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Research Article

Using Cell-free Matrix Derived from Ostrich Plantar Ligament for Repair of Articular Cartilage Defect of Rabbit

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Abstract

Articular cartilage lesions are most common injury of the knee for which several repair methods have been described. We described *in-vivo* potential of collagen type I scaffold extracted fromknee of ostrich. Full thickness defects were created bilaterally in the weight bearing area of femoral condyles of the both femurs of 7 white New zealand adult male rabbits weighting 1800±100 g. The six left medial condyles as experimental group I were filled by using cell-free tissue and six right medial condyles of the same rabbit were treated by cell-tissue as experimental group II and the right medial condyle defect of one rabbit as control group was left without treatment. Histopathology data demonstrated significant difference between three groups with respect to the cartilage repair indicators as the morphology of the cells, color matrix and cartilage thickness ($P<0.05$). Furthermore, sub-chondral bone formation showed a significant difference and relationship between two experimental groups and control group ($P<0.05$). The use of cell-free ligamentum plantar matrix showed markedly significant improvement in the processes of joint cartilage repair compared control group ($P<0.05$). Results support the use of cell-free ligamentum plantar can be useful and promising bio-engineering procedure as a scaffold for the treatment of the articular cartilage surface.

Keywords: *Cell-free matrix, Ostrich plantar ligament, Repair, Articular cartilage defect, Rabbit*

Tavşanlarda Artikular Kartilaj Defekt Onarımında Devekuşu Plantar Ligament Kökenli Hücre İçermeyen Matriks Doku Kullanımı

Özet

Artikular kartilaj lezyonları dizin en sıklıkla gözlenen yaralanması olup birkaç onarım metodu tanımlanmıştır. Bu çalışmada devekuşunun dizinden ekstrakte edilen kollajen tip I doku iskelesinin *in vivo* potansiyeli tanımlanmıştır. Ağırlıkları 1800±100 g olan 7 adet Yeni Zelanda ergin erkek tavşanın her iki femurunda femoral kondulusların ağırlık taşıyan bölgelerinde bilateral olarak tüm kat hasarı oluşturuldu. Altı sol medial kondulus hücre içermeyen doku ile doldurulurken (Grup I) aynı tavşanların altı sağ medial kondulus hasarı hücre içeren doku ile tedavi edildi (Grup II) ve bir tavşanın sağ medial kondulus hasarı tedavi uygulanmayarak kontrol grubu olarak bırakıldı. Histopatoloji bulguları kartilaj onarım belirteçleri olarak hücre morfolojisi, renk matriks ve kartilaj kalınlığı bakımlarından her üç grup arasında anlamlı derecede fark olduğunu gösterdi ($P<0.05$). Ayrıca, sub-kondral kemik oluşumu iki deney grubu ile kontrol grubu arasında anlamlı derecede fark olduğunu göstermiştir ($P<0.05$). Hücre içermeyen ligamentum plantar matriks kullanımının kontrol grubu ile karşılaştırıldığında eklem kartilaj onarımında belirgin derecede iyileşme oluşturduğu gözlemlendi ($P<0.05$). Elde edilen sonuçlar hücre içermeyen ligamentum plantar kullanımının artikular kartilaj yüzey onarımında bir doku iskelesi olarak ümit verici biyomühendislik prosedüründe yararlı olabileceğini göstermiştir.

Anahtar sözcükler: *Hücre içermeyen matriks, Devekuşu plantar ligament, Onarım, Artiküler kartilaj hasarı, Tavşan*

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INTRODUCTION

Cartilage defects have been indicated to being the most common injury of the knee joint caused by trauma, inflammation and/or biomechanical dysfunction. Chondral and osteochondral lesions related to injury or other pathologic conditions are mainly associated with the progression of osteoarthritis, leading to joint destruction. The articular cartilage defects are one of the main challenges in orthopedic surgery and may progress symptomically to severe osteoarthritis in the stifle joint [1,2].

Several approaches have been described for cartilage repair such as bone marrow stimulation via micro-fracture or drilling, arthroscopic resurfacing, autologous chondrocyte implantation (ACI), and osteochondral trans-plantation. The repair of articular cartilage remained as a challenge for many years particularly because of cartilage's hypocellularity and insufficient nutrient supply. Furthermore, other factors responsible for delayed healing include the inability of bone marrow stem cells or resident chondroprogenitor cells for hyaline cartilage formation played role [2-10].

ACI has been demonstrated to be promising when implanting culture-expanded chondrocytes alone in clinical trials [7,8]. The disadvantages of autologous or allogeneic osteochondral transplants are shown to be the limited amount of grafts and donor site morbidity and also the incongruence of the surface. The implantation of autologous chondrocytes has been used for the treatment of full-thickness cartilage lesions. It is worth noting that this method is not effective for treatment of many types of lesions such as deep defects involving the subchondral bone [9,10].

Different scaffolds with osteochondral regenerative potential have been provided in order to overcome problems of joint treatment, and promising findings were achieved using these scaffolds [11,12]. In recent years, matrix-induced autologous chondrocyte implantation has successfully been used in animal models [13] and even in clinical trials [14]. However, mentioned technique is time-consuming and implicates the risk of potential donor-site morbidity because of chondrocyte harvesting [15,16]. Collagen-based materials have been already used for cartilage repair in rabbits with satisfying findings [17,18].

Various matrices were applied in orthopedic applications including polylactic acid based matrices, fibrin glue, hyaluronic acid, alginate, polyglycolic acid, and collagen [19-23]. It has been indicted that collagen-based matrices may be satisfying in this respect because of their biocompatibility, biodegradability and mechanical integrity.

Cell-free collagen type I scaffold has been reported to be correlated with high-quality tissue repair in defects up to 12 mm in diameter [6,22]. Moreover, a new technique using a cell-free collagen type I matrix in human have been suggested promising findings.

This study was aimed to investigate the effectiveness of a cell-free scaffold derived from ostrich type I collagen in healing of loading aspect of cartilage defect in rabbit.

MATERIAL and METHODS

This study was carried out on rabbit as an animal model after approval by ethical committee of the Iranian laboratory animal ethic frameworks under the reference code IAEC-205-P.

Tissue Restoration

A total of five pairs of ostrich's limb with an average age of 11 months were collected from a slaughterhouse in Iran. Metatarsophalangeal fibrocartilage tissue of limbs were used for tissue engineering and creating extracellular scaffold in order to replace a cartilaginous defect in rabbit femoral condyle. Histological examination on this tissue revealed fibrocartilage tissue rich in collagen fibers.

Briefly, the tissue samples were harvested and placed in the plantar surface of the metatarsophalangeal joint of 3rd and 4th digits. The tissues were transferred to the laboratory of Faculty of Specialized Veterinary Sciences, Science and Research Branch of Islamic Azad University. After washing with sterile sodium chloride solution and 10% povidone iodine, specimens were fixed with 10% neutral buffered formalin for histological evaluation and stored in PBS (Phosphate buffered saline) solution for decellularization process.

For preparing a cell- free tissue, a combination of physical and chemical methods were applied. The protocol used in this study was a modification of the method described previously by Tischer et al.[24]. An equal approximately 5 mm thick specimens were placed in deionized water for 48 h. Then sonicator was used as a mechanical stage of decellularization process. It was followed by ionic detergent (Sodium dodecyl sulfate (SDS) 2%) in a vacuum for 10 days. This process continued by placing them in deionized water and 70% ethanol for 24 h separately. Finally, they were washed and put in PBS.

In order to confirm decellularization process, the tissues were sent to laboratory for DAPI (4,6-diamino-2-phenylindole) staining. To determine types of bacteria in the fibrocartilage tissue and select effective post-operative antibiotics, bacterial cultures were performed on Blood-agar and Mac-conkey medias. A sample was also cultured on Muller-Hinton agar and antibiotic discs including Ciprofloxacin, Oxytetracycline, Chloramphenicol, Cloxacillin, Ampicillin, Gentamicin, Penicillin, Lincomycin, Enrofloxacin and sultrim were used to test bacterial sensitivity. The cultures were incubated in 37°C for 48 h.

To inhibit bacterial growth in the acellular tissue, an antibiotic cocktail containing 100 mg streptomycin, 80 mg of gentamicin and 100 units of penicillin was added to PBS solution. The *in-vivo* study was performed on seven white Newzealand adult male rabbits with average weight of 1800±100 g. In order to respect ethics, we involved less animals in the study. Therefore left and right limbs of each rabbit were prepared for surgery. The rabbits were randomly divided into three groups (two experimental and one control). The left limbs of six rabbits designed as experimental group I and were implanted with acellular scaffold, the right limbs of same rabbits were designed as experimental group II and were implanted with unprocessed scaffold. Both limbs of another rabbit were used as control group with no treatments. In order to achieve environmental adaptation, all animals were kept for a week before initiating the study under appropriate temperature, humidity and diet. Animals were kept NPO (Nil Per Os) for 6 hours and were prevented from drinking water for 2 h before surgery. They were anesthetized with intramuscular injection of acepromazine (1 mg/kg) and ketamine hydrochloride (40 mg/kg) cocktail. After aseptic preparation of stifle joint, animals were positioned in dorsal recumbency. Arthrotomy of each stifle was performed via lateral approach and medial femoral condyle was exposed *(Fig. 1-A)*.

A 4 mm osteochondral defect was made on the weight bearing surface of medial femoral condyle with orthopedic drill. Cell-free and cellular plantar ligament were trimmed to a diameter equal to the defect size and implanted with press fit technique in left and right femoral medial condyle defects in experimental group I and II, respectively *(Fig. 1-B)*. Both condylar defects in control group was left untreated. The joint was irrigated with diluted Gentamicin. Afterward, the joint capsule was sutured with 4-0 polyglactin910 with simple interrupted suture pattern. Subcutaneous tissue was sutured with the same suture material with continuous suture pattern. The skin was sutured with nylon suture material with simple interrupted suture pattern in all rabbits.

In first 48 h, ice pack was placed over the surgical site twice a day for 15 min. For the next 72 h, warm compress was placed on the surgical site every 12 h for 15 min. All the animals received a daily dose of Enrofloxacin (5 mg/kg) and Flunixin meglumine (1 mg/kg) two times a day for 3 days. Clinical assessment was also done. Both groups were evaluated for walking pattern, pain, joint effusion and range of motion (ROM) and infection. The animals were euthanized with intravenous injection of high dose thiopental sodium 12 weeks after surgery and specimens were collected and fixed in 10% Buffered formalin. Decalcification process was performed with EDTA 5.5% solution. The specimens were embeded in paraffin, cut with 5 micrometer microtome and stained with

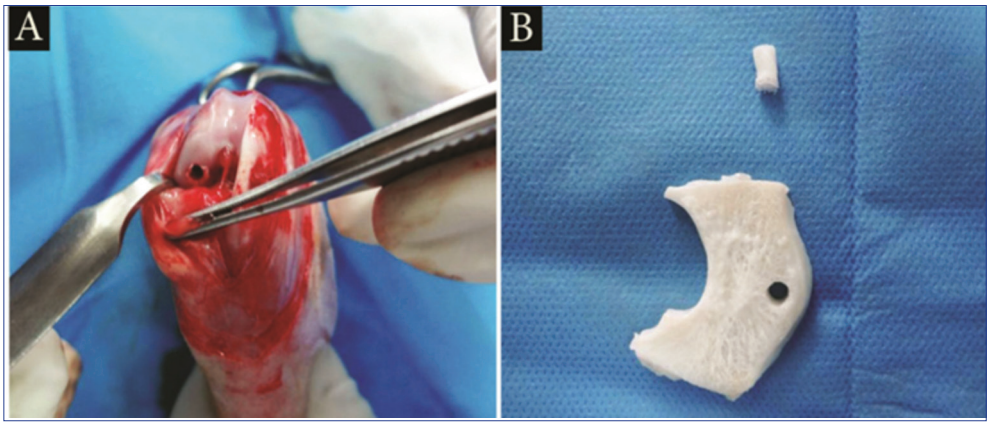

Fig 1. A- Osteochondral defect in the femoral medial condyle, **B-** Preparing blocks from decellularized Plantar ligament to press-fit into the defect

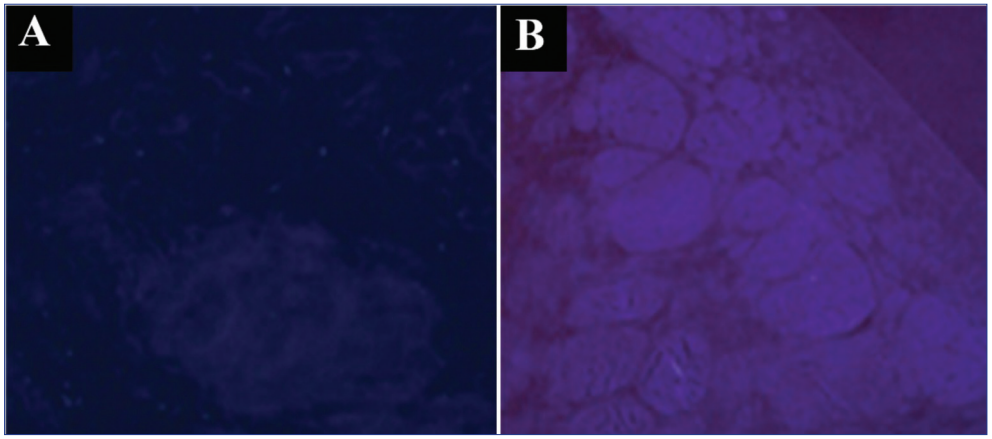

Fig 2. Morphology of Plantar ligament before **(A)** (×10) and after **(B)** (×40) Decellularization process with DAPI staining

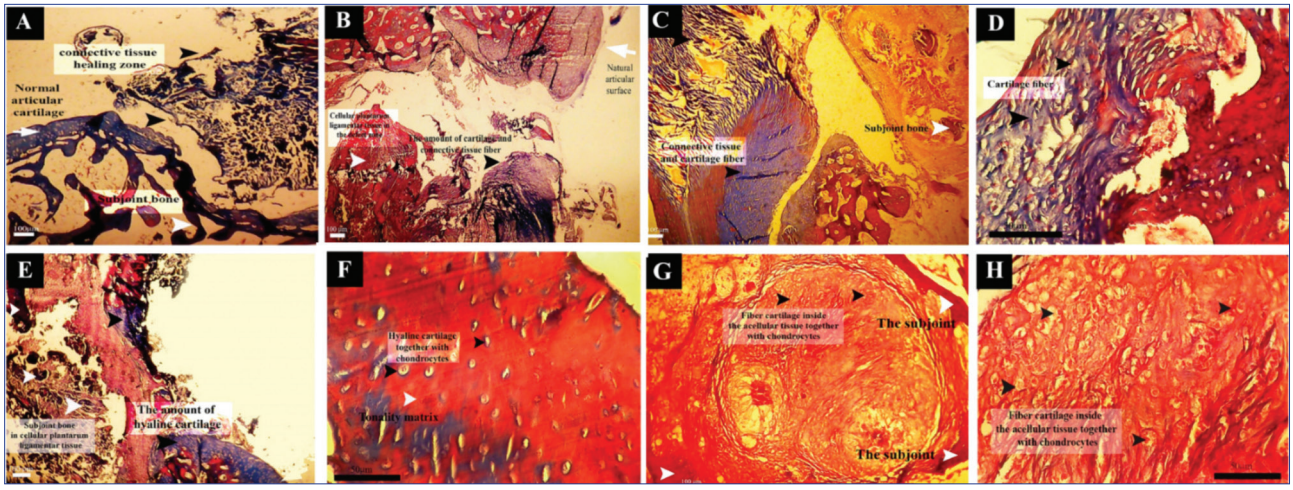

Fig 3. Histopathological samples stained with Trichrome staining. **A-** Control group; **B,C,D-** Un-processed (cellular) group; **E,F,G,H-** Acellular group

Table 1. *Evaluation and scoring of histologic factors of experimental groups*

Parameters	**Parameters**	**Score**	**Experimental Groups**													
			1		**2**		**3**		**4**		**5**		**6**		**7**	
			R	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**
The morphology of the cell	Hyaline cartilage	4	1	2	1	2	2	2	1	2	1	2	1	3	0	0
	Mostly hyaline cartilage	3														
	Fiber cartilage and transparent	2														
	Mostly cartilage fiber	1														
	Mostly without cartilage	0														
Color matrix	Like natural areas	4	1	2	1	2	0	3	2	3	1	2	2	3	0	0
	Slightly dimmer	3														
	Decreased	2														
	Highly decreased	1														
	Without color	0														
Regular levels	Smooth	2	0	1	0	1	1	0	1	0	0	0	1	1	0	0
	Slightly irregular	1														
	Irregular	0														
Thickness of cartilage	100%	4	1	2	1	2	0	3	1	1	0	2	1	3	0	0
	75%	3														
	50%	2														
	25%	1														
	0%	0														
Connecting two levels	Both integrated edge	2	2	2	1	2	0	2	2	2	1	2	2	2	1	1
	An integrated edge	1														
	Both non-integrated edge	0														
% [The formation of new bone under the cartilage under the original trade mark; (indicating normal bone repair following a similar cartilage)]	90-100	3	1	2	1	2	0	1	1	0	0	1	1	2	0	0
	75-89	2														
	25-74	1														
	Less than 25	0														
Total			6	11	5	11	1	11	8	8	3	9	8	14	1	1

hematoxylin-eosin (H&E) and Trichrome.abbits.staining for histopathological evaluation.

Statistical analysis was performed using SPSS 16 software package. Difference between groups was analyzed using the Mann-whitney test and $P<0.05$ was considered statistically significant.

RESULTS

DAPI staining demonstrated that almost 95% of the cells were removed and the native structure of tissue was preserved without any destruction as well *(Fig. 2-A,B)*.

In clinical assessment, no signs of pain was observed in about 4-5 days after surgery. The quality of walking was reported favorably in all animals. Weight bearing on operated foot was seen immediately after recovery. All rabbits were able to walk freely in their cages and natural ROM of stifle joint was seen. Macroscopically, xenografts were completely in their place in both experimental groups and tissue necrosis or transplant rejection was not grossly observed. Pathologic changes were observed only on one of the femoral condyle of rabbits in the control group.

H&E and Trichrome stained slides *(Fig. 3)* were evaluated microscopically. The results are shown in the following table *(Table 1)*. Based on four indices including cellularity, the predominant cell type, the morphological matrix and collagen formation. We scored specimens using some components of the International Cartilage Repair Society Visual Assessment Scale and a total score was obtained [25]. Histopathology data demonstrated a significant difference between acellular and cellular groups with respect to the cartilage repair indices, such as the morphology of the cells, color matrix and cartilage thickness ($P<0.05$). Furthermore, a significant difference was present between experimental groups I and II in respect to subchondral bone formation ($P<0.05$). Moreover, there were significant correlations among the 2 experimental and control groups ($P<0.05$). Cell-free plantar ligament matrix showed markedly significant improvement in articular cartilage repair compared to control group ($P<0.05$). As the matter of fact, a similar pattern to normal tissue was seen in terms of the type of cartilage formation (hyaline cartilage).

DISCUSSION

Hyaline cartilage as an avascular, alymphatic and aneural tissue is susceptible to trauma because of the current life style and activity of human societies [26]. Over 151 million people suffer from osteoarthritis worldwide, indicating a massive clinical and socioeconomic load [26]. Several strategies are being studied for restoring of articular cartilage defects and the scaffold-based cartilage treatments have been addressed as a fascinating treatment option. Investigations are mainly focusing on simplifying the surgical methods and adopting more effective agents to stimulate tissue regeneration.

The most important aspect of our study was using a new biomaterial derived from ostrich plantar ligament as an acellular scaffold which can facilitate repair of the articular cartilage defects of up to 4 mm in diameter in femoral condyle surface of rabbits.

Several studies focused on evaluating cartilage repair using different animal models including sheep, horse and goat [13,27-29]. A specialized characteristic of rodents and lagomorphs is that they possess auto-intrinsic repair of cartilage which is not present in larger animal and human [30].

Scaffold-based cartilage treatments have been reported to be effective in the improvement of cartilage repair processes [31,32]. The preclinical and clinical researches of scaffold therapy indicated that it had superior results in cartilage healing [4]. The advantage of Cell-free matrices is avoiding of cell manipulation and its regulatory obstacles with good clinical findings [33]. Additionally a cell-free one step procedure is time saving and also cost effective. Current studies showed that new generation of biomaterials are able to exploit the intrinsic tissue regeneration potential.

Many kinds of matrices have been used in reconstruction of bony and cartilaginous defects including polylactic acid, fibrin glue, hyaluronic acid, alginate, polyglycolic acid, and collagen matrices [19-23]. It has been suggested that collagen-based matrices may be promising sources due to their biocompatibility, biodegradability and mechanical integrity.

It is clear that transplanting unprocessed tissues is associated with immune reactions. Therefore, tissue-engineering methods such as decellularization are used to diminish the immune response and elimination of post-operative suppressive therapies. Therefore, the main goal of our study was to investigate the bio-behavior of a xenogenic decellularized scaffold originating from Ostrich in the repair of rabbit stifle osteochondral defects. Metatarsophalangeal fibrocartilage tissue of 11-months-old ostrich was used for tissue engineering and creating alternative extracellular matrix in osteochondral defects. Histological examination conducted on this tissue revealed a fibrocartilage ground substance rich in collagen fibers. Other studies assessed microstructure, mechanical properties and collagen content of ostrich plantar ligament highlighting that it has a good mechanical strength and it may be appropriate for application in tissue engineering. Since it is considered as a slaughterhouse waste, it is very cost-effective and has resolved the problem of expensive tissue procurement. On the other hand, as ostrich still is not the main source of humanitarian needs in the world, common and serious zoonosis disease has not been

reported, so risk of disease transmission is low.

In literature it has been documented that collagen has well biocompatibility and a number of advantages for use in tissue repair. Greater cellular interaction because of the presence of ligands in cell adhesion, ease of integration *in vivo* without any resultant adverse response, and great capacity to be co-polymerized with other biological materials to increase their bio-functionality are some of the aforementioned advantages. Schneider et al.[34], indicated that implantation of a cell-free collagen type-I gel consisted of 4.8 mg/mL rat tail collagen was associated with a high-quality tissue repair in the Goettinger minipig articular defect which can be considered equal to cell-based procedure one year postoperatively. They showed the high chondrogenic potential of collagen gel, which may be useful for *overcoming inherent disadvantages* of using in conventional cartilage tissue engineering techniques [34]. Another study evaluated a cell-free collagen type 1 matrix *in vitro* and also on nude-mouse as an animal model and their results revealed that this cell-free scaffold offered a suitable environment that leads to chondrocyte colonization and subsequent transformation of the former matrix into repair tissue [35]. Many studies have been evaluated different cell-seeded collagen matrices in cartilage repair because of their high biocompatibility [36-39]. In addition collagen gels increase chondrocyte proliferation and proteoglycan synthesis *in vitro* [40].

Ostrich acellular plantar ligament as a source of collagen in this study had milky appearance after processing and maintained its 3D structure and viscoelasticity following decellularization procedure and sterilization. Furthermore, immune response similar to what is present in porcine decellularized matrix was not observed in our decellurized ostrich collagen matrix.

The presence of fibroblasts and fibrocytes in decellularized matrix was the most interesting finding in this. Decellularization has opened new insights in trans-plantation of xenogenic tissues, that cost-effectiveness and the availability of extracellualr matrix are two significant advantages of them. The specific tissue after decellularization process is resilient and stiff, crucial to proper functioning of the fibrocartilage tissue. In Microscopic overview of tissue, presence of inflammatory mononuclear cells was reported only in experimental group II and a single rabbit of experimental group I with joint effusion.

In conclusion, the extracellular matrix of ostrich plantar ligament rich in collagen type I and proteoglycans is a complex 3D structure that as an acellular scaffold could be a potential bioactive bed in mimicking the natural tissue behavior. It can promote cell proliferation, differentiation and enhance the healing of osteochondral defects in rabbit stifle and therefore it might be a very valid option in regenerative medicine in future.

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