Tissue Engineering of Cartilage from Human Adipose Tissue

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Tissue Engineering of Cartilage from Human Adipose Tissue

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June 2005

감사의 글

감사합니다.

초여름 싱그러운 녹색이 산천에 물드는 아름다운 계절입니다.

여기 이 자리에 오기까지 여러 면에서 도와주시고 이끌어주신 정윤규 선생님, 개인적으로도 앞으로 살아가야할 제 삶의 방향을 몸소 보여주시고 계셔서 항상 존경과 흠모의 염을 가지고 살고 있습니다.

막바지에 미국에서 연구와 강의를 하고 있는 성도유 선생님은 논문 작성과 교정에 열 일 마다하고 무한한 도움을 주셨습니다.

이 자리를 빌려 도움을 주신 여러분들의 관심과 애정에 감사드립니다.

개업해서 많은 어려움을 겪으면서도 학위논문을 준비할 수 있었던 것은 가족들의 믿음과 성원이 없었다면 견디기 힘든 나날들이었을 겁니다.

앞으로도 이런 여러분들의 기대와 성원을 저버리지 않도록 열심히 성실히 살겠습니다.

민 완 기 올림

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Abstract

The fact that cartilage has little self-healing potential reflects the reason for vast research in this field. Recent evidence indicates that processed lipoaspirates (PLA) in adipose tissue may be a source of chondrocytes which produce matrix proteins of cartilage. The present study was to confirm the above observation and to investigate the feasibility of fibrin glues as a scaffold for cartilage tissue engineering approach. The results showed that the cells differentiated from PLA in fibrin glue were positive for proteoglycan, cartilage matrix protein, using Alcian Blue staining technique. Moreover, these cells also expressed type II and X collagen, aggrecan, and phenotypes of chondrocytes by reverse transcriptase-polymerase chain reaction technique. The findings of this study indicate that fibrin glue may be a successful carrier of chondrocytes in well-defined nodules from PLA.

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Introduction

Cartilage, a combination with chondrocytes and extracellular matrix, is widely distributed in the human body. Unfortunately, the remodeling rate of cartilage is so slow that the damaged cartilage has been shown to have a limited potential of repair. Eventually, the extended injury area of cartilage to the subchondrol bone is substituted by fibrocartilage, which has inferior quality to the original cartilage. Thus, it is necessary to explore a promising method for the repair of cartilaginous defects.

Within decade, cartilage tissue engineering is most like to benefit from a source of autologous multipotent stem cells such as bone marrow stroma.

Among the many cells in the bone marrow, mesenchymal stem cells (MSCs) are capable of differentiating into chondrogenic cells. However, the clinical use of mesenchymal stem cells (MSCs) has several obstacles, including pain and incontinence in aspiration of bone marrow from spine and low cell yield upon harvest. Recently, the human adipose tissue has been reported to have multi-lineage cells (processed lipoaspirate, PLA).[1-5] The adipose tissue can be significant as a source for mesenchymal stem cells due to its volume and accessibility.

Furthermore, if the tissue-engineered cartilage can be produced in an injectable form, minimal invasive chondrocyte implantation would be possible. The ideal, injectable, tissue-engineering polymer should be non-reactive, maintain a three-dimensional configuration, and degrade at the appropriate rate. Fibrin glue, a non-reactive and rapidly degrading biologic polymer, offers several advantages over various polymers. By using a thrombin glue, which has decreased concentration of the thrombin component on the fibrin glue, the fibrin substrate remains liquid enough to allow percutaneous injection of the polymer. As the glue polymerizes, it should mold into any form regarding its surrounding structure. It has been shown that fibrin-based polymers produce high quality neocartilage when seeded with chondrocytes,[6] This would be ideal for the neocartilage to be delivered in a minimal invasive fashion.

This paper examined the chondrogenic potential of multipotential cells from human adipose tissue and to develop a tissue-engineered cartilage using fibrin glue.

Materials and Methods

Fat harvest:

After informed consent and approval, fat was harvested from transverse rectus abdominis flap, abdomen liposuction, or abdominoplasty procedures.

Extraction of stem cells:

The raw fat was washed extensively with sterile phosphate buffered saline (PBS) to remove blood cells, saline, and local anesthetics. The extracellular matrix was digested with 0.07% collagenase (Sigma. St. Louis, MO, USA) at 37°C for 30 minutes to release the cellular fraction. And then collagenase was inactivated with an equal volume of Dulbecco Modified Eagle Medium (DMEM: GibCo, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS: GibCo, Carlsbad, CA, USA). The infranatant was centrifuged at 250 g for 10 minutes to obtain a high density PLA. The pellet was resuspended in DMEM and 10% FBS and then mixed with an erythrocytes lysis buffer (0.16M NH4Cl) for 10 minutes to lyse contaminating erythrocytes. After an additional centrifugation step, PLA was resuspended in DMEM and 10% FBS and plated in 100mm tissue culture dishes at a density of 1×10^7 cells per plate. PLA was maintained in control medium (control medium was composed of Dulbecco Modified Eagle Medium, 10% fatal bovine serum, 1% antibiotic/antimycotic).

The culture medium was changed twice weekly. Confluent PLA (approximately 80% confluence) was passaged at a ratio of 1:3 in trypsin/ethylenediaminetetra -acetic acid (GibCo, Carlsbad, CA, USA).

Chondrogenesis:

1×10^5 PLA was plated into the center of each well (a 6 well plate) and allowed to attach at 37°C for 2 hours. PLA was maintained in chondrogenic medium (CM) consisting of DMEM, 10% FBS, 100 ng/ml of insulin (Sigma, St. Louis, USA), 50 ug/ml of ascorbic acid-2-Phosphate (Sigma, St. Louis, USA), and 5 ng/ml transforming growth factor β-1(Biosource, Carmarilo, CA, USA) for a week. After one week, cells differentiated from PLA was harvested with collagenase and collected by centrifugation at 250 g for 10 minutes. The supernatant was removed and the cell pellet was added with 10 ml of CM to make a concentration of 1×10^6 cells/ml. The cells in 10 ml of CM were overlaid on prepared fibrin glue (80 mg/cc, a mixture of 0.25 cc of thrombin and 0.25 cc of fibrinogen, Baxter, Deerfield, IL, USA) in 50 ml of tube and then were centrifuged to allow the cells to enter fibrin glue. Entrapped cells were centrifuged everyday and incubated with CM at 37°C with 5% CO_2 for 2 or 6 weeks for the analyses.

Analysis to confirm chondrogenesis:

-Staining

At 2 and 6 weeks of incubation period, fibrin glue containing cells were

fixed with 4% paraformaldehyde for 15 minutes at room temperature, washed with several changes of PBS, embedded in paraffin, and cut with the size of 6μm. Sliced paraffin were mounted on slides and stained using standard hematoxylin and eosin (H&E) according to the instruction of protocol or incubated with 1% (wt./vol.) Alcian Blue (Sigma, St. Louis, MO, USA) in 01.N HCl (pH 1.0) for 30 minutes. Digital images were obtained using a Zeiss Axioskop II microscope and Spot software.

-Extraction of RNA/gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR)

At 6 weeks of incubation period, total RNA was isolated from PLA and differentiated chondrocytes from PLA in fibrin glue using Trizol (Invitrogen, St. Louis, USA). Isolated total RNA was denatured at 70°C for 5 minutes and cooled on ice. And then incubated with dNTP and MMLV-RT (Timesaver cDNA synthesis kit, Amersham biosciences, Piscataway, NJ, USA) for a minimum of 2 hours at 42°C. For PCR amplification of the cDNA, used primer pairs designed to the following genes were presented in Table 1 (F=forward oligo, R=reverse oligo). cDNAs using PCR were amplified by 95°C for 1 minute, 53°C for 1 minute, 72°C for 1 minute with 35 cycles and then the PCR products were extended at 72°C for 5 minutes for the end of reaction. PCR using primers to ß-actin was used as a positive control. PCR products were resolved using convectional agarose gel electrophoresis.

Table 1. Primer sequences of gene products for RT-PCR

Gene		Sequence	Size(bp)
ß-actin	F	5'-TGTGATGGRGGGAATGGGTCAG-3'	360 bp
	R	5'-TTTGATGTCACGCACGATTT-3'	
Type ll collagen α1	F	5'-CCAAGTACTTTCCAATCTCAGTCAC-3'	328 bp
	R	5'-ACAGAAAGCACCATTGTGTAGGAC-3'	
Aggrecan	F	5'-GCGATATCATGACCACTTTACTC-3'	500 bp
	R	5'-CCTGTCAAAGTCGAGGGTGT-3'	
Type X collagen	F	5'-TGGAGTGGGAAAAAGAGGTG-3'	600 bp
	R	5'-GTCCTCCAACTCCAGGATCA-3'	

Results

This study examined the chondrocyte lineage potential of mesenchymal stem cell population obtained from human fat tissue. Raw human fat tissues were used to obtain stem cell fraction, PLA (Fig-1). The PLA was cultured in CM at 1 day and 6 days (Fig-2) The nodules stained with H&E contained chondrogenic cells within lacunae surrounded by abundant gel-like extracellular matrix at 2 weeks (Fig-3). At 2 weeks, nodules also positively responded with the staining of Alcian Blue, an indicator of proteoglycan which is a cartilage matrix protein (Fig-4). At 6 weeks, a enhanced definition of PLA derived cartilages are shown using H&E and Alcian Blue staining (Fig-5 and 6). The RT-PCR analysis confirmed the presence of phenotype of chondrocytes, e.g., type II collagen and aggrecan in all nodules (Fig-7). Type X collagen, which is an indicator of progression change into hypertrophic chondrocytes was also present (Fig-7). On the other hand, PLA did not express any phenotype of chondrocytes (Fig-7).

Discussion

The results in this paper confirm chondrocyte formation from adult stem cells in fat tissue. Also this study shows the possibility of successfully producing a tissue engineered cartilage within liquid polymers. The healing of articular cartilage defects with bone marrow derived stem cells has been successfully demonstrated in small animal models.[7-9] However, the use of bone marrow may have several disadvantages compared to that of fat tissue for stem cells. Harvesting stem cells from fat tissue reduces pain and morbidity of the donor site associated with harvesting of autogenous chondrocytes or bone marrow. The abundant number of cells in fat tissue eliminates a second stage procedure for reimplantation of the induced cells. Previous studies have shown that a bone marrow aspirate of 30ml produces approximately 1×10^5 cells, while a liposuction aspirate yields approximately 3.3×10^4 cells/mm³ of fat.[10] Furthermore, the fat cells have high differentiation capacity, leading to relatively easily transferred to chondrocytes. Nonetheless, further laboratory and clinical work would be needed to evaluate the efficacy and density of the actual cartilage formed.

Cartilage is composed of a mixture of collagen fibrils and proteoglycans, which has the high tensile strength.[11,12] Alcian Blue staining confirmed the presence of proteoglycan within the processed lipoaspirates in the first two weeks. The expression of type II collagen in RT-PCR analysis is highly suggestive of cartilage produced in a nonchondrogenic cell type origin. The

expression of type II collagen in processed lipoaspirate cells is consistent with its detection in embryonic tissue and precartilage mesenchymal precursors before chondrogenic differentiation. Aggrecan, as shown in the analysis, has been also demonstrated to accumulate at the onset of overt chondrogenesis, coincident with cellular condensation. Taken together with these expression, it is well indicative of chondrogenic capacity of processed lipoaspirate.

The initial studies on the formation of neocartilage have been reported that the possible use of solid polymers such as polyglycolic acid polymer with chondrocytes was necessary to surgically place the solid polymer/chondrocyte into the nude mouse in the purpose of neocartilage construction. With a recent tissue application, this would reveal additional scars from surgical incisions. Recent advanced tissue-engineering techniques have led to the use of liquid form of biodegradable polymer, fibrin glue in the formation of neocartilage. By using polymers in liquid form, it will become possible to inject the mixture of chondrocytes and fibrin glue into various parts of the body and then to mold the preexisting anatomical form. Previous study demonstrated that subcutaneously implanted fibrin glue with articular or non-articular chondrocytes into nude mice resulted in the formation of cartilages. The finding of the present study for the first time shows that differentiated chondrocytes from PLA in adipose tissue are also able to form neocartilage in liquid form of fibrin glue. Further studies in an ***in vivo*** model will solidify its efficacy.

In conclusion, chondrogenic capacity of the adult fat cells was confirmed

using a liquid based polymer. Further study will reveal its clinical efficacy.

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국문요약

인간 지방 체세포에서 연골조직배양

<지도 교수 **정 윤 규** >

연세대학교 대학원 의학과

민 완 기

연골이 자기 회복 가능성을 적게 가지고 있다는 사실은 이 분야에서 많은 연구가 이루어져야 하는 이유를 반영한다. 최근 논문들에서 인간 지방체 세포의 processed lipoaspirates (PLA)가 연골의 matrix protein을 생성하는 연골 세포의 근원일 가능성들을 시사하고 있다. 이 연구는 위 가능성을 확인, 증명하기 위한 것이고 fibrin glue가 연골 조직 배양의 scaffold가 될 수 있다는 가능성을 연구한 것이다. 결과는 fibrin glue에 있는 PLA에서 분화된 세포가 proteoglycan, cartilage matrix protein을 생성한다는 것을 Alcian Blue staining을 통해 확인할 수 있었다. 이 세포는 또한 reverse transcriptase-polymerase chain reaction technique에 의하여 type II, X collagen, aggrecan, chondrocytes의 표현형을 발현했다. 이 연구 결과는 fibrin glue가 PLA에서 well-defined nodules에 있는 chondrocytes의 성공적인 carrier 일 수 있다는 것을 보여준다.

Key word: 연골 조직배양, 인간 지방 체 세포

Figure-1.

Processed human fat tissue into putative stem cell. 2-D (×100)

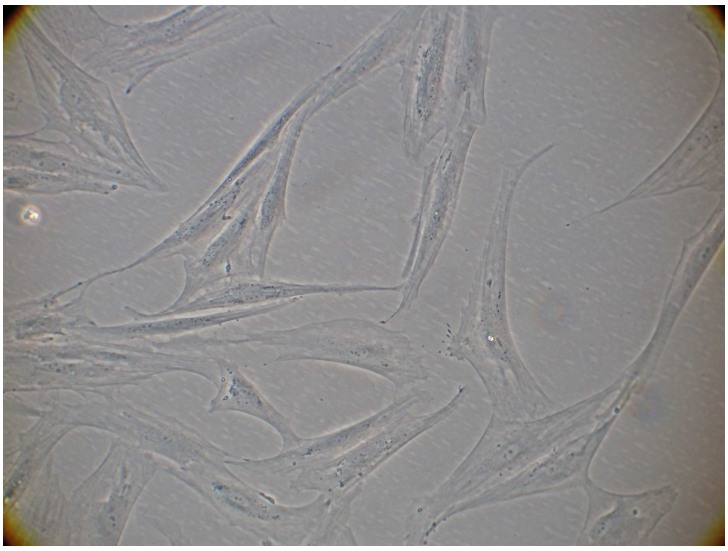
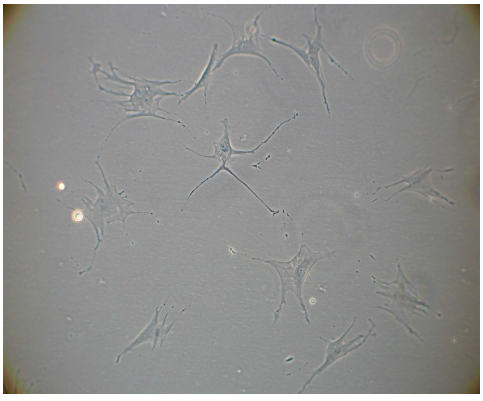


Figure-2.

Plated PLA transforming into chondrocytes in chondrogenesis medium (×100) after 1 day (left) and 6 days. 2-D (Right)



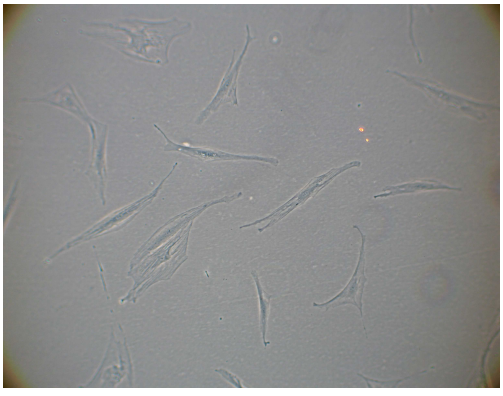
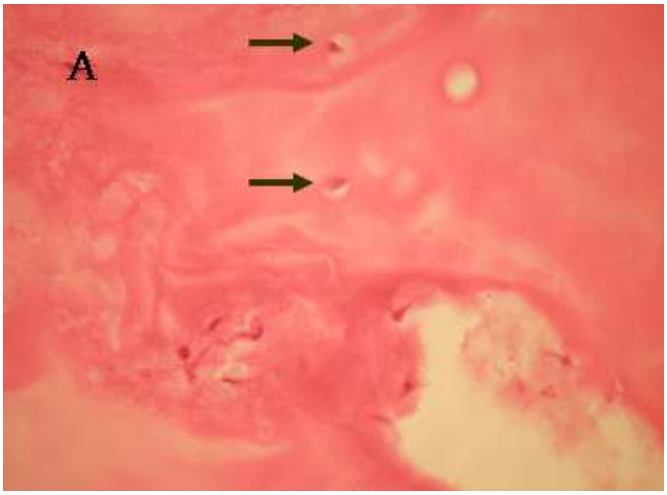


Figure-3.

Photographs showing ***in vitro*** chondrogenic induction of fat cells resulting in chondrocyte formation. 3-D

A: H&E ×200

B: H&E ×400



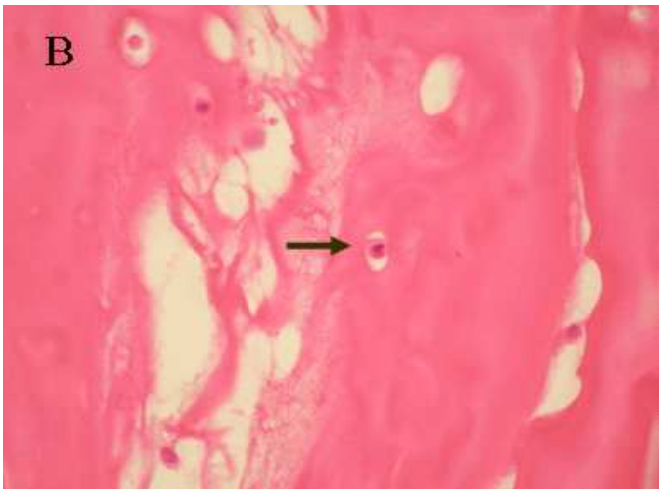
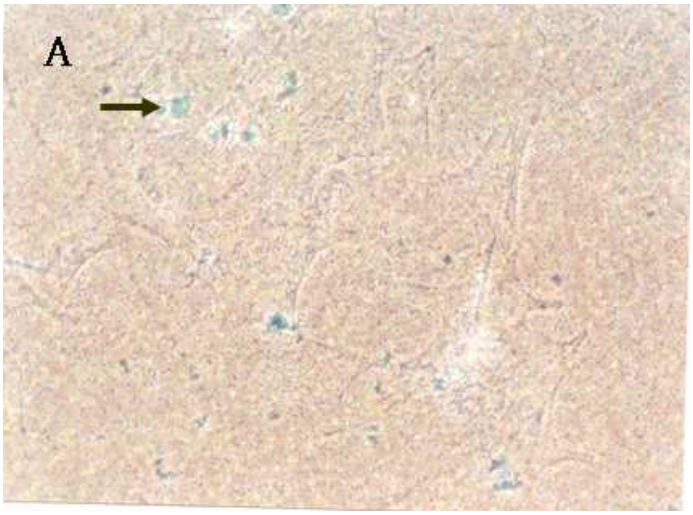


Figure-4.

Chondrogenic induction of fat cells confirmed by Alcain Blue at pH 1.0. 3-D

A: ×40

B: ×100



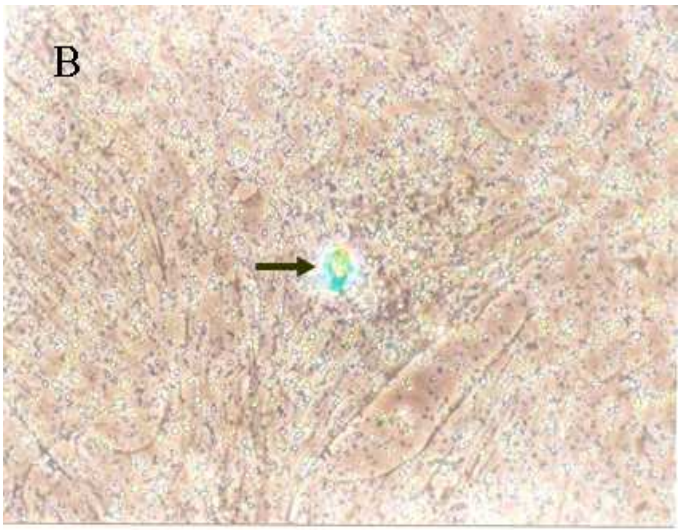


Figure-5.

PLA derived chondrocytes shown at 6 weeks. Note ***in vitro*** chondrogenic induction of PLA cells resulting in a transitory fibrohyaline cartilage. 3-D (H&E, ×200(A), ×400(B))

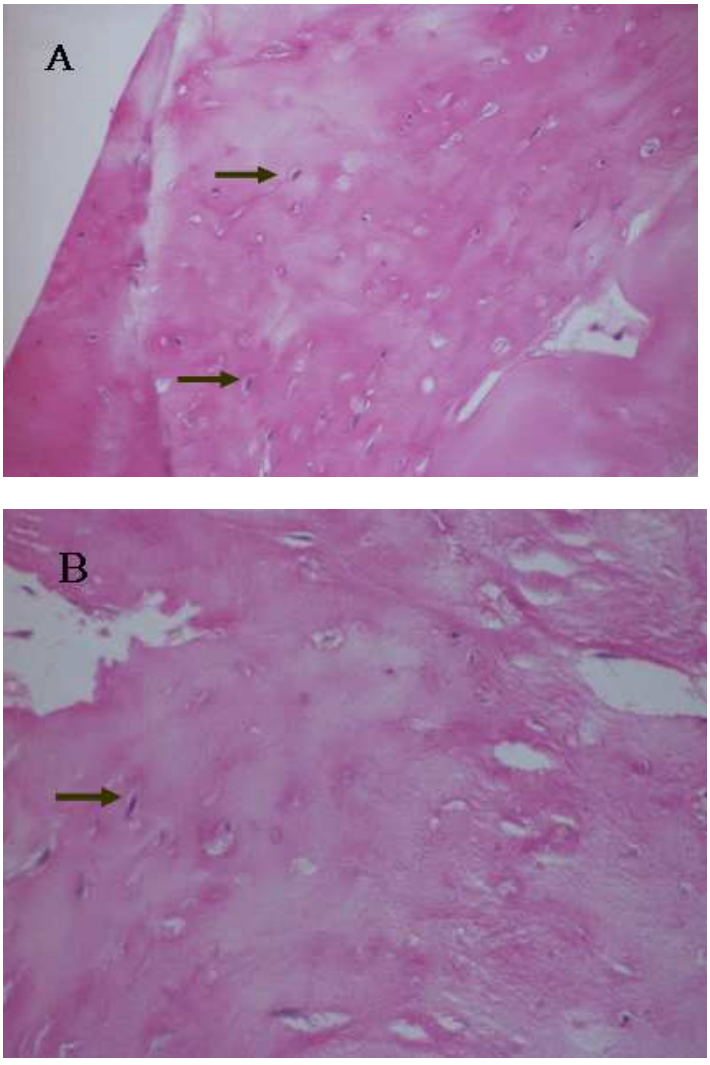
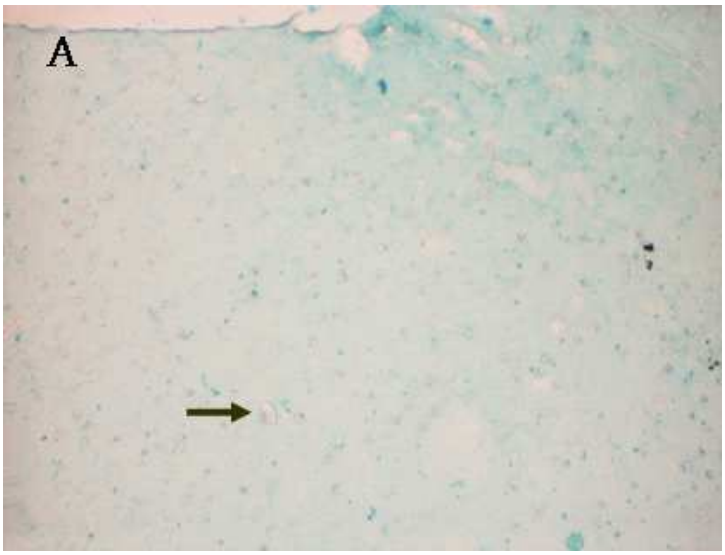


Figure-6.

Alcian Blue staining at 6 weeks. 3-D (×100(A), ×400(B))



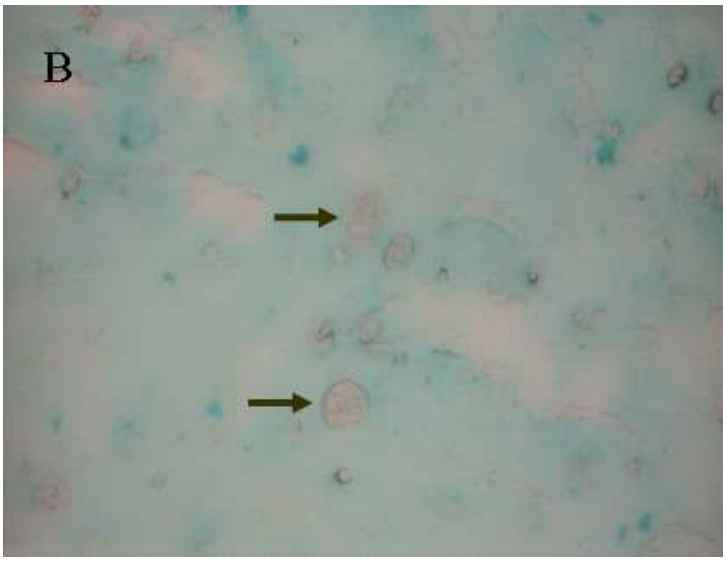


Figure-7.

RT-PCR analysis of cartilage at 6 weeks.

1. β-actin
2. Human type II collagen α 1
3. Aggercan
4. Human type X collagen