AUTOLOGOUS CHONDROCYTE TRANSPLANTATION
(An experimental study in rabbit knee joints)

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SUMMARY

Purpose: To repair cartilage and to restore joint surface by transporting chondrocytes into osteochondral defects.

Materials and Methods: The chondrocytes were isolated from cartilage tissue samples taken from the shoulder joints of 20 New Zealland rabbits, fed by GMMA Research Center Laboratory Animals Unit, by mechanical segmentation and enzymatic digestion performed at GMMA Department of Genetics. After the viability test and counting of the cells, chondrocytes were reproduced in a monolayer system in an atmosphere of 5 % CO_2 and 90 % humidity at 37 °C. Then chondrocytes that were induced to injectable form were transplanted to the defect caused in the rabbit knee and washed with hyalurinidase, and after that it was covered with periosteal graft taken from tibia proximal. Left knees of the rabbits were taken as the control group.

Results: The subjects were controlled for 12 weeks after the operation. After the fifth week, considerable improve in their walking was noted. In the 1st, 2nd, 4th, 6th, 8th, 10th, and 12th weeks 2 rabbits were sacrificed one at a time. In macroscopic findings, the surface of the joint of the newly formed tissue was smooth and transparent in appearance in the 12th week. In the same week, in histologic findings, it was seen that the defect was replaced by the hyaline cartilage. There was no significant difference between the control and study groups in synovial inflammation.

Conclusion: It can be concluded that autologous chondrocyte transplantation is a new treatment method in joint cartilage defects but more research is needed to improve this method.

Key Words: *Articular Cartilage, Autologous Chondrocyte, Cell Transplantation.*

ÖZET

OTOLOG KONDROSİT TRANSPLANTASYONU

(Tavşan diz ekleminde deneysel çalışma)

Giriş: Her yıl çok sayıdaki insanı etkileyen eklem kıkırdak defektlerinin tedavisinde hala tatmin edici sonuçlara ulaşılamamıştır. Biz bu çalışmamızda kondrositleri kullanarak kıkırdak tamirini ve eklem yüzeyi restorasyonunu amaçladık.

Gereç ve Yöntem: GATA Araştırma Merkezi deney hayvanları bölümünde bakılan, 20 adet Yeni Zelanda tavşanının omuz ekleminden steril koşullar altında alınan kıkırdak doku örneklerinden GATA Tıbbi Genetik BD'da mekanik parçalama ve enzimatik digesyonu takiben kondrositler izole edildi. Canlılık testi ve hücre sayımının yapılmasından sonra monolayer sistemde 37°C'de, %5 CO_2 ve %90 nem içeren inkübatörde kondrositler çoğaltıldı. Daha sonra enjektabl hale getirilen kondrositler, tavşan dizinde oluşturulan ve hyalurinidaz ile yıkanan defekte transplante edilerek üzeri tibia proksimalinden alınan periosteal greft ile kapatıldı. Sol diz kontrol grubunu oluşturdu. 1., 2., 4., 6., 8., 10., ve 12. haftada ikişer tavşan sakrifiye edildi.

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Bulgular: 12 hafta boyunca takip yapıldı. Beşinci haftadan sonra tavşanların yürüyüşlerinde belirgin düzelme görüldü. Mikroskobik olarak 12. haftada yeni oluşan dokunun eklem yüzeyi düzgün ve şeffaf görünümdeydi. Histopatolojik olarak 12. haftada defektin hyalin kıkırdak ile replase edildiği gözlendi. Sinovyal enflamasyon açısından kontrol grubu ile anlamlı farklılık saptanmadı.

Sonuç: Sonuç olarak otolog kondrosit transplantasyonu eklem kıkırdak defektlerinde yeni bir tedavi alternatifidir ve yöntemin geliştirilmesi için daha geniş çalışmalar gerekmektedir.

Anahtar Kelimeler: *Eklem kıkırdağı, Otolog Kondrosit, Hücre Transplantasyonu.*

INTRODUCTION

In contrast to other tissues originated from epithelial and mesenchimal, highly differentiated mature joint cartilage does not have the capability of replacing new cells and tissues damaged by synthesized extracelluler matrix because it lacks vascular blood support and has a limited regeneration ability. Thus, it is insufficient in restoring its normal functions. Cartilaginous injuries, which are not penetrated to subchondral bone, cannot improve and develop and as a result this leads to the degeneration of articular cartilage. Therefore, developed therapy methods are needed in cartilaginous tissue damages[1]. Insufficiency (loss of function) and pain induced by joint cartilage damages led researchers to find ways to facilitate and increase cartilage repair. In order to have repaired or regenerated cartilage to function as joint tissue sufficiently, normal painless motion of synovial joint should be restored. Realization of this requires that; structure, composition, mechanical properties and strength of the repairing tissue should be similar to the surface of natural joint[2]. Under the light of the latest developments in understanding of biology, composition, metabolism and biomechanical properties of joint cartilage, new researches on joint cartilage offer hope about biologically based procedures as alternative to conventional methods (subchondral drillisation, abrasion, and spongiolisation) in the treatment of joint injuries and degenerative joint diseases. Since many different results are reported, the most suitable method in treatment of focal defects is not exactly known[2,3].

In this study, we aimed at repairing osteochondral defects induced by us in the rabbit knees, by using autologous chondrocytes reproduced through monolayer system and transformed into injectable form to be used in transplantation.

MATERIAL AND METHOD

Twenty healthy white New Zealand Rabbits, 10 male, 10 female, which are 1 year old and weigh approximately 2.250 gr. (min. 1.900 gr. – max. 3.000 r.) were used. They were taken from Chicken Upbringing and Development Farm (Ankara, Turkey) and taken care by Gülhane Military Medical Academy (GMMA) Research and Development Center (Ankara, Turkey), and all of them passed through veterinary control.

a. Taking Cartilage Tissue Sample: The rabbits were observed throughout the experiment. They were left hungry and antibiotic prophilaxy (Maxiporin 15 ml/kg) was started 12 hours before the operation, it was applied so as to give 3 dosages a day until 96 hours after the operation. Anesthesia of the subjects was induced by giving 10 mg/kg Ketas (Ketamin hydrochlorur 50 mg/ml, Parke-Davis) intramuscularly after premedication by injecting 0.1 ml/kg Rompun (Xylazin hydrochloride 23,32 mg, Bayer). Dissosiative anesthesia provided by these two medications continued for 55-65 minutes. Average duration of the operation was 20 minutes. After the anesthesia, right shoulder area of the subjects were shaved and disinfected. Subjects were fastened to the operation table so as to keep their right shoulders upwards, and the regions to which operation will be applied were sterilized and covered. With clinical approach, skin and subcutan connective tissues were dissected by applying 2cm incision to the right shoulder regions. Humerus tuberculum majus level was accessed by incising M. deltoidoscarpularis acromialis. Connective tissue around capsule was separated after lig. glemohumerale craniale was cut by dissecting musculus (m). cleidobrachialis and m. infraspinatus. Tendon of m. biceps brachia was cut, to reach the inner part of the joint. When any limitation was encountered during manipulation, in splitting m. cleidobrachialis and m. infraspinatus, a pressure was created by inserting a bend ended homeostatic clamp or an ecarteur to the region from inside to outside and by positioning joint in luxe position, processes to be implemented in caput humeri was facilitated. After reaching caput humeri, using the lancet numbered 11, samples of joint cartilaginous tissue were taken from different points (Figure 1).

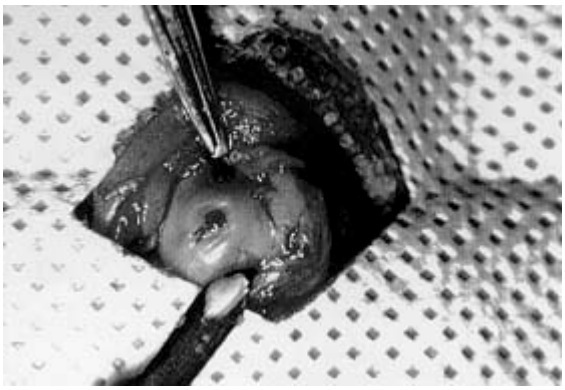

Fig. 1: Taking articular cartilage samples from rabbit shoulder joint.

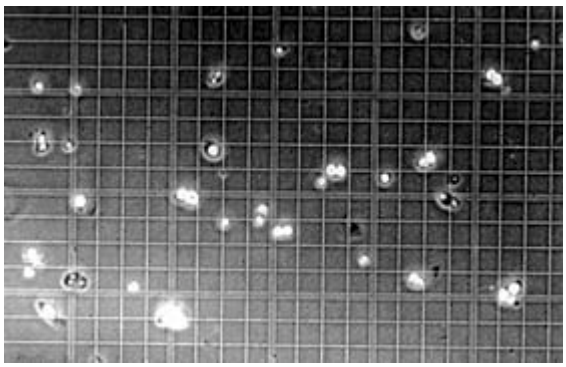

Fig. 2: The number of living cells were determined by the trypan blue viability test.

b. Transfer of Tissue Samples to Laboratory: Biopsy material was extracted from donor region under sterile conditions, and then it was taken to the laboratory in a sterile tube containing 10 cc transfer solution. Samples were washed 3 times with PBS or serum physiologic and then transferred to new tube. Until the experiment, it was kept in a refrigerator under +4°C[4].

c. Mechanical Segmentation Process: In a cabinet containing laminar air flow, cartilaginous tissue samples taken were firstly transferred to 35 mm petri dishes. Thereafter, they were neatly segmented into pieces smaller than 0,5 mm with the lancet numbered 15.

d. Decomposition of Chondrocyte with Enzymatic Digestion Process: Type II collagenase (Biochrom, Germany) and tissue culture medium were added onto the 35 mm petri dishes so as to have 1 mg/ml final concentration. It was kept in incubator at 37 °C, in an atmosphere of 5% CO_2 and 90% humidity for 12 hours. Then it was controlled using a Leica (Germany) inverted light microscope. If the decomposition was not completed totally, it was incubated for two or four more hours. When it was observed that all cells were decomposed, they were filtered using nylon filter and then after adding 10 ml culture medium centrifuged for 10 minutes at 1000 rpm. After disposing supernatant, 10 ml culture medium was added to the pellet part remaining at the bottom of the tube and it was carefully homogenized with a glass pipette and then mixed for a minute in vortex.

e. Determining Viability of Chondrocytes and the Number of the Living Cells: Trypan blue viability test was employed to estimate the number of the living chondrocytes (Figure 2).

f. Incubation of the Cells: Amount of the living cells were counted and; if there were less then 2 million cells, they were put into 25 cm^2 tissue culture flasks (Falcon, USA), if there were more than 2 million cells, then they were put into 75 cm^2 tissue culture flasks. Thereafter, they were placed in the incubator at 37°C, in an atmosphere of 5% CO_2 and 90% humidity with its cover half-closed.

g. Providing Subculture through Trypsinisation Process: Aim of this process was to decompose dead cells and to transfer produced living cells to bigger flasks. The material was first washed 7 times using sterilized PBS or RPMI 1640. Then, 3 ml Trypsin/EDTA (0,25/0,02) (Sigma USA) solution was added onto the material in order to cover flask base. It was incubated at 37 °C, in an atmosphere of 5% CO_2 in autoclave for a minute. Later on, 5 ml culture was added to it, and it was centrifuged at 1000 rpm for 10 minutes. Liquid left in the tube was disposed, and after adding 5 ml culture medium, it was homogenized with a glass pipette. This process was repeated 10 times and then suspension was put in the flask and placed in a 5% CO_2 incubator.

h. Providing Injectable Chondrocytes To Be Transplanted: In monolayer system, after 15 days chondrocytes started to cover the entire surface, and depending on the initial number of the cells, they became confluent in about 20 days (Figure 3). After this stage, culture flasks were taken out of the incubator and in the cabinet containing laminar air flow, top section of the plastic culture flask was cut using a cutter run by electricity which was made in GMMA Department of Orthopaedics and Traumatology Orthosis and Prosthesis Unit. Meanwhile, cover of the flask was kept open.

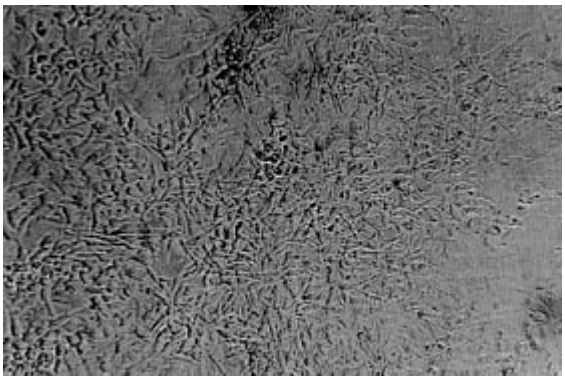

Fig. 3: The chondrocytes in confluent appearance, Zeiss inverted light microscope. X10

After the top cover of the flask was removed, chondrocytes adhered on the base of the flask were scraped using a cell scraper (Falcon, USA), and when it became jelly, it was aspirated with a pipette and collected in a 20 cc injector (Figure 4).

i. Inducing Osteochondral Defect in Rabbits' Knee Joint: On the 45th day of the postoperative period, subjects were prepared for the operation at the second stage of the study. They were left hungry 12 hours before the operation as it was done in the first operation, prophylactic antibiotic and anesthesia protocol were employed. Left and right knee regions of the subjects were shaved and disinfected, and they were positioned on the operation table so as to keep their right extremities upwards. Knee joint was reached using lateral parapatellar approach method. After 2,5 cm skin incision, subcutan connective tissue was passed. While dissecting as retinaculum bluntly, fascia lata was cut and after incision, through 0,5 cm lateral to lig. patellar, inner part of the knee joint was reached. While lig. collateral lateral was secured, through vastus

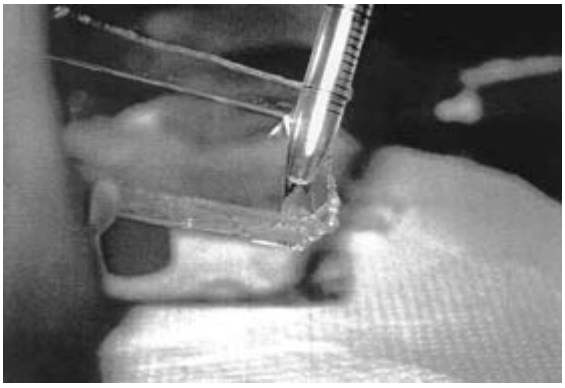

Fig. 4: Preparation of injectable chondrocytes.

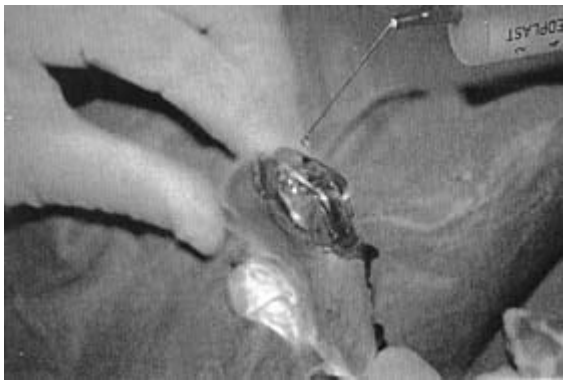

Fig. 5: Defect were washed using hyaluronidase (1U/ml) for 5 mins. in order to remove the antiadhesiveness.

lateralis of m. quadriceps and m. biceps femoris, patella mediale was deviated, so the joint was totally opened up. With the lancet numbered 11, an osteochondral defect with 3 mm diameter was induced on the lateral surface of medial condyle. The same defect was induced on the left knee joint.

j. Washing Cartilage Surface with Glycotic Enzyme. After inducing osteochondral defect in femur medial condyl bilaterally in all subjects, defects in the right knee joint of the subjects were washed using hyaluronidase (sigma, UK) for 5 minutes until its final concentration became 1 U/ml (Figure 5). Purpose of this process was to remove antiadhesive character of the region by depolymerising GAGs.

k. Transplantation of Autologous Chondrocytes to Osteochondral Defect: Jelly autologous culture, which had been induced to injectable form, was filled with chondrocyte graft. Periosteal graft with 4 x 4 mm diameter, which was taken from proximal tibia or lateral femoral condyl, was attached using Vicryl (Ethicon, USA) 4/0 suture material, facing the defect. Except for one subject, due to the small size of knee joint, suture could not be placed in the others. Therefore, in all subjects periosteal graft was attached using fibrin adhesive (Tisseel, Immuno AG, Austria) which freezes when its two parts come together. After two minutes, it was observed that the fibrin adhesive freezed (Figure 6). Having checked that the position of the defect was correct, knee joint was washed using serum physiologic and rifocine mixture. Then it was closed in accordance with the layer method. Control group was formed by leaving osteochondral defects induced in left knees of the subjects to self-cure without any process.

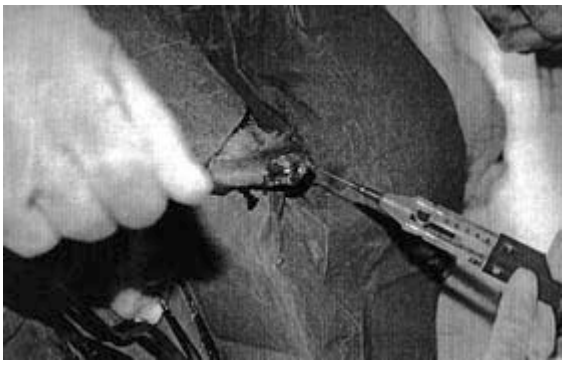

Fig. 6: Attachment of periosteal graft by using fibrin adhesive (Tisseel, Immuno AG, Austria).

Clinical, radiological and histopathological control of the subjects continued till the postoperative 12th week. At the end of 12th week, subjects were sacrificed, bilateral knee joints were disarticulated, and they were sent for histopathological examinations.

RESULTS

During the study, since one rabbit died because of pulmonary infection, one because of heavy infection, and two because of unknown reasons, they were not included in the research. Furthermore, since the culture of the cells in monolayer system, which were isolated from the samples of cartilaginous tissue taken from two rabbits, were infected on 12th and 14th days, these two rabbits were also excluded from the study.

In 1st, 2nd, 4th, 6th, 8th, 11th and 12th weeks, two rabbits were sacrificed at a time. Before the subjects were sacrificed, their walking was observed. Just after they were sacrificed, their knee joints were opened. Femur joint surface and synovial membrane were observed macroscopically. Then, femur distal section was resected as *en block* of femur. Fixation was employed in 10% formaline. Following this process, it was saved in 10 % formic acid for decalcification for 7 days. Cross sections provided were examined histopathologically using hematoxylen eosin dye (HE). Then they were transferred to sagittal plane so as to include longitudinal axis of femur. At the same time, intrapatellar section of synovial membrane of knee joint was also extracted and fixed in 10% formalin, then it was painted with HE.

In histological evaluation of the cross sections taken from distal femur, attention was paid to the following points. (1) Type of the tissue degenerated in the surface of cartilage: hyaline cartilage, fibrocartilage or fibrous tissue; (2) statue of surface of the cartilage; smooth, depressed or irregular; (3) existence or non-existence of regenerated subchondral bone; and (4) situation of the inflammatory cells around the regenerated tissue.

In order to decide whether the formed tissue is hyaline cartilage or not, O'Drisscoll, et all. criteria was employed[3,5]. If the surface of the regenerated tissue was smooth, if its surface was the same with that of the surrounding joint and if it was covered with hyaline cartilage, then the graft was considered successful. Furthermore, if infiltration of inflammatory cells were low, and if regeneration of the tissue surrounding subchondral bone was realized until osteochondral junction level, it was also considered successful.

Cross sections taken from synovial membrane were evaluated according to the modified Lukoschesk's Ranking Sequence: 0 (normal), 1 (very little inflammation), 2 (light inflammation), 3 (moderate inflammation) and 4 (serious inflammation)[3,5].

Macroscopic findings: All of the animals were living under normal conditions before they were sacrificed. In the 2nd week; the newly formed tissue was white and depressed in appearance. Its borders could be clearly differentiated from its environment. In the 4th week; although the newly formed tissue was still white and its borders could be differentiated, it was still smooth. In the 12th week; the surface of the joint of the newly formed tissue was smooth and transparent in appearance. Borders started to become uncertain.

In the control group:

In the 2nd week; the newly formed tissue was white and a little depressed in appearance. In the 4th week; the tissue was white or pink and some sections were deep red like granulation. In the 12th week; in appearance, the newly formed tissue was fairly similar to the joint cartilage in its environment.

Histological Findings: In the second week, after implantation, although the central area of the joint surface seemed to be depressed, a part of the defect was filled with hyaline cartilage. In its contour, a light inflammatory cell infiltration was observed. In the fourth week, there was still hyaline cartilage formation and very little depressed appearance in the central part of the defect. Around the defect, very light inflammatory cell

infiltration was observed. In the control knees, although there was little bone formation in the deeper part of the defects, they were still filled with fibrous tissue. In both subjects, surface of the joint appeared to be a little depressed. In the 12th week, deeper sections of the defect were about to transform into osseous tissue in which trabecular pattern close to normal was seen. In peripheral parts, cartilaginous tissue was observed. It was also observed that surface of the joint was covered with cartilaginous tissue and it was smoother, and that there was little inflammatory cell infiltration in peripheral parts (Figure 7). In the control defects, it was observed that bone formation was developed in deeper parts and surface of the joint was still covered with fibrous tissue (Figure-8).

Synovial Inflammation: According to the modified Lukoschek's Ranking System, points for the two knees were; in the 2nd week 2, in the 4th week 2, in the 6 week 1 or 2, in the 8th, 10th, and 12th weeks 0 or 1 points. No clear difference was found between the groups.

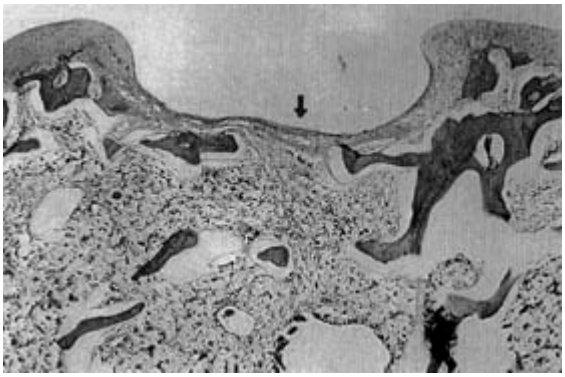

Fig. 7: In control group, in 4th week, granulation tissue, i. e. a tissue that ends with fibrosis and continues with cartilage tissue, is seen in the defect. Slight synovial inflammation can be seen around the defect. HE, X25.

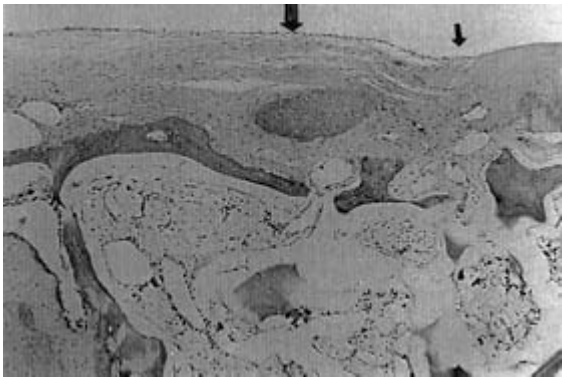

Fig. 8: Repair tissue in 8th week, the defect is replaced by cartilage tissue and around the defect a little fibrous tissue increase can be seen. HE, X50.

DISCUSSION AND CONCLUSION

The most appropriate method to recover chondral and osteochondral defects, which cause an important degree of pain, loss of function and human power, and high medical treatment costs by inducing osteoarthritis in old ages, is still not known[6]. Having an increasing interest, autologous chondrocytes transplantation (ACT) is a hope offering method in repairing osteochondral defects on femorotibial joint surface. In this method, cells isolated from the patient can be reproduced in vitro and autologous chondrocytes can be re-transferred to the defected area in order to repair patient's damaged cartilage[7,8].

One of the advantages of this technique is that although the number of cells obtained from small sample pieces of cartilage taken from rabbits is little, it can be increased in a short period of time. At this stage great care should be taken about the sterilization conditions otherwise culture flasks can easily be infected, as we experienced in our study[9,10].

We took cartilage samples from the shoulder joints of the rabbits in order not to damage their knees on which the study was performed. Their knees are very small, thinner layers of articular cartilage and vulnerable to damage compared to human knees (This is a challenge because there is no ideal animal model that duplicates completely human situation.). However, we should consider morbidity risk of donor in humans also[3,7].

In this study, we used autologous chondrocytes for transplantation because of their advantages like lower risk of transferring organisms and absence of immunological reaction.

However, when we look at literature, we can see that after 24 weeks of observation, Wakitani et all., recovered osteochondral defects in rabbits' knee joint using allogenic chondrocytes which were embedded into collagen gel, and they indicated that it recovered through hyaline cartilage formation. In their study, although defects were shown as filled with hyaline cartilage, a clear subchondral bone formation was not seen when histological photomicrographs were evaluated. At the same time, they reported that histological monolayer cell infiltration was not observed and no immunological reaction by transplanted

chondrocytes or collagen was seen[11]. On the other hand, in their study, Noguchi et all., repaired osteochondral defects using culture chondrocyte grafts and compared allogenic and isogenic grafts. They reported that in early stages of defects (in the second and fourth weeks), which were treated using isogenic or allogenic chondrocytes, they were filled with hyaline cartilage, and in peripheral sections there was a light inflammatory cell inflammation which was more clear in allogenic defects[12]. Since they increase subchondral bone formation and do not have the risk to transfer organisms like HIV from donor to receptor, autografts are relatively more superior than allografts. Besides, its donor maturity is automatically constant in clinical practices and the amount of cartilaginous tissue to be extracted is limited without causing any insufficiency in material[7]. Furthermore, isogenic chondrocytes have more definite regeneration in subchondral bone tissue than allogenics.

One of the problems we encountered was that we did not know the number of optimal cells that we had to place into the defected area, so we filled the defect until it was full[7,13]. Further research is needed to solve this problem.

The second problem we had was the difficulty of inducing a defect in the rabbit knee joint cartilage which is very thin. Moreover there are many factors that need to be standardized, for instance; geometric shape and volume of the lesions (the results in large lesions are less predictable), the depth of injury (penetration of the subchondral bone alters the response), the age of the animal and individual animal's biologic response and activity level[14].

We used hyaluronidase to wash the defects. Pretreatment of the articular cartilage lesion with enzymes such as hyaluronidase and chondroitinase has been advocated to enhance these cell based repair strategies. Such treatment is intended to improve cell attachment and possibly promote the integration of the regenerated repair tissue with the native cartilage[14].

Another problem in our study was closing the defects. We used periosteal graft since it is easy to get. With recent studies it has been understood that using periosteal graft is not effective on its own. In their study on rabbit knees, Brittberg et all., reported that, compared to the group in which chondrocytes were used, repairing tissues formed in the group periosteal grafting was employed, is histologically of lower quality[5]. This led the studies carried with ACT to come into agenda. Therefore, we did not use periosteal graft in the control group so the rabbit knees were damaged less and rehabilitation period was shorter. Because of their advantages like no donor morbidity, less invasive surgery and shorter operation time, biologic and synthetic polymers and tissue engineering products need to be used in closing and holding the cells in the defects instead of periosteal grafts[15,16].

Brittberg et all., carried out a research for the first time on the usage of ACT in the treatment of localized cartilage defects of patella or femur condyl in 23 patients, cultured for 14 and 21 days and they were injected to the defected region and covered with periosteal fleb. Two years after the transplantation, good or perfect clinical results were reported about 14 of 16 patients (who had condyler defect) and 2 of 7 patients (who had patellar defect). Hyaline cartilage was shown in the biopsies taken from one patellar defect and 11 femoral defects. Finally, they published results of a bigger group of patients. After the treatment of chondral and osteochondral defects in knee through chondrocyte transplantation, subjects were monitored for 2 or more months and in 47 of 66 subjects there was an increase in function. These results show that chondrocyte transplantation together with periosteal grafts increase restoration of joint surface in humans. But there is still a need for research to define newly formed tissue and to determine its functions[17].

To summarize, there are a lot of parameters that need to be explained, for instance; differences in the species, the age of the animals, extent of the lesion, depth of the defect, the lack of retention of the cells in the defect due to possible displacement of the periosteal flap, location of the defect (the patella compared with the trochlea), the numbers of the cells that can be injected, and immobilization of the animals.

Consequently, in order to prevent joint surface damages and degenerative joint defects to occur in the future, autologous chondrocytes transplantation to repair osteochondral defects is a new alternative treatment method. There is a need for further studies to develop this method.

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