

Design and Fabrication of Anatomical Bioreactor Systems Containing Alginate Scaffolds for Cartilage Tissue Engineering

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Abstract

The aim of the present study was to develop a tissue-engineering approach through alginate gel molding to mimic cartilage tissue in a three-dimensional culture system. The perfusion biomimetic bioreactor was designed to mimic natural joint. The shear stresses exerting on the bioreactor chamber were calculated by Computational Fluid Dynamic (CFD). Several alginate/bovine chondrocyte constructs were prepared, and were cultured in the bioreactor. Histochemical and immunohistochemical staining methods for the presence of glycosaminoglycan (GAG), overall matrix production and type II collagen protein were performed, respectively. The dynamic mechanical device applied a linear mechanical displacement of 2 mm to 10 mm. The CFD modeling indicated peak velocity and maximum wall shear stress were 1.706×10^{-3} m/s and 0.02407 dyne/cm², respectively. Histochemical and immunohistochemical analysis revealed evidence of cartilage-like tissue with lacunas similar to those of natural cartilage and the production of sulfated GAG of matrix by the chondrons, metachromatic territorial matrix-surrounded cells and accumulation of type II collagen around the cells. The present study indicated that when chondrocytes were seeded in alginate hydrogel and cultured in biomimetic cell culture system, cells survived well and secreted newly synthesized matrix led to improvement of chondrogenesis.

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Introduction

Tissue engineering is a multidisciplinary field that aims to construct biological tissues such as cartilage⁽¹⁾. Tissue engineering strategy generally involves the expansion of cell lines *in vitro*, followed by seeding the cells onto a three-dimensional (3D) biodegradable and biocompatible scaffold that provides structural support and can also act as a reservoir for bioactive molecules such as growth factors. Bioreactors and scaffolds including hydrogels play critical roles in tissue engineering,

former by provision of the physiological environment to control environmental conditions such as oxygen, pH, temperature, and aseptic operation, and latter by acting as temporary artificial extracellular matrices⁽¹⁻³⁾.

Several bioreactors are used for cartilage tissue engineering that can be categorized based on stimuli into several groups such as direct compression, hydrostatic pressure, shear bioreactors (direct flow and fluid perfusion) and hybrid bioreactors incorporating

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multiple loading regimes ⁽⁴⁾. Because perfusion culture method facilitates the continuous exchange of medium and the constant removal of metabolic wastes, it has been used by several investigators to improve the extent of proliferation and differentiation of MSCs ⁽⁵⁾. Also combination of plasmid DNA impregnated PGA-reinforced collagen sponge and the perfusion method promoted the *in vitro* gene expression for MSC ^(6,7).

Alginate is the most frequent biomaterials used to fabricate hydrogel scaffolds. Alginate is extracted from certain seaweeds or produced by some bacteria. Alginic acid is a family of linear copolymers of 1,4-linked b-D-mannuronic acid (M) and a-L-guluronic acid (G). Gelation of alginate is based on the affinity of alginic acid towards certain ions and the ability to bind these ions selectively and cooperatively. Alginate is used in the food industry as thickening and emulsifying agents (ice creams, fruit drinks, and so forth), textile and paper industries, water treatment processes. For biomedical application alginate has been widely used for drug delivery and encapsulation ⁽⁸⁻¹⁰⁾. Cell encapsulation involves the entrapment of living cells within hydrogels and provides cells with a three-dimensional environment mimicking the *in vivo* conditions. Cell encapsulation has been used in cartilage tissue engineering due to its ability to support chondrocytes phenotype ⁽¹¹⁻¹³⁾.

Several investigators examined methods of fabrication macroscopic shape of the encapsulated chondrocytes in alginate to generate stratified articular cartilage constructs ⁽¹⁴⁾. One of desired macroscopic shape of the hydrogels is sheets, by depositing multiple layers when used as a graft. The advantage of these methods of fabrication is the formation of 3D cell-gel constructs to function as a platform for therapeutic applications. Many methods have been proposed for alginate sheet fabrication ⁽¹⁵⁾. Also a membrane material was used as a cover or part of the mold to allow calcium ions to diffuse into the alginate solution ⁽¹⁶⁾.

Engineering anatomical shaped of con-

structs such as incase of cartilage grafts need to develop appropriate culture system such as bioreactor. Bioreactors are means and model systems that used to cell proliferation, seeding of cells on scaffolds, generation of 3D tissue constructs and enhance nutrient diffusion ⁽¹⁷⁾. Several biomimetic bioreactors have developed for tissue engineering to mimic physical and chemical *in vivo* environment of the cartilage tissue to scale-up of bioengineered grafts toward clinical applications and generate anatomically shaped tissue constructs for the potential replacement. However, these bioreactors should be customized to support the cultivation of anatomically shaped grafts to match anatomy of the specific implantation site with the size and shape of the scaffold ⁽¹⁸⁻²⁰⁾.

Therefore, aim of the present study was development of new tissue-engineering approach to create cartilage tissue in a three-dimensional culture system. This study is an attempt to address concerns about the cell culture and transplantation of cartilage including the maintenance of the chondrocyte phenotype, overcome on leakage chondrocyte from site of graft.

Materials and Methods

Materials

Phosphate Buffered Saline (PBS), Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), antibiotic (penicillin-streptomycin), amphotericin B, collagenase type 2, alginate and trypsin, HEPES, Ham F-12, ascorbic acid and L-glutamine were purchased from Sigma-Aldrich (MO, USA). Anti-type II collagen (COL2A1 (M 2139): sc-52658) were purchased from Santa Cruz Biotechnology, Inc., USA.

Bioreactor description

The biomimetic bioreactor was designed with several parts including culture chamber and mechanical device (Figures 1 and 2).

Briefly, the culture chamber was made from medical stainless steel (316 L), it has two inlets/outlets, one for the culture medium

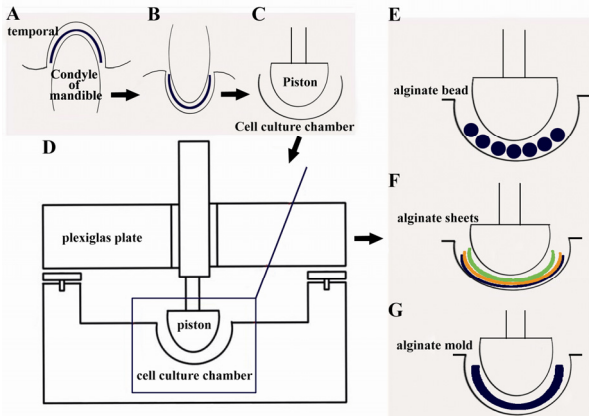


Figure 1. Study design for development of biomimetic bioreactor and scaffolding methods. A) Schematic sketch of natural TMJ, temporal and mandibular bones. B) Modification of TMJ as desired shape. C) Design of culture chamber and piston as TMJ analogue. D) Sketch of closed cell culture system including piston. E) Culture of alginate/chondrocyte beads in bioreactor. F) Culture of alginate/chondrocyte sheets in bioreactor. G) Culture of alginate/chondrocyte mold in bioreactor

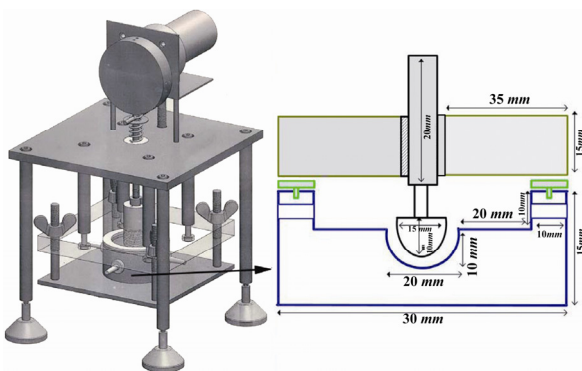


Figure 2. Complete assembled bioreactor including cell culture chamber, mechanical device and Plexiglas cover (left). Measurement of biomimetic cell culture system including piston (right)

and the other for the gases (O_2 , CO_2). Medium recirculation was provided by a multi-channel peristaltic pump (Sabz Zist Kimia Co. Iran) in the range of 1 ml/min. In design of culture chamber, we attempted to provision of the joint parts analogue *in vitro*. Therefore the inside geometry of central part of culture chamber was designed to mimic ball and socket joints such as Temporomandibular Joint (TMJ). A sketch of the geometry of the chamber with scales could be seen in figure 2. Tap of chamber has a machined circular groove with 0.2 mm depth for the O-ring. The chamber was closed tightly by a Plexiglas plate cover with sterility of the culture environment. Center of Plexiglas plate has a

hole with tight screw to permit movement of stainless steel piston. The piston could be moved vertically by an electromotor (Bouhler Germany, with maximum power 23 watt) mounted top. The maximal frequency of the motor movement for linear displacement piston experimentally measured and amounted to 50/60 Hz. For mechanical stimuli; we have programmed the input to be in the range of 20, 35, 50 and 70 N. After complete assemble, bioreactor was placed in incubator.

Computational fluid dynamics

The shear stresses at flow rates 1 ml/min exerting on the surface of the bioreactor chamber wall was simulated by Computational Fluid Dynamics (CFD) modeling, calculated by FLUENT software (Fluent Corp). The meshes were created by Gambit software as described by Cinbiz et al, 2010⁽²¹⁾.

Scaffolding techniques and cell culture

Chondrocyte isolation and expansion were performed as described by Masuda et al, 2003⁽²²⁾. Briefly, cartilage was acquired from a local abattoir (Ahwaz, Iran). Nasal septum was obtained within 1-2 hr of death. The connective tissues/perichondrium was removed to expose the white glossy nasal septal cartilage and pieces about 4-5 cm height and 1 cm thickness of tissue were removed. Cartilage slices were treated first with 0.25% trypsin for two hr at 37°C and subsequently, for 16-20 hr in a 37°C, 5% CO_2 incubator in high glucose DMEM supplemented with 0.2% collagenase type II and antibiotic/ antimycotic (100 U/ml penicillin, and 0.1 mg/ml streptomycin/ 250 µg/ml amphotricine B). The digest was filtered through 70 and 100µm nylon filters (BD Falcon, USA), washed 2-3 times with PBS and the filtered solution centrifuged at 2500 rpm for 10 min in room temperature.

The obtained cells ($2-4 \times 10^5$) were cultured in 25-cm² T-flasks in 5 ml of complete culture medium (DMEM/Ham's F-12 supplemented with 5% FBS, ascorbic acid (50 g/ml), L-glutamine (29.2 mg/ml). The cells were harvested, counted with hemocytometer, and then seeded into alginate scaffolds. Viable cell in 3D scaffold

fold in static/dynamic cultures was analyzed by trypan blue testing.

The preparation of chondrocytes in alginate gel was performed as described by Masuda et al, 2003⁽²²⁾. Briefly, 2.0 % alginate solutions were prepared by adding alginic acid sodium into NaCl, and HEPES in deionized water. The solutions were sterilized via 0.45- μ m filter; then isolated cells ($2-4 \times 10^6$) were resuspended in and then slowly expressed through a syringe needle (21-gauge needle and 20 ml syringes) in a dropwise fashion into a 102 mM CaCl₂ solution. The beads were allowed to polymerize, washed in 150 mM NaCl, and finally placed in complete culture medium. 50-60 beads were cultured in each flask.

Alginate sheet preparation

Alginate sheet preparation was performed by modification of previously reported methods⁽¹⁴⁾, and we developed a new novel method to fabricate of alginate/chondrocyte sheet. The working principle is based on the use of sterile filter paper. Tow layer sterile filter paper was prepared and soaked in 102 mM CaCl₂. The 2.0% alginate solutions and chondrocytes (with $2-4 \times 10^6$ density/ml alginate solutions) were mixed in 20 ml syringes. Once mixed, one hydrogel sheet was cast between two-layer filter papers soaked with CaCl₂. After instantaneous gelation, casting was transferred into CaCl₂ solution. During polymerization of gels, the casting two-layer filter papers were separated spontaneously. The sheet was allowed to polymerize further for a period of 10 min, resulting in a desired thickness such as 0.5-1 mm-thick sheet of alginate. Alginate sheets were finally placed in complete culture medium and cultured. The other sheets can be cast in similar method to generate stratified engineered tissues.

Preparation of macroscopic shape of alginate

Desired molded shape of sterile filter paper was prepared and sealed to avoid contamination and leakage. The previously prepared alginate/cells mixture expressed through a syringe needle into mold. Then, mold containing alginate/cells mixture was transferred into

CaCl₂ solution and was allowed to polymerize. Then, construct was removed from filter paper which could be cut to desired shape or diameter.

All cell-seeded scaffolds (beads, sheets and molded shape) with $2-4 \times 10^6$ density/ml alginate solution after 2nd passage were cultured for 5 days in culture flasks and then transferred to the bioreactor and dynamically cultured for 3-5 additional days under direct perfusion of 1 ml/min complete culture medium with controlled environmental conditions such as oxygen, pH, and temperature (37°C, 5% CO₂, 95% O₂ and 95% humidity). Cell proliferation was analyzed by hemocytometer. For comparison, as control groups tissue constructs (as beads, sheets and molded shapes) were cultured for 7-10 days in static conditions in culture flask.

Histological evaluation

Histochemical staining: After culture in bioreactor, all cell-seeded scaffolds were fixed in bouin fixative, dehydrated, cleared, and then embedded in paraffin wax. Five to seven μ m sections were cut. We performed histological and several histochemical staining methods (alcian blue/ nuclear fast red, hematoxylin/ safranin O/fast green and toluidine blue)^(13,14). The sections were stained with hematoxylin & eosin (H&E) for cell morphology and alcian blue, hematoxylin/safranin O/fast green staining methods for the presence of GAG. Toluidine blue staining method was performed for assessment of overall matrix production by cells of constructs. When matrix production by the chondrons is visualized with an alcian blue and nuclear fast red technique, cartilage matrix containing sulfated proteoglycans appears blue, and cell nuclei appear red. Histochemical examination of chondrocyte redifferentiation was assessed as proteoglycan accumulation, as measured by staining with alcian blue/neutral red and hematoxylin/ safranin O/fast green.

Immunohistochemical analysis: Sections of all construct examined for production of collagen II with anti-type II collagen (COL2A1

(M2139): sc-52658) according to manufacture protocol. Final dilution was 1:100 and sections counterstained with hematoxylin⁽²³⁾. Negative control sections did not receive primary antibody, otherwise they were treated identically (for all the antibodies tested) or using an isotype-matched control. Natural cartilage was utilized as positive control to verify the expression and production of collagens.

Statistics: Statistical significance was determined by one-way ANOVA with s post hoc test or multivariate ANOVA with the Tukey test. Correlations among groups were assessed using Pearson's test (two-tailed). A value of $p=0.05$ was selected as the threshold of statistical significance.

Results

Bioreactor

The biomimetic bioreactor was portable and easy to handling and assembling/ disassembling. The system mimicked anatomical analogue of TMJ. When tissue construct was placed on cell culture chamber, the dynamic mechanical device applied a linear mechanical displacement of 2 mm to 10 mm at various frequencies while keeping the humidity and temperature (37°C, and 95% humidity) inside the chamber constant (Figures 1 and 2).

Computational fluid dynamics (CFD)

Results of CFD indicated very low wall shear stress on surface of culture chamber at flow rate 1 ml/min. Peak velocity and maximum wall shear stress were 1.706×10^{-3} m/s and $0.02407 \text{ dyne/cm}^2$ ($1 \text{ Pa} = 10 \text{ dyne/cm}^2$), respectively. Contour of pressure (Pascal) and velocity magnitude (m/s) are depicted in figures 3 and 4.

Chondrocyte distribution and morphology within alginate hydrogel

When chondrocyte distribution in alginate hydrogels has been examined by invert microscopy and compared with static control groups, the results revealed clearly that for alginate sheet, cell distribution was more homogeneous than the other constructs. It

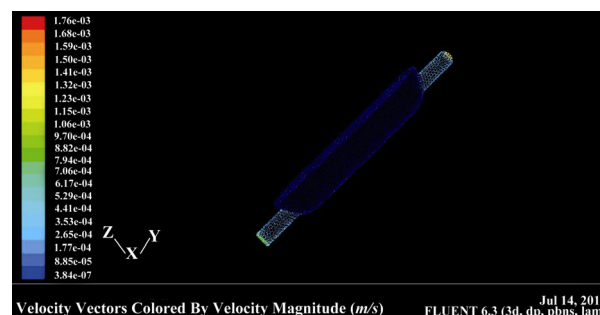


Figure 3. Contours of velocity magnitude (m/s) calculated for the culture chamber provided with an inlet flow velocity of 1cm/s

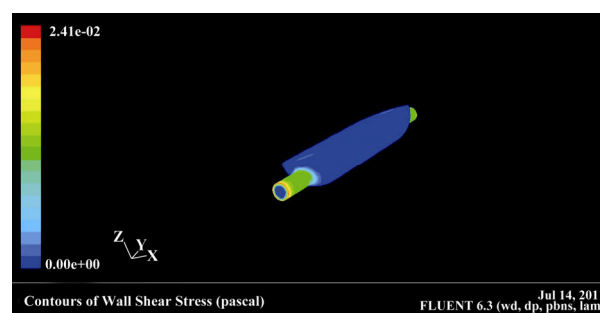


Figure 4. Contours of wall shear stress (Pascal). Colors represent wall shear stress levels (Blue, low, Yellow-Green, Intermediate)

may be due to control on thickness of sheets (Figures 5 and 6). In all casting methods in bioreactor, viable and round chondrocytes were observed. Alginate/chondrocyte sheet, bead and molded shape of alginate/chondrocyte successfully were cultured in bioreactor and have strength architecture to potential implantation. When compared, sheet and macroscopic shaped constructs could be cut to desired shape for cartilage implant (Figures 5 and 6).

Analysis of viable cells test revealed 60-70% for static and 80-90% for dynamic culture. There was no difference between alginate scaffolds.

Histology

Hematoxylin & eosin: Typical histological appearance of sections revealed evidence of cartilage-like tissue, such as lacuna and chondron formation. The lacuna housed mature and round-shaped chondrocytes. The morphology of these lacunas was similar to those of natural cartilage.

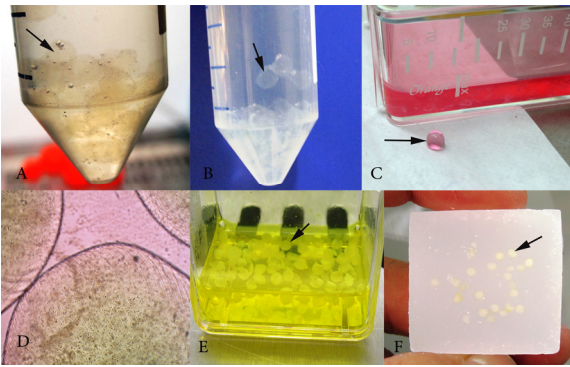


Figure 5. Preparation and culture of alginate/chondrocyte beads. Arrows indicate single bead. A) Polymerization of alginate/chondrocyte beads in CaCl_2 . B) Washing of alginate/chondrocyte beads in NaCl . C) Culture of alginate/chondrocyte beads in flask then in bioreactor. D) Invert microscopic observation of alginate/chondrocyte beads (three beads). E) Fixation of alginate/chondrocyte beads in bouin fixative. F) Embedding of alginate/chondrocyte beads in paraffin wax

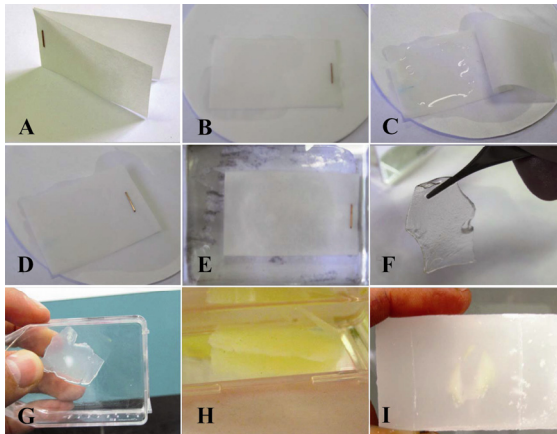


Figure 6. Preparation and culture of alginate sheets. A) Tow layer sterile filter paper was prepared. B) filter paper was soaked in CaCl_2 . C) hydrogel sheet was cast between two layer filter papers. D) sheet was allowed to instantaneous gelation. E) casting was transferred into CaCl_2 solution. F) single alginate/chondrocyte sheet. G) Alginate sheets cultured in flask. H) Fixation of alginate/chondrocyte beads in bouin fixative. I) Embedding of alginate/chondrocyte beads in paraffin wax

Alcian blue/neutral red: On histological examination, the newly formed matrix was stained blue with Alcian blue, at low pH, showing sulfated glycosaminoglycan secretion (such as chondroitin-6-sulfate) (Figure 7A).

When sections stained by hematoxylin/safranin O/fast green method, the cationic dye safranin O stained the produced sulfated glycosaminoglycan of matrix by the chondrons intensely red (Figure 7B).

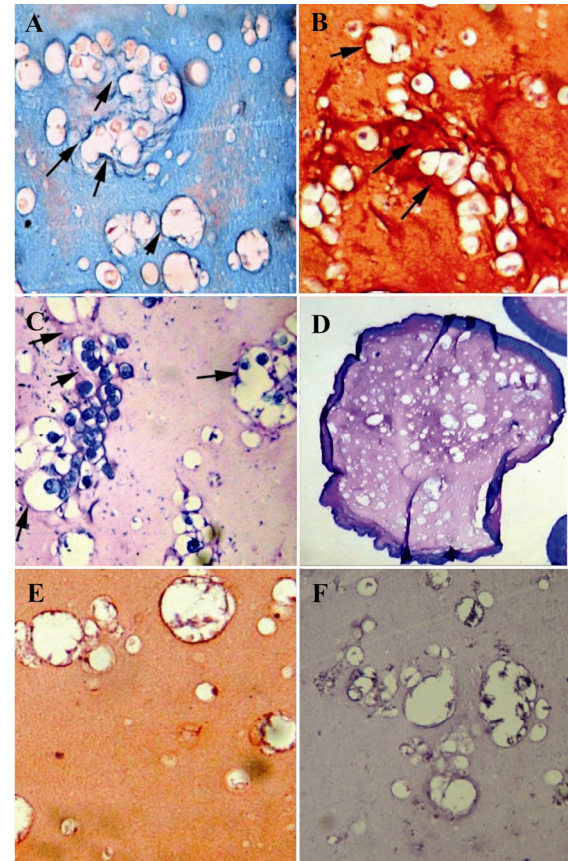


Figure 7. Histochemical examination of tissue constructs cultured in biomimetic bioreactor and culture flask. Arrow indicate newly formed matrix (magnification of A, B, C, E and E 20 \times , for D 5 \times). A) After 2 weeks cultivation of alginate/chondrocyte sheet in biomimetic bioreactor, newly secreted matrix with lacuna formation around chondrocytes as shown by alcian blue staining. Nuclei appear red. B) After 2 weeks cultivation of anatomic shaped of alginate/chondrocyte in biomimetic bioreactor. The cationic dye safranin O binds to stained glycosaminoglycan intensely red. C-D) The histological examination of the alginate/chondrocyte beads cultured in bioreactor with toluidine blue revealed that it contained a cartilage-like matrix that accumulated within the lumen of clusters of round cells and chondron were surrounded by remnants of territorial matrix. E-F) Control group (static culture in culture flask) when stained with safranin O/ hematoxylin and hematoxylin/eosin, respectively

Toluidin blue: The histological examination of the all tissue constructs with toluidine blue revealed that they contained a cartilage-like matrix that accumulated within the lumen of clusters of round cells. And chondron were surrounded by remnants of territorial matrix. Because of their high content of acidic radicals in their sulfated glycosaminoglycans, chondrocyte granules displayed metachromasia. Sections showed the strong staining of metachromatic territorial matrix-surrounded

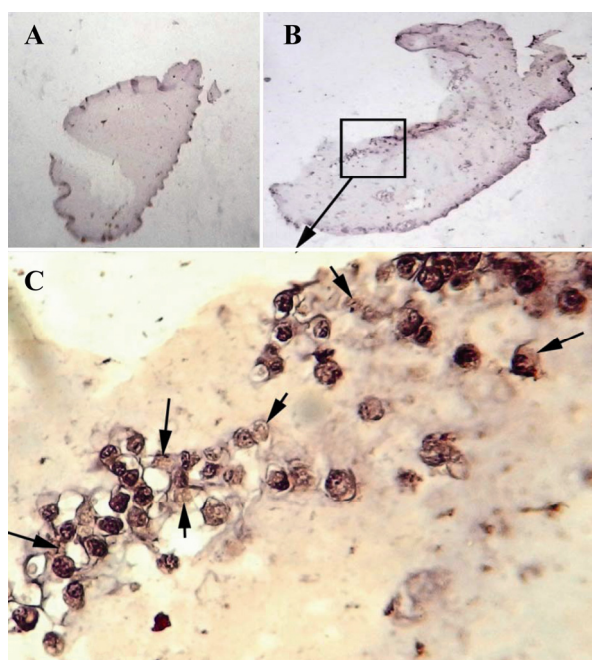


Figure 8. Effect of perfusion bioreactor culture on collagen type II. The control A) exhibited less immunostaining for type II collagen then bioreactor group B, C) (magnification of A, B are 5× and C 20×)

cells and they changed the color of toluidine blue to purple/ red (Figures 7C and 7D).

Sections of bioreactor cultured constructs showed the strong staining of metachromatic territorial matrix-surrounded cells, produced sulfated glycosaminoglycan of matrix by the chondrons when compared with control static groups (Figures 7E and 7F).

Immunohistochemistry

The control group exhibited less immunostaining for type II collagen then bioreactor group. Immunohistochemistry showed a strong presence of cells staining for type II collagen in alginate from all tissue constructs. There was no difference between alginate constructs (Figure 8).

Discussion

We report an approach for tissue engineering of anatomically shaped cartilage grafts, starting from mature chondrocyte. This approach is based on a bioreactor capable of (i) housing construct sheet and anatomically shaped tissue, and (ii) providing controlled interstitial flow through the tissue chamber. Results of our study demonstrated that when

chondrocyte is encapsulated in alginate hydrogel and cultured in biomimetic anatomically shaped cell culture system, cells survived well and secreted newly synthesized matrix consisting of GAG and collagen, leading to improvement of chondrogenesis *in vitro* to potential cartilage implant.

The advent of tissue engineering provides generation of tissues outside the body by using cell, scaffolds and cell culture systems. Because of its mild gelling and biocompatibility and biodegradability properties, alginate has long been used in cell microencapsulation for the fabrication of tissue *in vitro* ^(8,9).

But for transplantation, these tissues should comply some criteria such as accordance with shape and exact geometry in natural body. Several organs and tissues in the body such as articular cartilage, skin, and *etc.* have multi-layered structures. Therefore many studies investigated ability to recapitulate stratified tissue of cell-based construct ^(24,25).

Several methods to fabric sheet scaffolds have been proposed. In a study by Park et al, 2004 alginate gel-coated paper was prepared by using a filter paper of a diameter of 5.5 cm immersed in a sodium alginate solution ⁽²⁶⁾. Also Ladet et al, 2008 produced layered, multimembrane hydrogels from alginate and chitosan using start-stop, interrupted gelation techniques. They proposed that these so-called 'onion' structures can be used in tissue engineering for various layers for different drug concentrations, cellular encapsulation, bioadhesives applications ⁽²⁷⁾. Richard et al, 2010 showed a method for sheet tissue engineering strategy using thin and elastic Crosslinked Urethane-doped Polyester (CUPE) scaffolds ⁽²⁸⁾.

Cartilage, in particular articular cartilage has zonal structure (superficial, middle, and deep). But few investigators such as Han et al, 2008, Lee 2007 and Ng et al, 2005 examined the organization of cartilage as a basis for the development of the stratified tissue engineering ⁽²⁹⁻³¹⁾. Therefore, in an attempt, we introduced a scaffold fabrication method and cultivation of cells in biomimetic bioreactor as an

approach in cartilage tissue engineering. By this method, also diverse tissue and organs such as skin, blood vessel and trachea can be engineered. In a study, Tritz et al, 2010 built up the biomaterials in the form of thin alginate sheets through progressive cells and hydrogel spraying method⁽³²⁾. Gleghorn et al, 2008 created laminated alginate gels through a several step process⁽³³⁾.

In previous studies the cell-sheet tissue engineering strategy were proposed to regenerate several types of tissues, however for generation of anatomically shaped construct of a large size, methods of scaffolds fabrication and cell culture system needs to be developed and customized. There are a limited applicable methods to reform the complex geometry of anatomically shaped constructs and previous investigation have documented the production of cartilage by injection of alginate *in vivo*, but in irregular and irreproducible shapes. We introduced a cheap, simple and reproducible molding method of alginate for large size with anatomically shaped construct for cartilage tissue engineering. When tissue construct can be fabricated in large macroscopic shape, it can be cut to desired complex geometry such as meniscus to correct grafts with shapes exactly matching those of the patient. Therefore, one of the main advantages of the introduced molding is they can be cut to desired anatomical shape after growing tissue construct in bioreactor. Chang et al, 2001 developed a method to create structures of complex geometry to form cartilage in specific shapes. They used several standard facial implants (nose bridge, chin, malar, and nasal septum) as templates and chondrocyte/alginate constructs were molded in these shapes⁽¹²⁾. Also Alhadlaq A, et al 2003, Abukawa H, et al 2004 and Weng et al, 2001 investigated generation of craniofacial bone grafts such as mandible condylar reconstruction⁽³⁴⁻³⁶⁾.

To maintain, promote construction maturation, and to match anatomy of specific implantation site, cell culture system should be developed. Therefore several biomimetic bio-

reactors were designed and focused on the ability to recapitulate the anatomical shape of cell-based constructs *in vitro*. Our attempt to design of temporomandibular joint analogue was in accordance with this need. Grayson et al, 2010 introduced an approach for creating *in vitro* entire bone condyle containing viable cells by human Mesenchymal Stem Cells (hMSCs) on a decellularized bone scaffold that had the exact geometry of the TMJ, using an “anatomical” bioreactor⁽³⁷⁾. Also Stojkowska et al, 2010 utilized a biomimetic bioreactor with mechanical stimulation for evaluation of alginate hydrogels as cell supports in cartilage tissue engineering that resulted in cell proliferation⁽²⁰⁾.

One of the major advantages of the novel bioreactor is maintaining the sterility inside the bioreactor during construct growth and development. We tested the operation and sterility before tissue construct fabrication. In addition, assemble of bioreactor performed in sterile laminar hood after autoclave of all parts. The tissue chamber has multiple pores and an inlet for the media to exactly replicate the perfusion process in the body. Our introduced scaffolding molding methods are cheap, simple, reproducible, and applicable for diverse tissue and organs such as cartilage, skin, blood vessel and trachea.

With a few design modifications and additions, the bioreactor will be much simpler and more efficient. All parts inside the bioreactor can be made from Plexiglas to avoid any contamination and easy handle. The tissue chamber can be redesigned to mimic anatomically shaped tissue and organs. Bioreactor can be redesigned to incorporate linear motor control and data acquisition.

Taken together, our results indicated that, biomimetic bioreactor increased efficacy of cell culture technique and scaffold methods maintained phenotype of chondrocytes *in vitro* to produce a matrix, consequently improved fabrication of cartilage tissue to implants.

Conclusion

In summary chondrocyte encapsulation in alginate hydrogel and culture in biomimetic bioreactor that apply several biomechanical stimuli such as direct compression and fluid flow induced shear stress, improve synthesis of GAG and collagen by mature chondrocyte.

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