ISSN: 2639-6866

Research Article

Stem Cell Research International

Repair of Articular Cartilage Defects using Bone Marrow Derived Mesenchymal Stem Cells in Rabbits

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Submitted: 04 Nov 2018; **Accepted**: 15 Nov 2018; **Published**: 22 Jan 2019

Summary

The present study was performed to examine the effect of intra- articular injection of bone marrow mesenchymal stem cells (BM-MSCs) and chondrogenic differentiated mesenchymal stem cells (CD- MSCs) on the repair of articular cartilage defects in rabbits.

Twenty-five adult female baladi rabbits were used in this work. 5 rabbits were used for preparation of bone marrow mesenchymal stem cells (BM-MSCs) and their left knees were not subjected for the surgical procedure and used as normal control group. The remaining twenty rabbits were subjected for surgically induced cartilage defects in their left knees through a small medial para-patellar incision using bone curette. In the next day, the rabbits were divided into four groups: group I was not injected intra-articularly, group II injected intra-articularly by a single dose of saline, group III injected intra-articularly by a single dose of BM-MSCs and group IV injected intra-articularly by a single dose of CD-MSCs. After 8 weeks from the time of intra-articular injection. On time the rabbits were sacrificed and the entire knee joints were excised and examined.

Groups I and II showed marked degenerative changes in their articular cartilage. The articular surface healed by fibrocartilage in group III, while in group IV the articular surface healed by hyaline cartilage. Treatment by CD-MSCs promotes a better healing effect on the articular cartilage defects of injured knee joints in rabbit's model and has a remarkable superiority of repair than BM-MSCs. This can prevent the progress of cartilage defect into osteoarthritis which was a harmful disease.

Keywords: Cartilage Defects, Knee, BM-MSCs, CD- MSCs. Osteoarthritis

Introduction

Articular cartilage is a connective tissue composed of specialized cells called chondrocytes, unlike other connective tissues; cartilage does not contain blood vessels. The chondrocytes are supplied by diffusion, thus compared to other connective tissues, cartilage growth and repair becomes more slowly [1].

Articular cartilage is the shiny white surface structure that covers the ends of most bones. Articular cartilage protects the ends of bones and allows the joints to glide smoothly with less friction. It also helps to spread the loads applied to a joint. This covering is only a few millimeters thick and it has no blood supply to facilitate the healing process. Therefore if it gets damaged, there was a very little capacity for healing [2].

Articular cartilage injury or chondral injury may occur as a result of a pivot or twist on a knee similar to the motion that can cause a meniscus tear. Damage may also be the result of a direct blow to the knee joint. Chondral injuries may accompany an injury to a ligament, such as the anterior cruciateligament. Small pieces of the articular cartilage can actually break off and float around in the knee joint as a loose bodies which causing locking, catching and swelling of the joint. The patient's condition may result from a series of minor injuries that have occurred over time and wears of articular cartilage down as a person ages [3].

Articular cartilage injury following joint trauma was a major risk factor for the development of osteoarthritis (OA) which was a condition that results in significant patient morbidity and substantial cost to healthcare systems. Approximately 10 to 25% of the population suffers from OA with increased prevalence noted in older age groups. OA is irreversible and eventually requires joint replacement for alleviation of pain and restoration of function as it progresses to end-stage disease. Due to the limited capacity of articular cartilage injury to repair, early intervention was required to prevent progression to OA. Effective management options are limited at present, resulting in a drive to develop novel tissue engineering techniques to articular cartilage surface defects [4].

Current treatment modalities aim to restore articular cartilage defect through primary repair, stimulation of adjacent tissue and graft implantation. Primary repair involves rigid fixation of osteochondral fractures in an acute setting. Microfracture and subchondral drilling breach subchondral bone to allow migration of cells and chemical mediators into defects. Although this leads to defect filling with repair tissue that is predominantly fibrocartilage, reasonable results can be obtained in the short- to intermediate-term with proper rehabilitation [5].

The management of articular cartilage defects presents many clinical challenges due to its avascular, anural and lymphatic nature. Bone marrow stimulation techniques, such as micro fracture were the most frequently used method in clinical practice however the resulting fibrocartilage tissue which was inferior to native hyaline cartilage. Other methods have shown promise like cell therapy and tissue engineering. There was an unmet need and growing interest in regenerative medicine and tissue engineering to improve the outcome for patients requiring cartilage repair [6].

Properties and functions of stem cells have been extensively studied in the development of organisms, cancer, wound healing, and regenerative medicine. Later, it has been investigated for tackling complex pathogenic conditions such as neurodegenerative diseases, hematopoietic impairment, and musculoskeletal degeneration [7]. Mesenchymal stem cells (MSCs) from bone marrow are well known to have a strong capacity to differentiate into various types of cells such as cartilage, bone and adipose tissue so there is significant interest in mesenchymal stem cells (MSCs) as cell sources of tissue engineering [8].

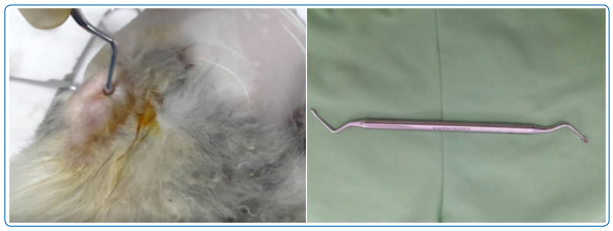
When the organ is injured, transplanted bone marrow cells are mobilized into the injured organ to repair the defect, based on this fact the intra-articularly injected (MSCs) can mobilize into the injured tissue and contribute to their regeneration [9].

Transplantation of mesenchymal stem cells (MSCs) is a cell-based strategy that has the potential to resurface articular cartilage defects while avoiding the downsides of autologous chondrocyte implantation. MSCs have an enhanced proliferative capacity and can be reproducibly differentiated into chondrocytes [10].

Material and Methods

This work was carried out on 25 adult female baladi rabbits at the animal house of Anatomy Department, Faculty of Medicine, Tanta University, Egypt. 20 rabbits subjected to surgery. 5 rabbits were used for preparation of bone marrow mesenchymal stem cells (BM-MSCs) and their left knee were not subjected for surgical procedure and used as normal control group.

The twenty rabbits were subjected to surgically induced articular cartilage injury. The surgical procedure done under general anesthesia by Ketamine (25 mg/Kg IM) .The surgically induced cartilage injuries carried out on their left knee through a small medial parapatellar incision. The lower end of the femur is exposed and articular cartilage injuries created in the femoral condyles of the knee using bone curette (fig. 14). The rabbits received declofenac potassium injection (15 mg/Kg IM) for pain relief and each rabbit received Cefotaxim (20 mg\kg IM) to avoid post-operative infection [11].



Bone curette used in formation of articular cartilage deficit

In the next day of surgical procedure, the twenty rabbits were classified into 4 groups (5 Rabbits each) and treated as follows:

- **Group I:** The rabbits were left without treatment (Sham operated).
- **Group II:** The left knee of each rabbit was subjected for intra-articular injection by a single dose of 0.5 ml saline (Sham operated treated with saline).
- **Group III:** The left knee of each rabbit was subjected for intra-articular injection by a single dose of bone marrow mesenchymal stem cells (BM-MSCs).
- **Group IV:** The left knee of each rabbit was subjected for intra-articular injection by a single dose of chondrogenically differentiated mesenchymal stem cells (CD-MSCs).

All rabbits were allowed unrestricted cage activity without immobilization with free access to food and water until sacrificed after 8 weeks from the time of intra- articular injection. On time the rabbits were sacrificed and the entire knee joints were excised and subjected to the following:

- Macroscopic examination.
- Light microscopic examination.
- Transmission electron microscopic examination.

Preparation of bone marrow samples

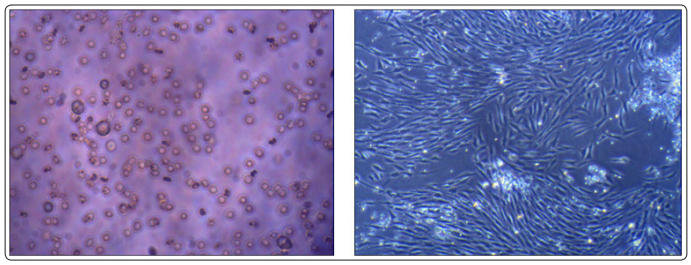
Five Rabbits were sacrificed and their normal left knees were taken as a normal control group, their long bones (femur and tibia) of left knee were dissected and removed. The bones were washed by saline, the bone ends were separated by bone cutter and the diaphysis were collected in Petri dish.

Separation and culture of BM-MSCs

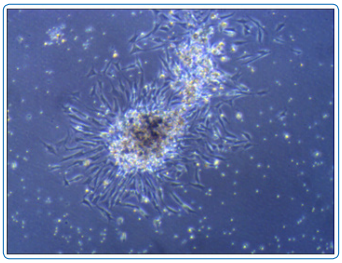
10 ml of the basal media were added to the samples of BM inside the Falcon tubes and centrifuged with 1600 rpm for 10 minutes at 25°C. The supernatant which contains few bone marrow stem cells and other components was discarded. The cell pellets which contain mainly the MSCs were formed at the bottom of the Falcon tubes.

Characterization of undifferentiated BM-MSCs:

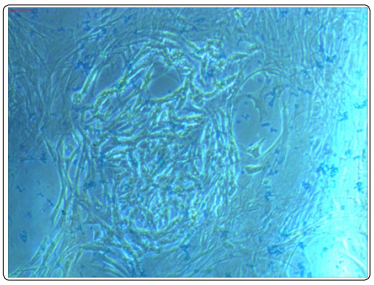
Daily examination of the cultured cells was done during primary cultures and the subsequent passages using phase contrast inverted microscope. These cells were characterized by formation of spindle fibroblast- like cells with long processes and vesicular nuclei attached to the floor of the flask.



After isolation After 5 days of culture



At day nine formation of colony



Formation of cartilage plate

Macroscopic examination

The samples were taken and examined by the naked eye for detection of the cartilage defects and healed lesions.

Light microscopic examination

The specimens were taken and immediately fixed in 10% formol saline solution for 24 hours washed by tap water for half an hour then the specimens was decalcified in the chelating agent disodium EDTA solution 4%.Decalcification lasted for about 4 weeks, during which the solution was renewed every 2 days until the tissues had softened. The decalcified knee joints were cleaved longitudinally in a sagittal plane along the central portion and specimens were processed to form paraffin blocks.

1. Haematoxylin and Eosin [H&E]

The sections were stained in haematoxylin for 15 minutes and washed in tap water for 10 minutes. Then they were stained in eosin for 1 minute then mounted and covered to protect it before examination. The nucleus is stained blue while cytoplasm and fibers stained deep pink.

2. Immunohistochemistry study: this method was used to:

A- Assess the relative proportions of collagen in the regenerated tissue. The samples were processed for immune staining using antibodies to:

1. Type I collagen.
2. Type II collagen.
3. Type II A Procollagen.

B- Assess one of the glycosaminoglycan components of the regenerated matrix using specific anti- aggrecan anti body.
C- Detect the characterization of BM-MSCs using CD 44 antibody.

Transmission electron microscopic Examination

After dehydration samples were embedded in Araldite 502 resin. The plastic molds were cut in the LEICA Ultra cut ultra-microtome, stained with 1% toluidine blue. After examination of semi-thin sections ultra-thin sections were cut, stained with uranyl acetate. Then counter stained with lead citrate and examined and photographed using JEOL-JEM-100SX electron microscope, Japan.

Results

Normal control group

Macroscopic examination showed normal smooth contour and translucency of the articular cartilage surface (Figure 26).The chondrocytes in the tangential zone were flattened, condensed and arranged parallel to the surface. The cells in the transitional zone lied in lacunae appeared larger, rounded, randomly spaced and the matrix homogenously stained eosinophillic. The cells in radial zone were arranged in vertical irregular columns with lack of colonies formation (Figure 28). Immuno-histochemical examination showed no brownish discoloration in the matrix of the articular cartilage which negative reaction for anti-collagen I (Figure 29). Also, the matrix showed brownish discoloration staining for the matrix which was a positive reaction for anti- aggrecan (Figure 30). While there was diffuse brownish discoloration staining for the matrix of the articular cartilage which was a positive reaction for anti-collagen II (Figure 31). The articular cartilage showed no brownish discoloration staining for the cytoplasm and nucleus of chondrocytes in radial zone which was a negative reaction for anti- procollagen IIA (Figure 32). The articular cartilage showed no brownish star shaped cells which was negative reaction for CD 44 anti- body (Figure 33). Ultrastructural examination of the articular cartilage showed a spherical chondrocyte present inside its lacuna with rounded heterochromatic nucleus and normally stained cytoplasm (Figure 34).

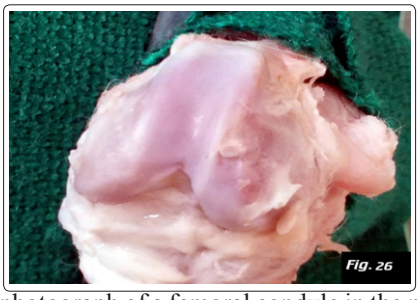


Figure 26: A photograph of a femoral condyle in the normal control group showing smooth, regular and transparent articular cartilage

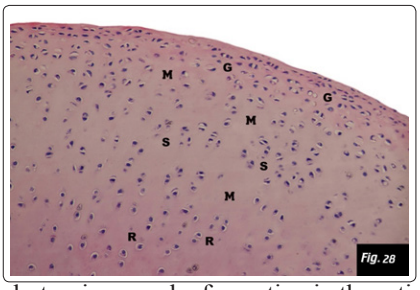


Figure 28: A photomicrograph of a section in the articular cartilage of a femoral condyles of normal control group showing flattened condensed and parallel chondrocytes in the tangential zone (G). Larger, rounded cells inside their lacuna present in eosinophlic matrix (M) in the transitional zone (S).The cells are arranged in vertical irregular columns with lack of colonies formation in radial zone (R). (H&E x400).

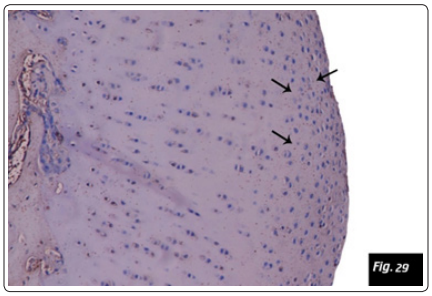


Figure 29: A photomicrograph of a section in the articular cartilage of a femoral condyle of normal control group showing no brownish discoloration in the matrix for anti-collagen I (➞).(Anti-Collagen I x200)

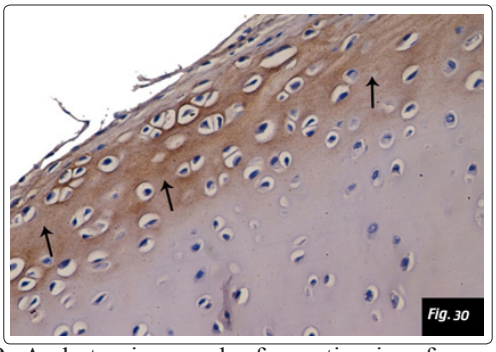


Figure 30: A photomicrograph of a section in a femoral condyle of normal control group showing brownish discoloration for anti-aggrecan in the matrix of articular cartilage (➞).(Anti- Aggrecan x400)

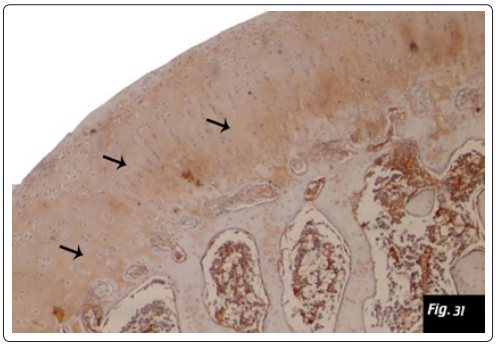


Figure 31: A photomicrograph of a section in the articular cartilage of a femoral condyles of normal control group showing diffuse brownish discoloration for anti-collagen II in the matrix of articular cartilage (➞) (Anti- Collagen II x200)

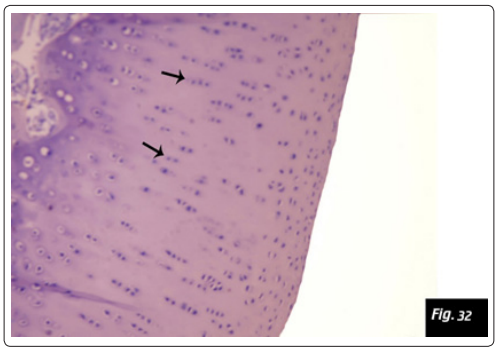


Figure 32: A photomicrograph of a section in the articular cartilage of a femoral condyles of normal control group showing no brown discoloration for anti- procollagen IIA in the cytoplasm and nucleus of the chondrocytes (➞). (Anti-Procollagen IIA x200)

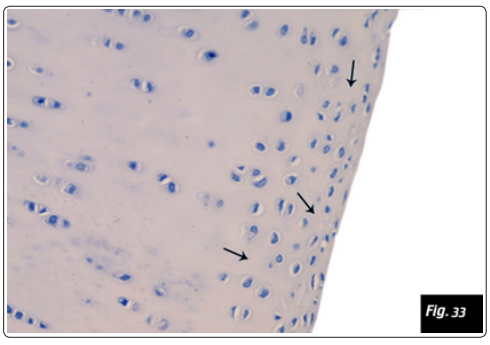


Figure 33: A photomicrograph of a section in a femoral condyles of normal control group showing no brownish star shaped cells in the matrix of the articular cartilage which is a negative reaction for CD 44 antibody (➞). (Anti- CD 44 x400)

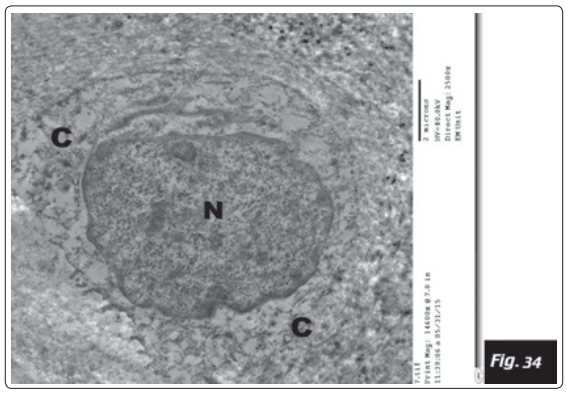


Figure 34: An electron micrograph of ultrathin section of an articular cartilage of normal control group showing a spherical chondrocyte within its lacuna with rounded heterochromatic nucleus (N) and normally stained cytoplasm (C). (Uranyl acetate & Lead Cit. x2500)

II- Group I & II (Sham operated and saline treated left knee)

Group I & II were showed the same results. The macroscopic examination showed opaque irregular surface of the articular cartilage with a defect which exposed the under lying bone without any evidence of repair and restoration of articular surface contour. The cells in tangential zone formed many chondrocyte colonies. The cells in the transitional zone were small in size with shrunken pyknotic nucleus and not present inside their lacuna. The cells in the radial zone had no distinct columnar order of distribution in comparison with the normal cartilage surface showed large crack defect which extended from the surface of the cartilage to the underlying bone. Immuno-histochemical examination showed diffuse brownish discoloration for the matrix of the articular cartilage which denotes positive reaction for anti-collagen I and showed no brownish star shaped cells at the site of defect which negative for CD 44 antibody. While the articular cartilage showed negative reaction for anti-collagen II, anti- aggrecan in the matrix of articular cartilage and anti- procollagen IIA in the cytoplasm and nucleus of chondrocytes at site of defect. The ultrastructural examination of the articular cartilage showed chondrocytes with shrunken, irregular and condensed nucleus surrounded by vacuolated and intensified stained cytoplasm.

III- Group III (Operated left knee treated by MSCs-Injection)

Macroscopic examination showed irregular and opaque pitting on the surface of the articular cartilage of femoral condyles, with evidence of scaring, retardation of the progressive lesions and partial restoration of cartilage contour (Figure 46). The chondrocytes in the tangential zone were flattened and parallel to the surface. The cells in the transitional zone were larger, rounded, situated in their lacunae and randomly spaced homogenously stained eosinophillic matrix. The cells in radial zone were arranged in vertical irregular columns with lack of colonies formation with presence of surface fibrillations (small irregularities of the surface) (Figure 48).Immuno-histochemical showed diffuse brownish discoloration in the matrix of the articular cartilage which positive reaction for anti-collagen I (Figure 49) and anti-aggrecan (Figure 50). Also, the matrix showed multiple brownish star shaped cells at site of the defect which positive reaction for CD 44 antibody (Figure 52), while showed

negative reaction for anti-collagen II (Figure 53). The articular cartilage showed brownish discoloration in the cytoplasm and nucleus of chondrocytes at radial zone which positive reaction for anti- procollagen IIA (Figure 54). Ultrastructural examination of the articular cartilage showed chondrocytes with lessirregular, less condensed nucleus surrounded by less intensified stained cytoplasm withdecreased number of vaculations inside it in comparison with group I & II (Figure 55).

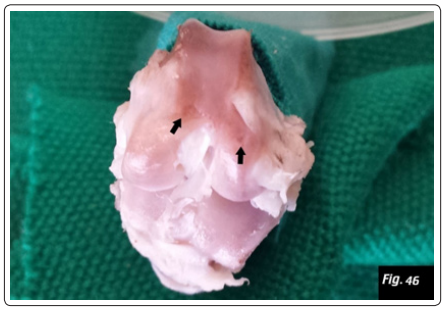


Figure 46: A photograph of the articular cartilage of group III showing irregular and opaque pitting on the surface of the medial and lateral femoral condyles (➞).

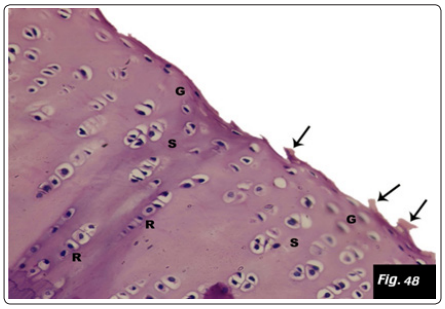


Figure 48: A photomicrograph of a section in the articular cartilage of group III showing surface fibrillations (➞). The cells in the tangential zone (G) flat and parallel to the surface. The cells in the transitional zone (S) larger, rounded, situated in their lacunae and randomly spaced in the matrix which becomes more eosinophillic. The cells in radial zone (R) arranged in vertical irregular columns with lack of colonies formation.
(H& E x400)

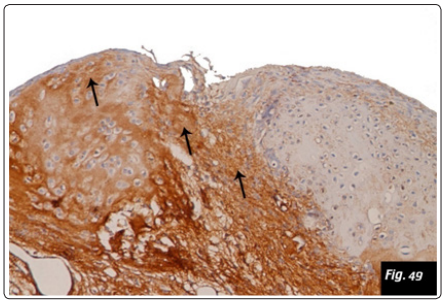


Figure 49: A photomicrograph of an articular cartilage of the femoral condyles of group III showing brownish discoloration in the matrix of the articular cartilage for anti-collagen I (➞). (Anti- Collagen I x200)

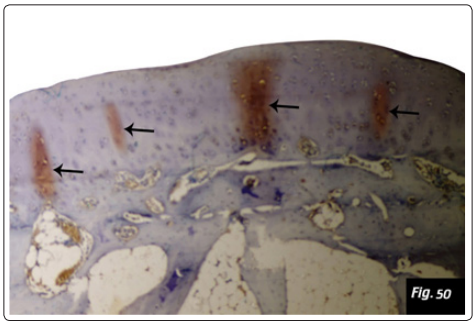


Figure 50: A photomicrograph of a section in the articular cartilage of the femoral condyles of group III showing brownish discoloration in the matrix for anti- aggrecan in different sites of healing (➞). (Anti- Aggrecan x100)

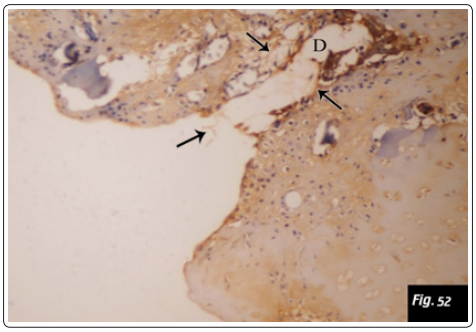


Figure 52: A photomicrograph of a section in the femoral condyles of group III showing multiple brownish star shaped cells at site of defect (D) in the matrix of the articular cartilage which positive reaction for CD 44 antibody (➞) .(Anti- CD 44 x 200)

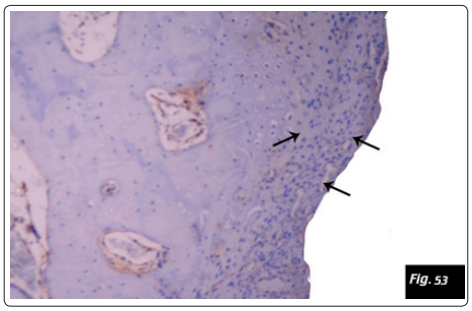


Figure 53: A photomicrograph of a section in the articular cartilage of group III showing no brownish discoloration of the matrix for anti-collagen II (➞).(Anti- Collagen II x200)

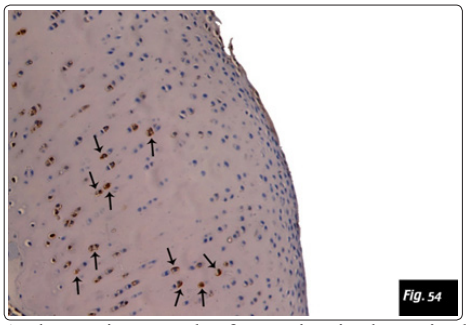


Figure 54: A photomicrograph of a section in the articular cartilage of group III showing no brownish discoloration for anti- procollagen IIA in the cytoplasm and nucleus of chondrocytes at radial zone of the articular cartilage. (➞).(Anti- Procollagen II A x200)

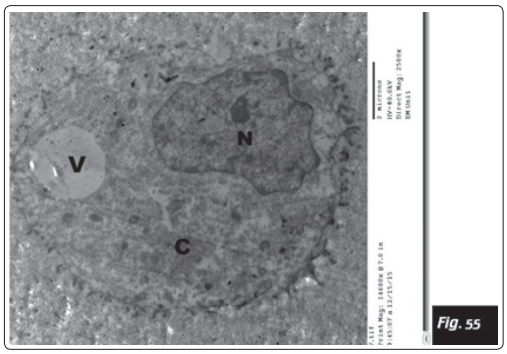


Figure 55: An electron micrograph of ultrathin section of articular cartilage of group III showing chondrocyte with less irregular, less condensed nucleus (N) surrounded by less intensified stained cytoplasm (C) with presence of one vacuole (V) inside it in comparison with group I & II. (Uranyl acetate& Lead Cit. x2500)

IV Group IV (Operated left knee treated by CD-MSCs Injection)
Macroscopic examination showed healed defect of femoral condyles leading to complete restoration of articular cartilage contour with normal transparent appearance (Figure 56). Histological examination showed smooth intact surface of the articular cartilage with apparent normal thickness and marked increased intensity of eosinophilic staining. The cells in the tangential zone were flattened and arranged parallel to the surface. The cells in transitional zone were large, rounded cells present inside their lacunae. The cells in radial zone were arranged in columns without colony formation. (Figure 58). Immuno-histochemical examination showed no brownish discoloration in the matrix of the articular cartilage which negative reaction for anti-collagen I (Figure 59). Also, the matrix showed few brownish star shaped cells which positive reaction for CD 44 antibody (Figure 60). While the articular cartilage showed diffuse brownish discoloration of the matrix which positive reaction for anti-collagen II (Figure 61) and strong brownish discoloration showed in the matrix which positive reaction for anti- aggrecan (Figure 62). The cytoplasm and nucleus of chondrocytes in radial zone showed brownish discoloration which positive reaction for anti- procollagen IIA (Figure 63).Ultrastructural examination of the articular cartilage showed spherical chondrocytes present inside their lacunae with rounded heterochromatic nucleus surrounded by normally stained cytoplasm without vaculations (Figure 64).

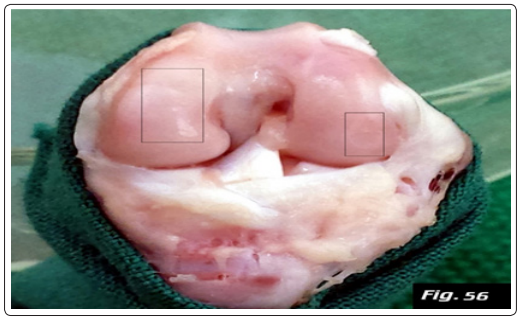


Figure 56: A photograph of the articular cartilage of group IV showing smooth and transparent surface with healed erosions of both femoral condyles (➞).

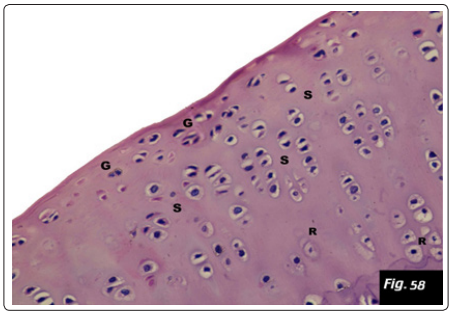


Figure 58: A photomicrograph of the articular cartilage of group IV showing cells in the tangential zone (G) are flattened and arranged parallel to the surface. The cells in transitional zone (S) are large, rounded cells present inside their lacunae. The cells in radial zone (R) are arranged in columns without colony formation.
(H& E x400)

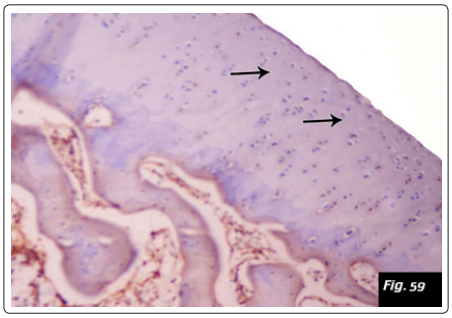


Figure 59: A photomicrograph of the articular cartilage of group IV showing no brownish discoloration in the matrix of the articular cartilage for anti-collagen I (➞). (Anti- Collagen I x200)

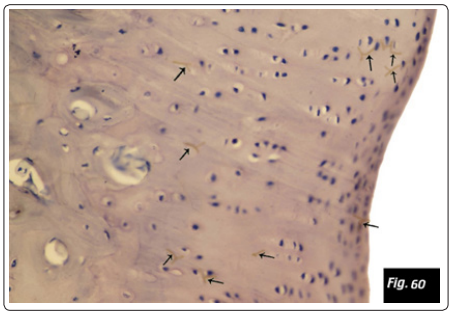


Figure 60: A photomicrograph of a section in the femoral condyles of group IV showing few brownish star shaped cells in the matrix of the articular cartilage which positive reaction for CD 44 antibody (➞). (Anti- CD 44 x 400)

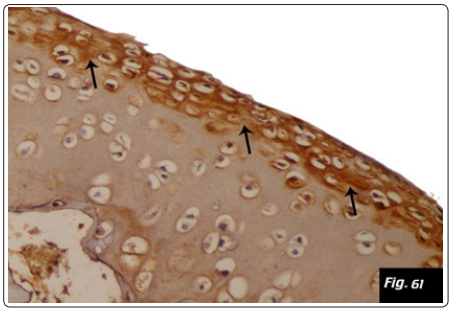


Figure 61: A photomicrograph of the articular cartilage of group IV showing brownish discoloration for anti-collagen II in the matrix of articular cartilage (➞). (Anti- Collagen II x400)

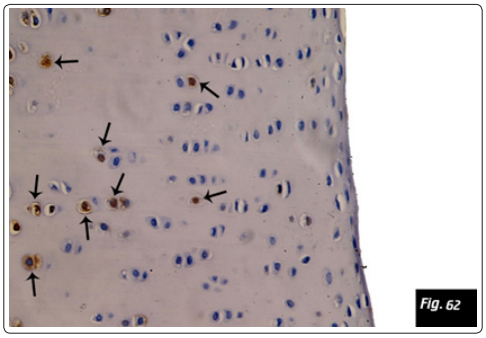


Figure 62: A photomicrograph of the articular cartilage of group IV showing positive reaction for anti- procollagen IIA in the cytoplasm and nucleus of chondrocytes in radial zone (➞). (Anti- Procollagen II A x400)

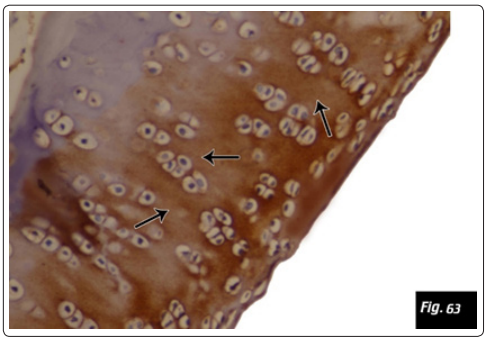


Figure 63: A photomicrograph of a section in the articular cartilage of group IV showing strong brownish discoloration for anti- aggrecan in the matrix of articular cartilage (➞). (Anti- Aggrecan x400)

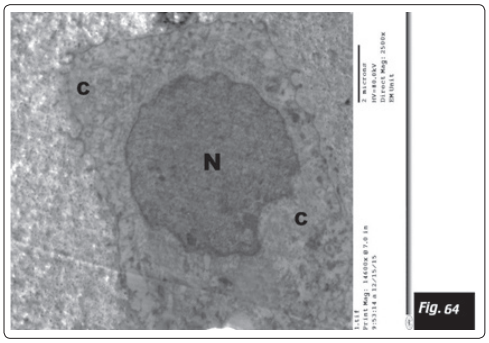


Figure 64: An electron micrograph of ultrathin section of articular cartilage of group IV showing spherical chondrocyte inside their lacunae with rounded heterochromatic nucleus (N) surrounded by normally stained cytoplasm (C) without vaculations. (Uranyl acetate& Lead Cit. x2500)

Discussion

Articular cartilage is a load-bearing in the structural organization of the normal joint and an essential part of joint motion. However, due to the lack of blood supply, it has limited capacity for regeneration once it is injured and is usually repaired with fibrous tissue which does not have the durability and mechanics of normal hyaline cartilage [12].

Cartilage defects are still a challenging clinical problem because these defects were could not be restored successfully spontaneously leading to biologic regeneration for osteoarthritic joints mainly in young and active peoples due to increase the incidence of wear and tear [13].

Kalamegam et al. Mentioned that the repair of articular cartilage injury by several methods were functionally inadequate in the long term and increased the risk of further damage [14]. Based on these reasons, the repair methods of damaged articular cartilage are limited and the traditional treatments are not ideal. As a result, the treatment of cartilage defects has remained a severe challenge in the orthopedic clinic. The MSCs may be used as chondrocyte progenitor cells for cartilage healing after stimulation by cell-cell contact and soluble factors. MSCs seem to be the best candidates for cell therapy for regeneration of injured tissue [15].

The present study highlighted the role of bone marrow derived mesenchymal stem cell (BM-MSC) as a curative method for articular cartilage defects which when untreated progressed to joint osteoarthritis. The knee osteoarthritis was a major cause of disability causing considerable pain, loss of mobility and reducing the quality of life for a lot of people [16].

The aim of the present study was to test the power of cartilage regeneration from the intra-articular injection of BM-MSCs and chondrogenic differentiated MSCs (CD-MSCs) on articular cartilage defects. The rabbit is a suitable model for questions related to articular cartilage defects and yields adequate amounts of tissues to allow comprehensive histopathological assessment of structural changes associated with the disease process [17].

The surgical procedure was induced by small medial para-patellar incision then the lower end of the femur was exposed and articular cartilage defect created in the femoral condyle of the knee joint using bone curette in rabbit [18]. In this study the BM-MSCs were transplanted by heterologous manner from one rabbit to another. This was one of the accepted experimental procedures to assess the therapeutic potential of these cells in experimentally induced pathological conditions [19].

BM-MSCs express low levels of major histocompatibility complex-1 (MHC-1) molecules on their cell surface and don't express MHC-II molecules. Consequently, BM-MSCs not activate the allogeneic lymphocytes and lead to lack in immunogenicity. These characteristics support the possibility of universal donor BM-MSCs for therapeutic application such as umbilical cord- derived MSCs [20].

In this study the macroscopic examination of the articular cartilage of rabbit knee which have sham operation without treatment or treated with saline injection (group I & II) showed the lesion without any evidence of repair in the articular cartilage defect. The articular surface of femoral condyles were opaque, irregular with a defect expose the underlying bone without any evidence of repair and restoration of articular surface contour. This result was agreed with Mark et al. who mentioned that the affected intra-articular cartilage when left without treatment lead to formation of intraarticular adhesions with opaque and persistent defects without restoration of articular surface contour which progress to osteoarthritis [21]. Oliveira et al. were found also that the knee of induced articular cartilage defects showed signs of obvious macroscopic lesions [22].

Moreover, the specimens which were progressed to osteoarthritis characterized by extensive articular cartilage loss and erosion

or ulceration of the articular cartilage surface, this was recorded by Elmorsy et al. [23]. The explanation of OA which induced by untreated cartilage defects was stated by Sulzbacher who mentioned that the course of progressive OA is primarily characterized by softening and loss of cartilage tissue. Biochemically, the network of collagen fibers were altered with loss of proteoglycans and increased water content of the matrix [24].

Microscopic examination of the articular cartilage of femoral condyles of the control group showed significant degenerative findings. One of the prominent finding was matrix depletion evidenced by decrease basophilia in H&E stained sections. Also, there was marked surface irregularities, superficial fibrillation, pitting, cracks and fissures. Cartilage organization and cellular arrangement was disturbed.

These findings can be explained according to Okada et al. who observed in their studies that there was decreased proteoglycans in the articular cartilage defect models [25]. This might be due to decreased glycosaminoglycan concentrations. It was suggested that in progressive articular cartilage defects there were a major chondrocytes death which easily lead to failure of cartilage matrix turnover as chondrocytes are the only source of matrix components synthesis in the articular cartilage.

These results were coincided with Lannitti et al. who mentioned that all animals of untreated articular cartilage defects showed superficial fibrillation of articular cartilage, minimal chondrocyte clustering and fibrosis in the superficial zone, with presence of multiple cracks and fissures (clefts) [26]. This was also in agreement with Li et al. who observed a histological changes as hypocellularity, disorganization, proteoglycan reduction and denudation of articular cartilage surface and fissures [27]. In addition, Elmorsy et al. found sever changes, such as the loss of the superficial layer of the articular cartilage with fibrillation and fissures in rabbit's knee [23].

Sulzbacher was stated that the fissures occur as a result of reduction of proteoglycans which disturbing the balance between the internal swelling pressure of the proteoglycan and the tension of type II collagen fibers [24]. Also hypocellularity occur due to apoptosis and chondrocytes that do not die formed chondrocyte clusters in large lacuna with presence of fibrous connective tissue bridging across the fissures occur as a reaction. also revealed that the chondrocytes are not able to produce normal matrix. Because matrix production by these cells is disturbed by shifting the metabolism towards catabolic effects by producing matrix degrading enzymes especially matrix metalloproteinase (MMPs) like proteases, aggrecanase or collagenase which were be able to cleave the collagen fibers and help in breaking down of the cartilage matrix.

Immunohistochemical staining for collagen I was positive indicated the presence of collagen I fibers in the matrix of the articular cartilage which were the main component of fibrocartilage. Staining for collagen II was negative indicated the absence of collagen II fibers from the matrix of the articular cartilage raveled that there was no hyaline cartilage formed at the site of defect because the collagen II fibers were the main component of the hyaline cartilage. Staining for procollagen IIA which produced by chondroproginator cells (chondrogenic mesenchymal cells) which were the precursor of chondrocytes was negative also indicated that there was no developing cartilage and there was no process of healing occurred at the site of articular cartilage defect.

Regarding the anti- aggrecan immune stain which was negative also due to the destruction of aggrecan molecules by the effect of aggreganase enzyme which released as a result of inflammatory condition to the induced articular cartilage defect. This result coincided with Anik et al. who revealed that the repaired articular cartilage or diseased articular cartilage may have less glycosaminoglycans (GAG) such as aggrecan molecules components on its matrix [28].

Roughley and Mort mentioned that matrix metalloproteinases and aggrecanases played a major role in aggrecan degradation which upregulated by mediators associated with joint inflammation and overloading during walking with presence of articular cartilage damage [29]. Moreover, Zhang et al. mentioned that aggrecan degradation significantly enhanced in femoral and tibial articular cartilage defect areas in mice model as a result of its destruction by aggrecanases or MMPs compared to their respective control mice [30].

Negative reaction to CD 44 immunostain in the present study indicated the absence of star shaped chondrogenic progenitor cells at the site of cartilage defect. This result was coincided with Zschamack et al. who mentioned that the cartilage defects in adults do not show any spontaneous healing responsebecause of the absence of BM-MSCs. The healing only occurred in osteochondral defects when the defect reaches the underlying subchondral bone to allow the migration of local BM-MSCs to the site of cartilage defect [31].

Ultra-structural study of articular cartilage of the experimental control groups I & II (sham operated and saline treated) of the present work showed chondrocytes with shrunken, irregular and condensed nucleus surrounded by vacuolated and intensified stained cytoplasm. Also, spindle shaped fibroblasts were present surrounding the site of defect in the articular cartilage. These results were coincided with Zamli et al. who reported that there were increased chondrocytes apoptosis associated with untreated articular cartilage defects in guinea pig models which lead to progressive osteoarthritis [32]. Also, these findings were in alignment with Li et al. who reported the ultra-structural characteristics of apoptotic cells including the presence of apoptotic bodies and cell shrinkage, whereas intensified staining of the cytoplasm, nuclear and cell membrane blabbing and condensation of the chromatin [27].

Macroscopic examination of the articular cartilage of group III (BM-MSCs treated group) showed some evidence of repair and healing than group I & II. There was small area of cartilage discoloration and pitting surface at the site of articular cartilage defect with evidence of scaring showed partial restoration of articular surface contour. The same results were previously observed by Muhammad et al. who mentioned that the BM-MSCs were have an active mobilization effect into the injured tissues after intra- articular injection and their contribution of tissue regeneration [33]. Also, Anik et al. found that rabbits treated with BM-MSCs after articular cartilage defects on their knee exhibited a reduction in the severity of cartilage lesions and promote cartilage repair and regeneration [28]. Moreover, Mokbel et al. who revealed that the use of intra-articular injection of autologous MSCs is a viable option for treating partial cartilage defects This study was intended to verify the likelihood of homing of intra-articularily injected mesenchymal stem cells (MSCs) and

its involvement in the healing process of experimentally induced acute and chronic partial chondral defects in dogs [34].

Histological examination of group III revealed that the surface of repaired articular cartilage was less smooth with less irregularities. So the present study showed incomplete restoration of the thickness of the articular cartilage and the chondrocytes were more disorganized and not grouped into discrete columns with more chondrocyte colonies were present. In agreement to the current result, Oshima et al. observed irregularly distributed chondrocytes after BM-MSCs transplantation in a rat with articular cartilage defect [35]. Another study performed by Lotz et al. recorded that clusters formation might represented the first phase in the repair of cartilage lesions due to proliferation of some cells adjacent to the damaged area, migration, differentiation and new matrix formation might occur [36]. This was matched with the results of Shalabi et al. Who observed healed articular cartilage defects and presence of disorganized chondrocytes and clusters formation in the matrix of the articular cartilage which treated with BM-MSCs. Hamoud Al Faqeh et al. mentioned that the intra-articular injection of BM-MSCs in a sheep model after articular cartilage defects demonstrated a good articular cartilage histoarchitecture, thickness and quality [17].

Immunohistochemical study of the articular cartilage of group III (BM-MSCs treated group) showed that the immunostaine for anti-Collagen II was negative which indicated the absence of collagen II fibers which were the main component of articular cartilage. The immunostaine of anti-Collagen I was positive revealed the presence of collagen I fibers which were the main component of fibrocartilage. These results were coincided with Hollander et al. who recorded that the fibrocartilage tissues were rich in collagen I in contrast they may be either present in a very small amounts or absent from hyaline cartilage [37]. These findings proved that treatment of acute cartilage injury by BM-MSCs was healed by fibrocartilage not hyaline cartilage.

The stain of anti-procollagen IIA was positive this indicated the presence of procollagen IIA which produced by chondro-progenitor cells (e.g. chondrogenic mesenchymal cells) which enhance the process of healing at the site of articular cartilage defect. This result was agreed with Aszodi et al. whose revealed that the presence of type IIA procollagen was important in cartilage development mainly in early stages, the procollagen IIA act as indicative of chondrogenesis because it contains a chordin-like receptors which binds with growth factors including TGFB and BMP-2, there for its presence could potentially facilitate a speedier production of matrix molecules and in-filling of the articular cartilage defects [38].

These results was coincided with Roberts et al. who stated that the articular cartilage defects were replaced by fibrous tissue after treatment by BM-MSCs therapy in 3% of human specimens which indicated by the presence of positive immunestaine for type anti-collagen-I antibody and negative immunostaine for anti-collagen II, anti-procollagen IIA and anti-aggrecan antibodies [39].

Immuno-staining for anti-aggrecan which was considered as one of the glycosaminoglycan components of the regenerated matrix (GAG) in this group was positive in some areas of the tangential and transitional zones. This result indicated that the degradation of aggrecan by aggrecanase was lesser than group I & II due to the decrease of matrix metalloproteinase which is responsible for degradation of aggrecan molecule. These data confirmed the observation of Xie et al. who demonstrated that BM-MSCs promoted cartilage tissue regeneration with abundant sulfated GAG in the knee joint articular cartilage defect of pigs and rabbits respectively [40].

The results of this experiment revealed that the articular cartilage defects treated with BM-MSCs were healed by fibrocartilage. This result was coincided with Roberts et al. who founded about 65% of articular cartilage defect specimens were predominantly healed by fibrocartilage after treatment of these defects by cell therapy indicated by presence of collagen I fibers, aggrecan molecules and procollagen IIA [39]. Also, Hamoud Al Faqeh et al. who revealed that the intra articular injection of BM-MSCs had a major tendency for the repair of tissues riches in fibrocartilage as meniscus regeneration [17].

The regeneration of articular cartilage defects by BM-MSCs in our experimental study can be attributed to that the BM-MSCs were multipotent cells that can differentiated in to a variety of cell types, including osteoblasts and chondrocytes Baksh et al. [41]. Moreover this result was explained by Caplan and Dennis who showed that BM-MSCs secreted a variety of cytokines and growth factors that have both paracrine and autocrine effects including suppression of the local immune system and apoptosis thus modulate the inflammatory response and enhance the regeneration of the affected tissues [42].

The BM-MSCs also inhibit B-cell proliferation in vitro [43]. Additionally, the activation of monocytes into dendritic cells, and suppress the pro- inflammatory potential and antigen presentation of dendritic cells [44]. They also suppress the natural killer cells and restrain their cytotoxic activity this means that MSCs down-regulate the activities of all immune cells [45].

Vasconcelos et al. were added that after MSCs injection they were passively arrested in capillaries or microvessels including arterioles and post-capillary venules and then directly release a lot of soluble growth factors and trophic cytokines [46]. The cytokines, such as tumor necrosis factor alpha (TNF-a) which induce many destructive effects on case of progressive OA were decreased by the trophic effects of BM-MSCs hence its direct differentiation into repair tissues.

Recently, Fanglong et al. demonstrated in his study that up regulation of aggrecan, down regulation of MMP-13 and significant increase in GAG content in the cell therapy groups imply that bone marrow mesenchymal stem cells promote cartilage matrix synthesis and reduced inflammation of the microenvironment of the chondrocytes [47].

As regard to CD 44 immunostain, it showed positive immune reaction indicate the presence of star cells with bluish vesicular rounded nuclei with prominent nucleoli around the defect. Wang et al. were found that these star shaped cells had a significant renewal capacity and were capable of differentiating into oesteoblastic and chondrocytic cells [48].

Ultra- structural study of articular cartilage of group III showed nearly normal chondrocytes with heterochromatic nuclei. In some sections, the chondrocyte was nearly spherical with irregular nucleus and decreased vaculations in its cytoplasm. This results was

coincided with Mark et al. who demonstrated that the ultrastructural result of the rabbits with osteochondral defects which were allowed for continuous passive motion to facilitate the migration of BM-MSCs toward the defect showed cells in its matrix with collagen fibers oriented similarly to those of normal cartilage and the cells appear metabolically active [21].

Macroscopic examination of group IV chondrogenic differentiated mesenchymal cells MSCs (CD- MSCs) showed healed erosions of femoral condyles leading to complete restoration of articular surface contour with normal translucent appearance with significant amount of regeneration revealed good cartilage histoarchitecture, thickness and quality in comparison with normal cartilage.

The histological findings of the present study confirmed the macroscopic results. They revealed marked reduction in the severity of the femoral condyle lesions. The surface appeared intact with smooth irregularities, with normal thickness of the cartilage resemble that of normal one and the surface was covered with intact articular cartilage. This indicated the regenerating effects of CD-MSCs.

Immunostaining of the articular cartilage of this group was in line with the histological findings. Immune staining for anti-Collagen I was negative, while immune staining for anti-collagenII, anti-procollagen IIA and anti-arggrecan were positive. These findings revealed that the articular cartilage defects were healed by hyaline cartilage.

As regarding CD 44 immunostain, showed small amount of star shaped BM-MSCs in the regenerated cartilage tissue revealed the present of continuous healing process. This result was in agreement with Roberts et al. who added that the presence of BM-MSCs revealed progressive regenerative effect on the cartilage tissue via secretion of procollagen IIA which was the mesenchymal progenitor of the chondrocytes [39].

Ultra-structural study of articular cartilage of group IV showed the chondrocyte spherical in shape inside its lacuna with normal matrix and cytoplasm without vaculation. This results were coincided with Casaki et al. who mentioned that treatment by differentiated chondrocytes give chondrocyte with matrix and cytoplasm resemble to normal cartilage [49].

The macroscopic, light microscopic and electron microscopic findings of this work revealed marked reduction in the severity of cartilage injury. The cartilage structure more or less resembled the normal cartilage. This indicated that using of CD-MSCs had better regenerating effects. This was in agreement with Roberts et al. who found about 96% of articular cartilage defect samples were healed by typically hyaline cartilage indicated by presence of positive immune stain for collagen II fibers which were the main component of the hyaline cartilage and positive immune stain for procollagen IIA which present in hyaline cartilage indicating a high degree of activity in the process of cartilage repair after treatment of the cartilage defect by autologous chondrocyte implantation in human and positive for aggrecan molecules which indicated the presence of active regenerated chondrocytes [39].

Moreover, Hamoud Al Faqeh et al. revealed that the affected joints were injected with BM-MSCs in basal media showed no evidence of regeneration in contrast with the CM (chondrogenic media) in sheep model which demonstrated excellent cartilage surface comparable to normal contralateral knee joint [17].

Also, the positive immunostain for aggrecan was explained by the same author who mentioned that the MSCs which were cultured in a chondrogenic media contain growth factors such as (TGF-B), insulin like growth factor-1 (IGF-1) and dexamethasone, promote the synthesis of proteoglycans. TGF-B was capable of increased proteoglycan synthesis by neutralizing interleukin-1b (IL-1b) which is responsible for the suppression of proteoglycan synthesis. This effect was seen in our in vitro culture experiments.

Lutianov et al. findings coincided with our results as the stem cells need to differentiate into chondrocytes before forming an extracellular matrix and new cartilage to treat articular cartilage defects in the knee joint [50].

Also, Li et al. mentioned that the differentiated BM-MSCs were considered as a promising approach for articular cartilage repair they observed that co-culture of goat BM-MSCs and chondrocytes in vitro had a positive influence in inducing BM-MSCs chondrogenic effects they also observed an increase in GAG content, up regulated expression of chondrogenic genes and collagen II, and down regulation in the expression of the collagen I gene [27].

From the results of the present study and revision of the previous works we can concluded that the treatment by BM-MSCs could modify the microenvironment of injured tissue and protect damaged tissue through releasing anti- inflammatory and anti- apoptotic molecules which activate the healing process. Moreover, the treatment by CD-MSCs promotes a better healing effect on the articular cartilage defects of injured knee joints in rabbit's model and have a remarkable superiority of repairing effect than MSCs only, so it can prevent the progress of these defects into osteoarthritis which was a harmful disease [51].

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