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Original Research Article

Human Mesenchymal Stem Cells Impregnated with Autologous Cartilage Paste Repair Fresh Focal Osteochondral Defects in Rabbits

Hazem Mahmoud Atta[1,2,3], Mohammad Mohammedaly Abbas[4,5], Mohammad Hussein Alkaf[5,6], Mamdouh M Kotb[8], Laila A Damiati[7], Gamal Karrouf[9,10], Soheir Mahfouz[11]

[1]Professor of Clinical Biochemistry, Faculty of Medicine, Rabigh, Saudi Arabia.
[2]Stem Cell Unit, King Abdulaziz University, Jeddah, Saudi Arabia.
[3]Professor of Medical Biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt.
[4]Assistant Professor and Orthopedic Consultant,
Department of Orthopedics, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.
[5]Sheikh Salem Bin Mahfouz Scientific Chair for Stem Cell Treatment of Osteoarthritis, Jeddah, Saudi Arabia.
[6]Orthopedic Surgeon Consultant,
Department of Orthopedics, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.
[7]Lecturer, Department of Biology, University of Jeddah; Jeddah, Saudi Arabia.
[8]Associate Professor of Radiology,
Department of Radiology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.
[9]Professor of Veterinary Medicine, Experimental Surgery Unit, Faculty of Science,
Medical Physics Dept., KAU, Jeddah, Saudi Arabia.
10Anesthiology & Radiology Dept., Faculty of Veterinary Medicine, Mansoura University, Egypt.
[11]Professor of Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt.

ABSTRACT

Introduction: Several therapies have been attempted to treat focal osteochondral defects of the knee joint, but none of them resulted in full repair. We try in this study to determine the efficacy of human umbilical cord stem cell xenografts, on the healing of focal osteochondral cartilage defects, in rabbit's knee.

Material and Methods: Focal defect was created in the medial femoral condyle of the right knee of all rabbit groups. In one group the defect was left untreated. Human umbilical cord mesenchmal stem cells (MSCs) were placed in the defect, in a second group. A third group received MSCs on a fibrin sealant scaffold. A fourth group received minced cartilage paste impregnated with MSCs. Healing was assessed clinically, radiologically and pathologically.

Results: The animals were able to move normally after 8 weeks of surgery. Examination of the knee joints showed better healing of the defect in rabbits that received MSCs+ cartilage paste compared to other animal groups. Magnetic resonance imaging (MRI) showed persistent osteochondral defect in rabbits treated with MSCs, without and with fibrin sealant. Complete defect fill, intact cartilage surface, was observed in rabbits treated with MSCs impregnated with cartilage paste. Pathologic examination revealed that MSCs, alone, induced chondrocyte proliferation and stimulated cartilage repair. A better result was obtained with MSCs + fibrin sealant. The best repair was obtained when autologous cartilage paste was used.

Conclusions: Repair of focal osteochondral defects in rabbit knees using human umbilical cord MSCs impregnated with autologous cartilage paste appears to be successful as proven clinically, radiologically, as well as pathologically.

Keywords: Cartilage Defect, Mesenchymal Stem Cells, Xenotransplantation.

Abbreviations:

ACI: Autologous chondrocyte implantation; MSCs: Mesenchymal stem cells; PRP: Platelet-rich plasma; PR-FG: Platelet-rich fibrin glue; hMSCs: Human mesencymal stem cells; UC-MSCs: Umbilical cord mesenchymal stem cells; KAU: King Abdulaziz University; ATCC: American type culture collection; RARE: Rapid acquisition with relaxation-enhanced; MOCART: 2 dimensional magnetic resonance observation of cartilage repair tissue; HE: Hematoxylin and Eosin; MRI: Magnetic resonance imaging.

***Correspondence to:**

Hazem Mahmoud Atta, MD, MSc, PhD,
Professor of Clinical Biochemistry, Faculty of Medicine,
Rabigh and Stem cell Unit, King Abdulaziz University, Jeddah, Saudi Arabia
and Professor of Medical Biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt.

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INTRODUCTION

Defects in the articular cartilage of the knee are responsible for worldwide problems.[1] Articular cartilage lesions represent about 60% of cases of knee surgery.[2] Treatment modalities are limited for the treatment of focal osteochondral lesions. Techniques involving autologous tissue or cell grafts, autologous chondrocyte implantation (ACI) and minced articular cartilage allografts, are now being employed. However, their long-term results are variable.[3] Steadman et al[4] reported improvement in clinical knee scores following long-term follow-up of patients, after microfracture.[4] Minas et al[5] were against the technique for fear of interfering with subsequent healing. Mosaicplasty is complicated by improper healing of the donor site, and limited sources.[3] However, microfracture and mosaicplasty have the advantage of using the patient's own tissue. More recent ACI techniques use scaffolds of biomaterials impregnated with chondrocytes as a scaffold instead of a periosteal patch.[6] However, this technique is employed in 2 steps and the collection of chondrocytes from the patient may lead to focal damage. There may also associated problems at the donor site.[7] For this reason, Bone marrow–derived mesenchymal stem cells (MSCs) which are able to differentiate into different mesenchymal lineages may be a better source of cells for the treatment of osteochondral defects.[8,9]

Rabbit models for cartilage defects are characterized by being cost- effective, are easy to manipulate, and have a wide joint size. Their disadvantage is that they have thin cartilage, may spontaneously heal, and have different loading conditions, compared to humans.[10-12]

Platelet-rich plasma (PRP) and platelet-rich fibrin glue (PR-FG) were tested as MSCs scaffolds in clinical trials for cartilage repair. They are non-immunogenic, bio-absorbable, sterile, easily prepared, and can be set intraoperatively. PR-FG has also been shown to provide sustained release and protection against proteolytic degradation of endogenous growth factors.[13] Haleem et al[14] used autologous- culture expanded bone marrow MSCs on a scaffold of platelet rich fibrin glue to repair focal femoral defects using a periosteal flap seal. They reported remarkable improvement in the clinical and radiologic scores with 12 months follow up of the patients.

Jang et al,[15] reported the transplantation of human mesenchymal stem cells (hMSCs) with or without differentiation for the regeneration of osteochondral defects in rabbits using a construct composed of platelet-rich fibrin glue (PR-FG) and hydroxyapatite. Rabbits were sacrificed at 4 or 8 weeks post-surgery and to macroscopically and histologically evaluate and score osteochondral repair. Better healing was observed in the group in which defects were seeded with differentiated hMSCs compared to other groups. Defects were filled with more hyaline-like cartilage and were better integrated with the surrounding native cartilage. They concluded that xenogeneic transplantation of differentiated hMSCs using a biphasic composite construct effectively repaired osteochondral defects in the rabbit model.

Saulnier et al,[16] investigated the possible anti-inflammatory and anti-catabolic effects of equine umbilical cord Wharton's jelly MSCs, in a model of mild osteoarthritis in rabbits. They concluded that the Intra-articular administration of xenogeneic neonatal MSCs concomitant with meniscal injury decreased metalloproteinase gene expression in synovium, leading to decreased cartilage degradation.

Mobasheri et al,[17] in a review, stated that chondroprogenitors from articular cartilage are better than bone marrow-derived MSCs. They concluded that the use of these cells might provide a great advantage for the clinical trials related to cartilage repair.

In order to devise a novel therapy for fresh traumatic focal defects, associated with early degenerative lesions of hyaline cartilage, we proposed to transplant MSCs, obtained from human umbilical cord into a rabbit model of focal osteochondral defect. The use of human umbilical cord MSCs xenografts alone or in conjunction with fibrin sealant or autologous cartilage paste was investigated to find the best method for cartilage repair. The most successful procedure should be implemented to avoid the occurrence of irreversible osteoarthritis

MATERIALS AND METHODS

Animals

All studies were carried out at the Surgical Research Laboratory of King Fahd Medical Research Center, King Abdulaziz University (KAU), Jeddah. The Research Ethics Committee of KAU approved the protocol. The experiment was performed in strict compliance with the ethical guidelines for humane treatment of animals as defined by KAU. Twelve mature New Zealand white, clinically healthy, male rabbits 12 months of age, weighing 3.8-4.5 kg were used in this study. They were housed at 25°C, with a 12-hour dark/ light cycle. The rabbits were allowed several days to acclimatize to their cages and surroundings**.** Rabbits were divided according to treatment regime into: control group: 2 animals with focal defect but with no MSCs treatment, but received antibiotics), MSCs group: 2 animals with focal defect that received MSCs only, MSCs+ autologous cartilage paste group: 4 animals with focal defect that received MSCs impregnated with cartilage paste, cartilage paste group: 2 animals with focal defect that received autologous cartilage paste only, MSCs+ fibrin sealant (Tisseel®, Baxter, Deerfield, Ill, USA): 2 animals with focal defect that received MSCs mixed with fibrin sealant. The fibrin sealant was prepared in the theatre according to manufacturer's instructions. It is used as an adjunct to hemostasis in patients undergoing surgery when control of bleeding by conventional surgical techniques is ineffective or impractical.

Stem Cell Preparation

Normal Human umbilical cord-derived mesenchymal stem cells (UC-MSCs) were purchased from American Type Culture Collection (ATCC® PCS500010™, Manassas City, VA, USA), together with their growth-supporting media, growth factors, enzymes and antibiotics. The cells were prepared and sub-cultured according to the manufacturer's guidelines and procedures. Cells from the second passage were utilized for injection. Cells were suspended in 0.2 ml of PBS (GIBCO Invitrogen Corporation, Cat# 14190-094, United Kingdom) at a density of 1.2–1.5 × 10^6 cells/ml. The same cell number was used with the fibrin sealant and with the autologous cartilage paste.

Operative Technique

Each animal was anaesthetized using a combination of 50 mg/kg ketamine (Tekam 50, Hikma Pharmaceuticals, Amman, Jordan), 50 mg/kg xylazine (Xylaject, ADWIA Pharmaceuticals, ARE), both given intramuscularly, and 2% lignocaine at the operative site**.** Each rabbit was given Preoperatively 50 mg/kg cefazolin IM (cefamezin, Hikma Pharmaceuticals, Amman, Jordan). Following

induction of general anesthesia, the right knee was prepared with standard sterilization and draping for aseptic surgery then the animals were positioned in dorsal recumbence, the knee joint was opened through a medial parapatellar approach. Incision was taken down to the antero-medial joint capsule, with knee flexion to identify anatomical landmarks. The joint capsule was incised obliquely between the patellar tendon and medial collateral ligament and extensor mechanism dislocated laterally. The articular surface of the medial femoral condoyle was exposed totally and a surgical, focal, osteochondral defect was performed by surgical drill size 2mm in diameter and 4mm in depth. UC-MSCs with and without fibrin sealant or cartilage paste were implanted in the defect. Cartilage paste was obtained by scraping of the articular surface of the non-weight baring area of the same knee joint then used alone or after mixing it with MSCs. Following implantation hemostasis was attained. The extensor mechanism was relocated, the capsule was closed medially with 2-0 vicryl and the skin closed with interrupted 3-0 prolene sutures. The skin was then cleaned and dressed.

Post-operatively, antibiotic was given daily for five days. Temperature was maintained at 25°C (± 2°C) with a dark–light schedule of 12 hours (± 1hour). Humidity was maintained at between 55% and 65%. Bedding of paddy husk was provided on the floor. All the rabbits were properly fed with commercially available rabbit feed along with fresh leafy vegetables and water. Animals were allowed unrestricted activity and were closely monitored regularly for signs of wound infection, wound breakdown and other complications.

Radiological Evaluation (Magnetic Resonance Imaging)

The knees of the sacrificed rabbits were examined ex-vivo using a 9.4 T high field MRI scanner (Biospec Avance III 9.4 T/30, Bruker Biospin, Ettlingen, Germany). A circular polarized volume coil with receives and transmit configuration, was used. Morphological images of the cartilage were acquired using fat suppressed 3D fast low-angle shot (FLASH) and fat suppressed 2D rapid acquisition with relaxation-enhanced (RARE) sequences. Scan parameters for FLASH images: TR: 40, TE: 4.62, matrix: 256 x 256 x 128, averages: 1, FOV: 4 x 3 x 3 cm, resolution: 0.0156 cm/pixel in read and 0.117 cm/ pixel in P1 and 0.234 cm/pixel in P2, Flip angle: 40, time of acquisition: 16 minutes and 23 seconds, slice thickness: 0.234375 mm, interspace or gap: 0.234375 mm and bandwidth: 50000 Hz. While scan parameters for (RARE) images: repetition time (TR): 2278.3, echo time (TE): 8.5, matrix: 256 x 256, averages: 4, FOV: 4 x 3 cm, resolution: 0.0156 cm/pixel in read and 0.1117 cm/ pixel in P1, Flip angle: 180, time of acquisition: 7 minutes 17 seconds, slice thickness: 1 mm, gap: 0.5 mm, bandwidth: 89285.7 Hz. Biochemical T2 mapping of the cartilage was acquired using multi-slice multi-echo (MSME) spin echo based protocol.

The repair tissue was evaluated using 2 dimensional magnetic resonance observation of cartilage repair tissue (MOCART) scoring system developed by Marlovits et al,[18] Score 0 = no repair and score 100= excellent repair. The evaluation includes 9 variables (the degree of defect fill, the cartilage interface, cartilage surface, structure and signal intensity, the constitution of subchondral lamina and bone, possible adhesions and effusion).

Histology Findings

Animals were sacrificed. Distal femora were cleared of all soft-tissue attachments and fixed using a 1:10 dilution of formalin solution. Sagittal sections of thickness between 3 mm and 4 mm were taken through the distal femur.

Bone was decalcified in a solution comprising a mixture of hydrochloric acid, formic acid and formalin. The decalcified sections were embedded in paraffin and sectioned carefully at a thickness of 5 µ to 6 µ with a microtome and stained with haematoxylin and eosin (HE). Between four and eight sections from the distal femur and medial and lateral condyle of the articular surface of each rabbit were analyzed.

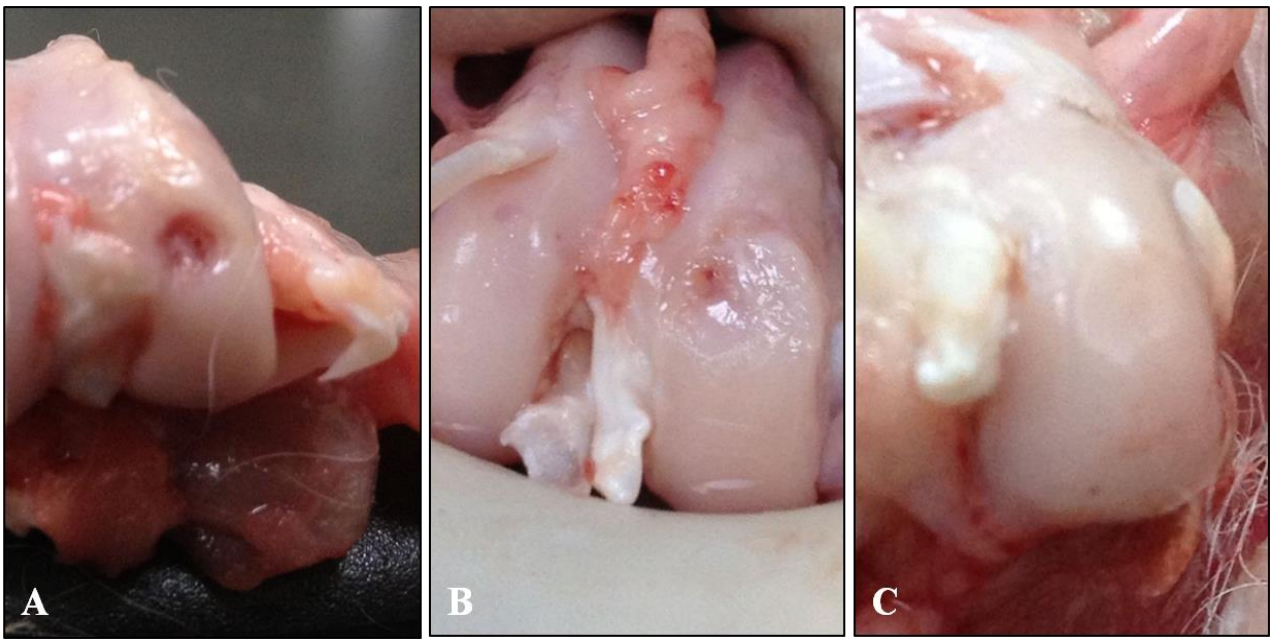

Figure 1: Gross Changes in the Articular Surface of the rabbit knee joint. Panel A shows the untreated defect. Panel B shows the defect treated with fibrin sealant. Panel C shows the defect after treatment with MSCs impregnated with autologous cartilage paste.

RESULTS

All the groups were stable after surgery with no signs of infection. The range of motion of all the operated knees in all groups was full with complete power and the rabbits mobilized well in the cage. Figure 1, shows the post-mortem gross appearance of the articular surface of the knee joint of the studied groups. Figure 1 (A) shows the focal defect that did not receive any treatment. No filling or repair of the defect is observed. Figure 1(B) shows partial filling of the defect in the rabbit group receiving MSCs and fibrin sealant. No evidence of healing of the articular cartilage surface. Figure 1 (C) show complete filling of the defect in the rabbit groups that received MSCs mixed with cartilage paste. The articular surface is smooth and the regenerated area was exactly similar to normal cartilage by palpation.

Magnetic resonance imaging (figure 2) showed persistent osteochondral defect in untreated rabbits (A) and in those treated with UC-MSCs only (B) without and with fibrin sealant (figure 2, A, B, D). The rabbit treated with stem cells with fibrin sealant; a large portion of the defect was filled with predominantly intermediate signal with associated low signal foci in both RARE and Flash images but the overlying cartilage and superficial subchondral bone were missing (Figure 2, C). MOCART score was 5 in the fibrin sealant and MSCs only groups, but the defect was much smaller when fibrin sealant was used. Complete defect fill, intact cartilage surface and complete integration with adjacent cartilage was observed in MRI in the rabbits treated with UC-MSCs and cartilage (figure 2, D); MOCART score was 80.

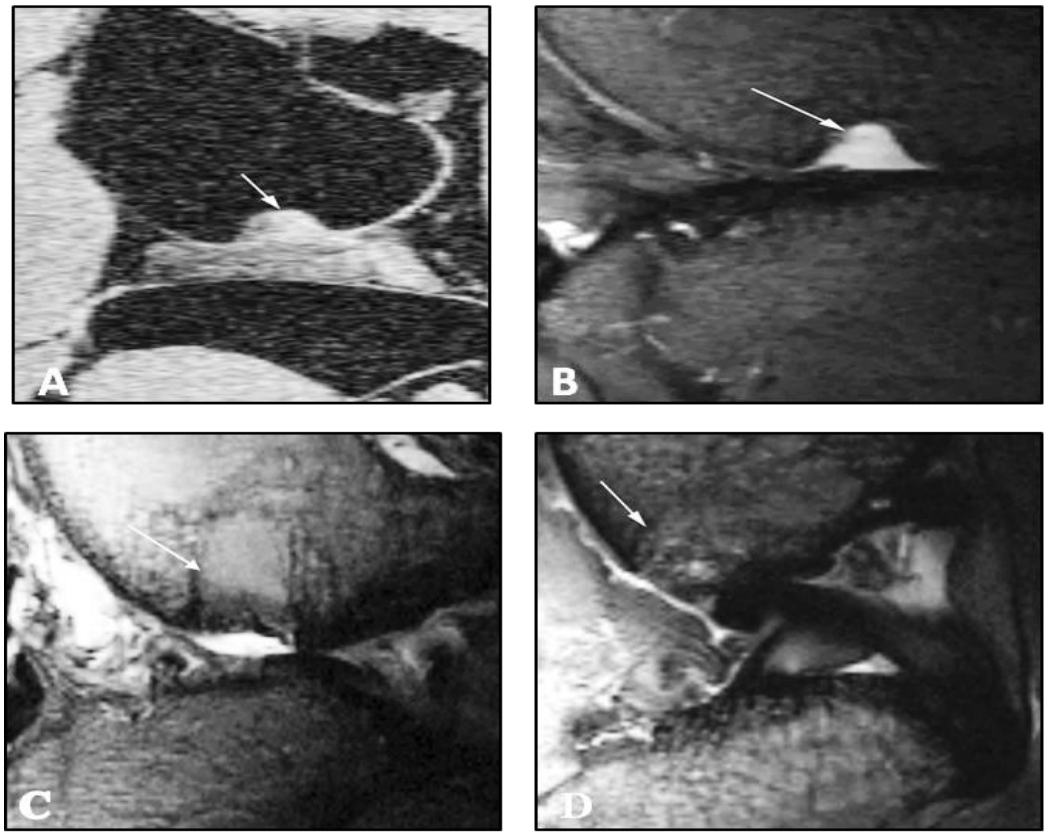

Figure 2: Magnetic resonace images: A: Untreated focal defect in the rabbit knee. B: rabbit treated with stem cells only. There is persistent 3 mm osteochondral defect in the medial femoral condyle. C: Rabbit that received MSCs on a fibrin sealant scaffold. D: Rabbit that received MSCs impregnated with cartilage paste. There is complete fill of the defect.

Histological Evaluation

The untreated defect (Figure 3 A&B): the surface cartilage appeared rough with superficial cracking and clefting with irregular thickness, near total loss of cartilage and areas of bone exposure. The cartilage was hypocellular and chondrocytes were few and single in arrangement.

The UC-MSCs group (Figure 3 C&D): irregular growth pattern of surface cartilage within the defect but not to the extent of the normal edges. Deep surface fissures are very apparent. There is increase in chondrocyte cellularity and the cells are mostly single, taking a more or less linear arrangement, surface is devoid of its flat layer of covering chondrocytes.

UC-MSCs and fibrin sealant (Figure 4, A-D): Surface cartilage is of irregular thickness with persistent evidence of surface clefts and hypertrophic chondrocytes mostly in clusters in a non-specific orientation but there is focal single linear cell arrangement resembling normal pattern in some areas., overlying thick trabecular bone . Underlying bone is still thick and the vascularity in adjacent connective tissue is increased.

UC-MSCs and cartilage paste (Figure 5, A&B and Figure 6, A, B, C, D): Defect in cartilage shows evidence of good healing activity with the edges of cartilage overhanging the defect. The new cartilage is of irregular thickness with obvious areas of hypertrophy and cells are arranged in linear manner similar to normal cartilage. There was also an observable increase in number of proliferating immature chondrocytes adjacent to thick mature cartilage, the deeper layers of which show mineralization. There are new proliferating chondroblasts. Bone is still dense and sclerotic but penetrated by many vascular connective tissue islands and tidemark is irregular and focally reduplicated in areas overlying a thick zone of mineralized cartilage.

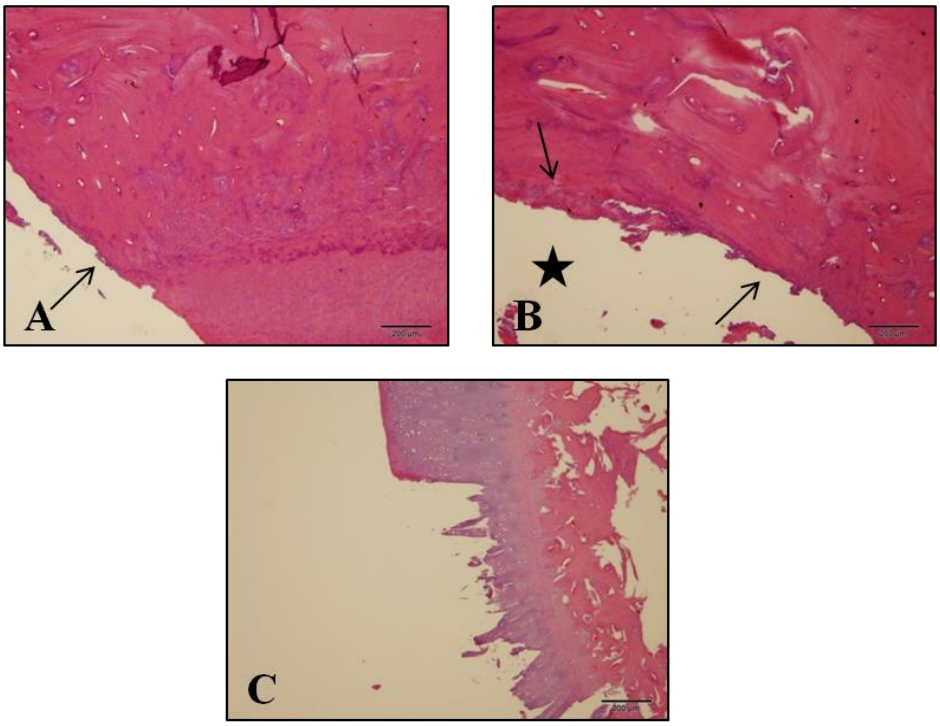

Figure 3: Photomicrographs of sections of the articular surface of the knee joint. A: Untreated focal defect showing irregular markedly atrophic rough surface with nearly totally loss of surface cartilage (arrow) overlying dense sclerotic bone (HEX100). B: Untreated focal defect showing irregular rough surface of markedly attenuated cartilage with focal fibrosis (star). Cartilage is markedly thinned out and mineralization is irregular (left arrow). Cellularity is low. There is an area of full thickness cartilage loss with focal exposure of bone (right arrow). Rest of bone is dense and sclerotic (HEX200). Defect and MSCs only (C and D): There is Irregular growth of cartilage in the defect but not to the extent of normal edges (star) with deep surface fissures and increase in cellularity. Cells are mostly single, round with a more or less linear arrangement. Surface is still devoid of its flat layer of chondrocytes. Bone still shows evidence of increased density and thickened trabeculae (HE X100).

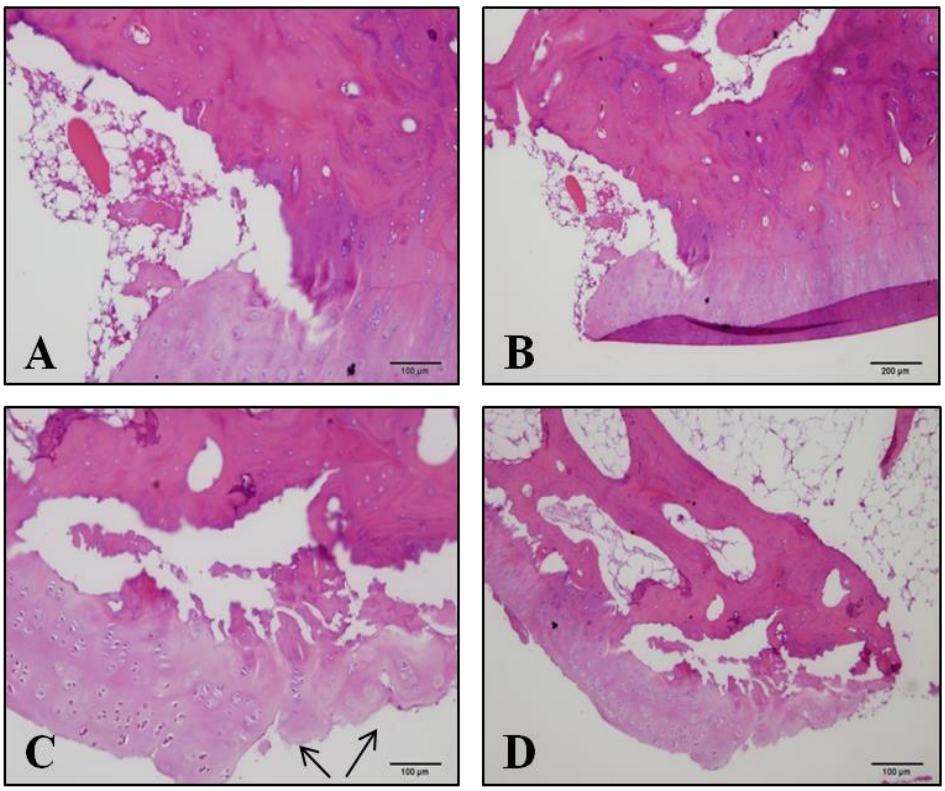

Figure 4: Photomicrographs of sections of the articular surface of the knee joint. MSCs and fibrin sealant: panels A and B show a wide area of thick regular cartilage with a smooth regular surface overlying dense, thick, markedly sclerotic bone with hypertrophic cartilage cells that started to show a more or less linear arrangement but proliferating cell clusters are still evident. Increased vascularity is also observed (arrow). (HE X400 & 100, respectively). Panels C & D show Irregular thickness of surface cartilage composed of hypertrophic chondrocytes mostly in clusters with a nonspecific orientation but there is also focal single linear cell arrangement resembling normal cartilage. The underlying trabecular bone appears still slightly thicker than normal (HEX 400 and 200, respectively).

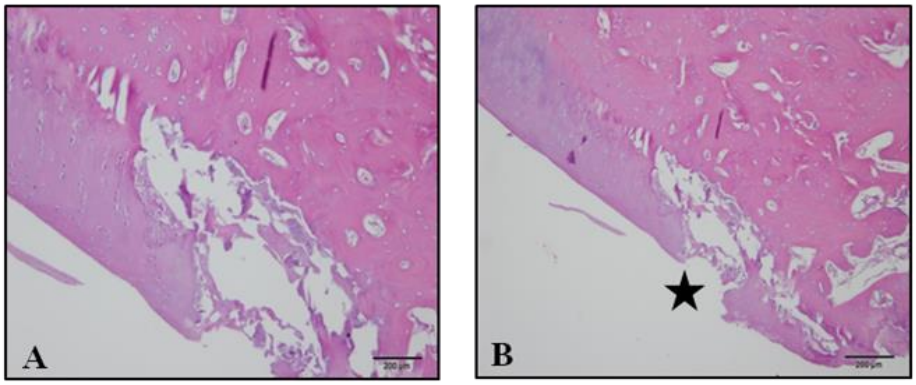

Figure 5: Photomicrographs of sections of the articular surface of the knee joint. MSCs impregnated with cartilage paste: panel A shows the defect in cartilage with healing activity with the edges of cartilage overhanging the defect .Cartilage is of irregular thickness with obvious areas of hypertrophy. Chondrocytes are arranged in linear manner similar to normal cartilage. Bone is still dense and sclerotic but penetrated by many vascular connective tissue islands (HEX400). Panel B: Bone appears thick and dense yet there is increased vascularity. The overlying cartilage is of irregular thickness with obvious areas of hypertrophy. Surface defect (star) shows healing activity with the edges of cartilage overhanging the defect (HEX200).

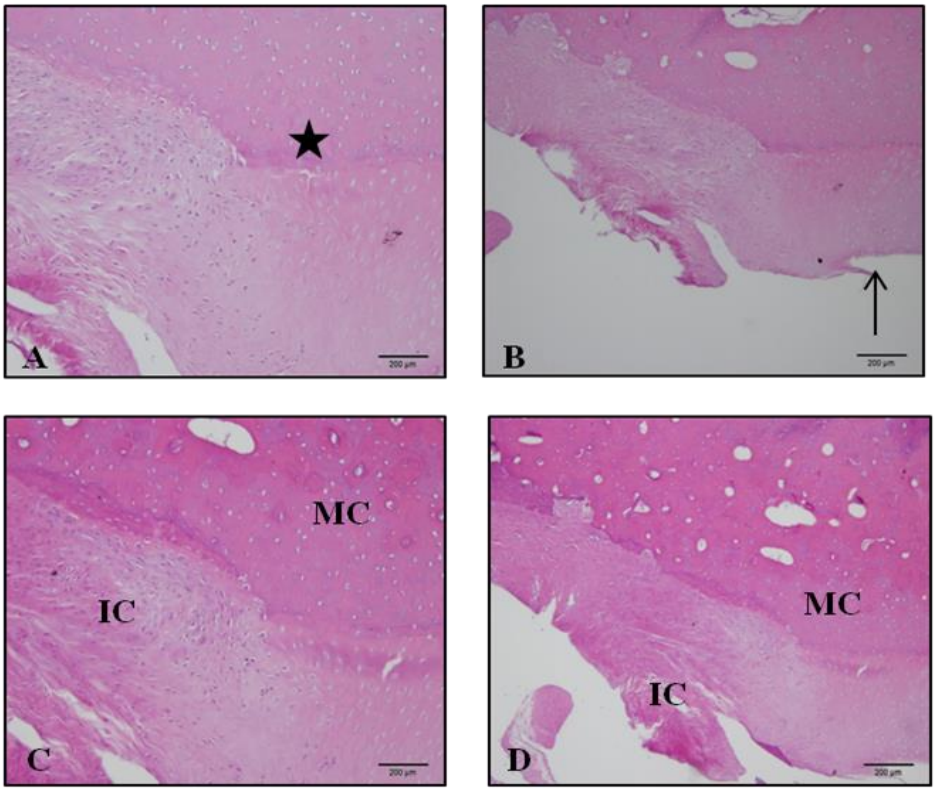

Figure 6: Photomicrographs of sections of the articular surface of the knee joint. MSCs impregnated with cartilage paste. A: Increased number of proliferating immature chondrocytes (left), adjacent to thick mature cartilage with diffusely arranged chondrocytes in no particular arrangement (arrow). There is a mineralized deeper layer of cartilage (star) (HEX400). B: Marked surface proliferation of immature chondrocytes adjacent to mature thick cartilage with diffusely arranged chondrocytes. The surface is smooth over the mature cartilage at edge (arrow) (HEX200). Panels C and D show immature proliferating cartilage cells (IC) adjacent to thick cartilage with diffusely arranged chondrocytes. Tide mark irregular and focally reduplicated (arrow) in areas overlying a thick zone of mineralized cartilage (MC). Remnants of dead cartilage can still be observed in lower right corner and are being replaced by the new proliferating chondroblasts (HEX400 and 200, respectively).

DISCUSSION

Cartilage is devoid of blood vessels, neurons, and lymphatics. It is formed of one cell type– the chondrocyte.[19] It is therefore very suitable for engineering and regeneration. Several investigators employed tissue engineering with chondrocytes and MSCs to repair articular cartilage defects.[17] The aim of cell-based therapies for cartilage defects is to obtain a fully functional hyaline cartilage able to function like the normal cartilage. Although ACI is the gold standard for the repair of chondral defects,[20] it has shown variable reports both clinically and experimentally.[21,22] Large numbers of undifferentiated cells are needed during repair of cartilage defects (1–5 cm^2), using ACI or microfracture. This is a major problem. Chondrocytes are removed from a non-weight bearing region of the cartilage, but they become fibroblastic-like upon implantation, resulting in fibrous and not the desired hyaline cartilage.[23] Some investigators report that the majority of ACI repair tissue has hyaline appearance.[24] The repair tissue may be comparable to surrounding cartilage.[25]

The structural and functional outcomes of the use of stem cells on cartilage repair were investigated in several animal studies.[1] There is a worldwide increase in the number of researchers that are studying the effects of human umbilical cord stem cells on cartilage repair.

In the present work, the effect of UC-MSCs, alone or combined with fibrin sealant or autologous cartilage paste on the repair of fresh, traumatic, focal osteochondral defects in the knee joints of rabbits, was investigated. Clinically, in the present study, all the animals did not show any local or systemic adverse reaction.

The use of xenografts was reported by many investigators who suggested that MSCs may function across species barriers.[26] Xenogeneic MSCs transplantation has been used for bone and cartilage repair without evidence of immunological rejection.[27,28] Equine umbilical cord xenografts were utilized by Saulnier et al,[16] to investigate their healing abilities in a rabbit model of mild osteoarthritis. They did not report any local or systemic adverse response after UC-MSCs injection.

In this work the use of UC-MSCs alone or combined with fibrin sealant did not have a remarkable healing potential on the focal cartilage defect. MRI showed persistent variable size defects. However, the defect was smaller when fibrin sealant was used but in either case MOCART score was low (5 out of 100).

Several investigators attempted before the use of platelet-rich preparations as fibrin glue as a scaffold for MSCs for chondrogenesis. Haleem et al,[14] used human autologous bone marrow MSCs seeded on platelet-rich fibrin glue (PR-FG) to repair human traumatic focal cartilage defects of the knee joints. The fibrin glue had no deleterious effect on cell viability. Healing of the defect by hyaline-like cartilage was reported, and clinical and radiological scores improved markedly. They stated that PR-FG has the advantage of being nonimmunogenic, biodegradable, and easily prepared intraoperatively. They postulated that the granules of platelets secrete transforming growth factor (TGF)-β1 and insulin growth factor-1 (IGF-1), which may stimulate cartilage regeneration. In the present study we used a patented fibrin sealant, prepared intraoperatively, and mixed with MSCs immediately before implantation into the defect. The fact that this sealant is used primarily for wound repair, and may be lacking certain needed cytokines due to the manufacturing process, might be behind the lack of proper healing of the cartilage defects.

On the other hand, combining UC-MSCs with autologous cartilage paste improved significantly MOCART score to 80 out of 100 as MRI showed complete defect and intact cartilage surface. Histologically, the defect showed evidence of good healing activity with the edges of cartilage overhanging the defect. The new cartilage was of irregular thickness with obvious areas of hypertrophy and cells were arranged in linear manner similar to normal cartilage. This study and others[29] showed that high-field 9.4 T MRI could be very valuable to objectively monitor and evaluate osteochondral repair in animal research.

Saulnier et al,[16] concluded that early intraarticular injection of equine UC-MSCs, in rabbit knees with osteoarthritis, was effective in decreasing the disease signs. UC-MSCs appeared to modulate the gene expression pattern of matrix-degrading enzymes in synoviocytes. The paracrine properties of MSCs were the main effectors in this process.

Mobasheri et al,[17] reported that cartilage repair requires the availability of large number of cells capable of chondrogenic differentiation. UC- MSCs are favored, because they can be easily obtained, possess high proliferation rate, do not trigger immune reactions or enhance carcinogenesis. UC-MSCs were reported by Fong et al[30] to differentiate into cartilage following culture in three-dimensional nanoscaffolds. However, there is still no solid agreement related to the ideal culture conditions needed to promote differentiation of MSCs to a chondrocytes.[17]

The results of the present work support the notion that combined therapies using UC-MSCs and chondroprogenitor cells or in our case, autologous cartilage paste, may show promise for use in repair of traumatic focal cartilage defects. Further clinical testing is needed. This requires the improvement of cell preparation techniques, use of biocompatible scaffolds and implementation of more trials using large animal models.

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ETHICAL APPROVAL

This study met the standards of and was approved by the Ethics and Research Committee of King Abdul- Aziz University, Jeddah, KSA, following the guidelines for surgical research laboratory in King Fahd Medical research center, King Abdul- Aziz University, Jeddah, KSA.

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