The short term clinical results of some of these techniques are promising, although data from microscopic analysis indicates that the repair tissue is fibrous in nature and does not have the same functional properties as native hyaline cartilage (1). Studies of normal chondrocyte metabolism and behavior under different conditions (i.e., different matrices) will provide information that is vital to achieving optimal repair of cartilage tissue.

At present, tissue engineering of cartilage appears to hold great potential. Autologous or allogenous chondrocytes can be cultured in biodegradable/biocompatible three dimensional matrices, where they proliferate and synthesize extracellular matrix proteins. Implantation of isolated chondrocytes in col-

lagen, hyaluronan, and fibrin matrices for the treatment of cartilage defects has been reported (21,26,33), although none of these matrices offers suitable mechanical stability against tensile and compressive forces encountered *in vivo*. We are interested in the feasibility of generating implants prepared by coculture of cartilage and DBM as a way to minimize the number of chondrocytes needed for grafting and save time. Here, we report the results of a comparative morphological analysis of chondrocytes cultured alone or in coculture with DBM.

DBM is a potentially viable scaffold for bone tissue engineering that has also been used to construct skeletal tissue (2,25,29). However, the supply of human bone is restricted somewhat by donor availability (16,31). Animal studies have demonstrated that DBM and other biomaterials can be used to effectively repair articular cartilage defects (4,7). Bone has the unique capacity to regenerate and repair without scar following injury, processes that can be enhanced by the use of autogenous bone grafts (18). In the field of bone and cartilage engineering, coculture systems have emerged as a potentially useful approach to cartilage tissue engineering that provides a more physiological environment for cell regeneration *in vitro* (32). The current study was designed to investigate the feasibility of *in vitro* chondrogenesis by coculture of chondrocytes with DBM, thereby potentially reducing the number of chondrocytes required while at the same time providing a simple method for tissue-engineered cartilage construction. We demonstrated that chondrocytes readily and efficiently proliferate in coculture with DBM without the addition of growth factors, thereby markedly reducing the number of total chondrocytes needed for new scaffold construction. Our results suggest that DBM enhances proliferation and provides a strong platform for chondrocyte growth.

In the current study, DBM was derived from cancellous bone of a calf and used in coculture with autologous articular chondrocytes in two different culture systems. In alginate bead cocultures, chondrocytes began to proliferate and grow starting at day 6 and continuing until the end of the experiment (8 weeks), consistent with other reports (34). After 8 weeks, homogenous mature cartilage with a degree of elasticity was achieved. Furthermore, histology revealed the presence of many mature cartilage lacunas in both coculture systems. Cell nuclei were more rounded in cocultures than in controls (chondrocytes alone), perhaps due to the effects of direct contact of chondrocytes with DBM. The fact that the coculture samples became harder with time may be due to the secretion of morphogenetic factors from DBM (12).

The culture of articular chondrocytes in alginate beads offers several advantages over monolayer cultures (5). In alginate beads, chondrocytes that have been stripped of native matrix during processing are able to reestablished a matrix in cell-associated matrix compartments, similar to previous results for BCC alone (22). A three-dimensional (3D) environment is apparently necessary for the production of type II collagen and for the maintenance of chondrocyte phenotype *in vitro*. Therefore gels such as alginate and agarose, as well as suspension cultures, which help maintain the rounded morphology of chondrocytes, have been developed (15,36). Several studies have shown that members of the transforming growth factor-β family play a major role in cartilage development. We used two different 3D culture systems without added TGF-β in the current study in order to specifically isolate the chondrocyte response to DBM in coculture. Interestingly, we observed that GAG was more strongly expressed in cocultures than in the controls. The proliferation of chondrocytes involves the deposition of GAG. The enhanced production of GAG in cocultures may be due to enhancing growth factors derived from DBM. Increased cell proliferation, more rounded cell shape, and a greater extent of matrix deposition were clearly observed in both coculture systems as compared to controls, which suggests that coculture with DBM is more conducive to chondrocyte growth and proliferation for new tissue engineering applications.

To date, the underlying mechanism by which DBM influences the proliferation of chondrocytes *in vitro* has not been elucidated, and the molecular details of the chondrocyte-DBM interaction have yet to be clarified. Continued research on the mechanisms of cell–DBM interactions may eventually enable us to specifically promote and direct DBM-mediated induction and proliferation. When fully realized, the ability to direct cell-DBM interactions to help to create stable sophisticated tissues or composite constructs could have important therapeutic implications. Ongoing studies in our laboratory are focused on identifying the factors in DBM coculture that could be retained after implantation in nude mice, and whether the extent of DBM influences chondrocyte proliferation.

In summary, we report here a simple method for coculture of chondrocytes with DBM in two different systems, alginate bead and pellet culture, which provides a ready means for chondrocyte proliferation and cartilage formation. The characterization of two standard 3D systems that are appropriate for testing implantable biomaterials in an *in vitro* environment will help in the development of appropriate differentiation protocols for better tissue engineering therapies.

Acknowledgments

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알긴산 배양과 펠렛 배양에서 소연골세포와 탈회골기질의 공배양

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요 약 : 연골밑뼈와 연골이식편 사이의 생유합성은 임상적으로 중요한 과제이다. 현재까지 탈회골기질의 이용은 생체내 뼈세포 증식에 있어서 가장 효과적인 방법이다. 본 연구에서는 연골세포와 탈회골기질의 공배양을 통해 뼈와 연골의 유도 목적에 부합되는 모든 필수적인 요소를 갖는 재료로 이용가능 여부를 확인하고자 실시되었다. 본 연구의 목적은 두 종류의 배양법 즉, 펠렛 배양과 알긴산 배양에서 탈회골기질과 공배양된 소 연골세포의 증식과 표현형을 비교, 평가하는 것이다. 알긴산 배양에서는 세포 군집의 형성 및 연골세포의 수적 증가가 관찰되었다. 전형적인 연골세포의 표현형이 시험기간인 8주에 걸쳐 유지되었으며, 조직학적인 검사에서 연골세포는 일반적인 원형의 형태를 유지하였고, 연골세포방과 연골세포가 점진적으로 증가하였다. 대조군(연골세포 단독배양)에 비해 탈회골기질과 공배양한 두군 모두에서 많은 세포증식이 관찰되었으며, 글리코사미노글리칸의 생성 또한 증가되었다.

주요어 : 공배양, 소연골세포, 탈회골기질.