Original Article

A Histological Study on The Effect of Bone Marrow Derived Mesenchymal Stem Cells Suspended in Hyaluronic Acid on Articular Cartilage of Osteoarthritic Knee joint of Adult Male Albino Rat

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ABSTRACT

Introduction: Current treatment strategies of osteoarthritis (OA) are restricted to short-term symptomatic relief by medical therapy. There is increasing evidence that bone marrow derived mesenchymal stem cells (BM-MSCs) represent a novel treatment for cartilage regeneration.

Aim of the Work: Is to evaluate the therapeutic effect of intra-articular injection of BM-MSCs on the experimentally induced OA in the knee joint of adult male albino rat.

Materials and Methods: Fifty five adult male albino rats were used and divided into five groups. Group I served as a control group. Group II for harvesting BM-MSCs from the tibia and femur. Group III for induction of OA by intra-articular injection of monosodium iodoacetate (MIA) to be sacrificed after 2 weeks from induction. Group IV: Hyaluronic acid (HA) was injected in both normal knees to be examined after 4 and 8 weeks of injection of HA. Group V: Knee joint OA was induced as in group III. After 2 weeks, rats were injected with BM-MSCs suspended in HA (MSCs HA) in their knees. Knee joint specimens were examined after 4 and 8 weeks of MSCs-HA injection. Knee joint specimens were processed for histological study and stained with Hematoxylin and eosin, Mallory trichrome and fast green Safranin O stains. For immunohistochemical study, the number of CD44 positive chondrocytes was evaluated and the obtained morphological data were statistically analyzed.

Results: OA knees treated with MSCs-HA showed well evidenced repair of the articular cartilage by hyaline-like cartilage in association with significant increase in the number of CD44 positive chondrocytes.

Conclusion: Intra-articular injection of in vitro expanded BM-MSCs suspended in HA promotes articular cartilage repair by hyaline-like cartilage in MIA induced OA.

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Key Words: BM-MSCs, CD44, hyaluronic acid, osteoarthritis.

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INTRODUCTION

Osteoarthritis (OA) as one of degenerative diseases in the joint is an important leading cause of long-term physical disability with severely impaired quality of life among elderly people globally. It is represented by gradual loss in the cartilage, concomitant with osteophytes, an expanded thickness of the subchondral plate, with formation of cysts in the subchondral bone. As the disease progresses, many vessels invaded the cartilage with more calcification of adjacent cartilage on the articular surface, driving to bone remodeling, diminished in the thickness of the articular cartilage, with enhanced cartilage deterioration[1,2].

In spite of the morbidity and high frequency of this degenerative disease, current treating strategies are restricted to short-term symptomatic relief by medical therapy and surgical procedures while a successful therapy up till now is missing[3]. Recently, however, a variety of strategies have been established to restore lesions in the articular cartilage. The advent of mesenchymal stem cells (MSCs) seems to be a promising solution to overcome cartilage destruction in osteoarthritis[4].

Mesenchymal stem cells as an adult stem cells are multipotent cells that can distinguish and differentiate to a many cell sorts, involving chondrocytes and osteoblasts[5]. The main resources of MSCs for tissue engineering and organ regeneration are bone marrow, adipose tissue and umbilical cord blood (UCB)[6].

MSCs mainly express on their cell surface low levels of major histocompatibility complex-I (MHC-I) molecules, but do not express MHC-II molecules. Subsequently, they will not make active the allogeneic lymphocytes with lack immunity. These features are more supportive for the possibility of universal donor MSCs for curative implementation, such as umbilical cord- derived MSCs[7]. In addition, MSCs could adjust the microenvironment of affected tissues, and safeguard the injured tissues via discharging anti-apoptotic and anti-inflammatory particles[8].

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Several experimental studies using animal models were conducted to examine the role of this type of cells (MSC) in treatment from osteoarthritis. However, there is controversy in their results. This work was performed to evaluate the therapeutic effect of intra-articular injection of BM-MSCs after suspension in hyaluronic acid on the experimentally induced OA in the knee joint of adult male albino rats.

MATERIAL AND METHODS

2.1 Animals

Fifty-five adult male albino rats were used in this study weighing 150-200 grams each. They were selected carefully free from any gait disturbance, limping, or swollen joints. The study was carried according to guidelines of the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). They were acclimatized for their environment two weeks before starting the experiment where food and water were available ad libitum with cycle of 12 hours dark and 12 hours light. The study was approved by local ethical Committee of Faculty of Medicine, Tanta University. They were randomly divided into five groups:

- Group I (10 rats) considered as control group and kept without treatment throughout the whole period of the experiment. They were subdivided into two equal subgroups to be sacrificed with their correspondents.
- Group II included 20 rats for obtaining bone marrow from both tibia and femur to be cultured for isolation of BM-MSCs in Tissue Culture Unit of Histology Department, Faculty of Medicine, Tanta University.
- Group III (osteoarthritis induced group) included 5 rats injected once intra-articularly with monosodium iodoacetate (MIA). They were sacrificed after two weeks from day zero of induction of osteoarthritis.
- Group IV (Hyaluronic acid injected group) included 10 rats. This group was subdivided into two equal subgroups; Subgroup IVa and Subgroup IVb in which knee joint specimens were examined after 4 and 8 weeks of injection of hyaluronic acid respectively.
- Group V (Stem cell treated group) included 10 rats that were exposed first to knee joint osteoarthritis induction as in group III. After 2 weeks, their knee joints were injected with bone marrow derived mesenchymal stem cells (MSCs-HA) suspended in HA. This group was further subdivided equally into subgroup Va and subgroup Vb to obtain their knee joint specimens after 4 and 8 weeks, respectively.

2.2 Chemicals

2.2.1 Monosodiumiodoacetate (MIA) for experimental induction of osteoarthritis was acquired as a powder then, it was dissolved as 5 mg of MIA in 1 ml saline (MIA; Sigma-Aldrich, St Louis, Missouri, USA; cat #I2512)[9].

2.2.2 Hyaluronic acid (HA): Hyalganis (Fidia Pharmaceutics S.p.A., Italy. Sanofi-Aventis) was administered by intra-articular injection of 0.1 ml HA solution (its concentration is 10 mg/ml)[10,11].

2.3 Induction of osteoarthritis in the knee joints of group III &V

The animals were anesthetized by ether, hair over knee joint was shaved and cleaned with Betadine solution. The knee joint was immobilized in full flexion while the rats on their back. A single intra-articular injection of 0.05 ml (0.25mg/0.05ml) of MIA through the infra-patellar ligament bilaterally using insulin syringes was performed[12]. After two weeks, rats were sacrificed at the scheduled time and the whole knee joint was dissected out and processed for histological study.

2.4 Preparation of stem cells derived from bone marrow from Group II

Isolation and initial culture of MSCs from the bone marrow of the rat long bones were done using Caplan's method[13]. The bone marrow of the femurs and tibias of rats was extracted, collected, and cultured for isolation of mesenchymal stem cells (MSCs). This was done by flushing with Dulbecco's modified Eagle's medium (DMEM, Lonza Company, cat. No.BE12-604F, Swiss.) supplemented with 10% fetal bovine serum (Gibco, Invitrogen Co., Cat. No10270-106, USA). Nucleated cells were isolated by centrifugation and suspended in complete culture medium supplemented with 1% penicillin–streptomycin (onza CO., Cat. No: 17–745E, Switzerland). Incubation of cells were performed in 5% humidified CO2 at 37°C for 7-9 days until large colonies were formed (70–80% confluence). Then, the culture was washed with PBS (Lonza Bioproduct, cat. No: BE17–512F, Switzerland) and released with 0.25% trypsin in 1 mmol/l EDTA (Gibco/BRL) (5 min at 37°C). MSCs in culture were distinguished by their morphological characteristics under phase contrast (inverted) microscope and Giemsa staining[14]. Together with immunocytochemical staining by CD44 and CD34[15]. The cell viability was performed by trypan blue and cell counting using a hemocytometer in the prepared cell suspension. At twelve days of culture when the cells reached 70-80 % confluence, trysinization was carried out to get passage 3 of MSCs. After their centrifugation, the cell pellet was added to hyaluronic acid (2 ml)[16].

2.5 Intra-articular mesenchymal stem cell suspension (MSCs-HA) & hyaluronic acid injection in group V

Rats were injected with 0.1 ml of MSC suspension intra-articularly through the infra-patellar ligament using insulin syringes into their right knees. The total intra-articularly injected viable cells to each knee joint were 1×10^{7}[10,11,16].

Processing of the knee joint specimens for groups I, III, IV & V

At the end of the experiment, the rats were sacrificed, the knee joints were dissected carefully, immediately fixed in 10% formal saline solution for 24 hours and washed by tap

water for half an hour then the specimens were decalcified by disodium EDTA solution (chelating agent) for 4 weeks[17]. During this period, the chelating solution was renewed every 2 days until the tissues became soft. The soft decalcified joints were cut in a sagittal plane and were processed to form paraffin blocks. 5 um serial sections were obtained, stained with hematoxylin and eosin, Mallory's trichrome, and Safranin O fast green stains[18].

2-6 Immunohistochemical staining for CD44

For immunohistochemical study, the sections were processed using the streptavidin–biotin immune-peroxidase technique for the CD44 antigen detection[18]. Sections were deparaffinized and rehydrated down to distilled water. The slides were covered by H_2O_2 (10%) for 15 minutes to block endogenous peroxidase. Antigen retrieval was done as follow; the sections were immersed in a preheated citrate buffer solution at PH 6 and keeping heat for 10-20 minutes by placing in a microwave at 2 watts. Sections were left for 20 minutes at room temperature to cool down, then washed in distilled water. The slides were gently blotted, and the sections were entirely covered by 50-80 µL of the primary antibody overnight in a humidified chamber. The slides were washed for 5 minutes by PBS and blotted. Sections were covered completely by 50-80 µL of the secondary biotinylated anti mouse antibody and incubated for 30 minutes. Then, slides were washed well with PBS and blotted. Sections were covered completely by 50-80 µL of Streptavidin horseradish peroxidase conjugate for 15 minutes. Then washed with PBS. Color was developed using 1-2 drops of 3, 3 diamino-benzidine (DAB) for 10 minutes. Then, slides were washed well with distilled water and blotted. The slides were then counterstained with hematoxylin for ½ minute. Then they were washed and dehydrated using, absolute alcohol then cleared and mounted. CD44-positive cells showed brown cytoplasmic deposits in the newly formed hyaline like cartilage[19]. Sections of tonsil were used as positive control. Negative control sections were prepared by applying all previous steps except that PBS was used in place of the primary antibody.

2.7 Histomorphometric assessment and Statistical analysis

For morphometric analysis, an Olympus microscope (Tokyo, Japan) linked to an Olympus digital camera (DXC-1850P, Tokyo, Japan) was used in histology department.

1. Histological scoring of the articular cartilage damage was measured in H&E-stained sections of all rats at a magnification of 200-fold. Grading was done according to Modified Mankin's grading scale on a scale of 0–10[20]. (Table 1)

Table 1: Modfied Mankin's grading scale

Category	Points
• Cartilage structure	
- Normal	0
- Surface irregularities	1
- Pannus and surface irregularities	2
- Clefts to transitional zone	3
- Clefts to radial zone	4
- Clefts to calcified zone	5
- Complete disorganization	6
• Cellularit	
- Normal	0
- Diffuse hypercellularity	1
- Cloning	2
- Hypercellularity	3
• Tide mark integrity	
- Intact	0
- Discontinuous	1
Total score	10

2. Evaluation of collagen fiber content: Evaluation of collagen fiber content was quantitatively measured in Mallory's trichrome stained sections, the software "ImageJ" (National Institute of Health, Bethesda, Maryland, USA) was used. Five non-overlapping fields at a magnification of 400 were examined from each slide, the color threshold was adjusted to select the proper field of the blue stained collagen, the image was then converted to a 8-bit image (gray scale) and the threshold was adjusted again till the selected field was covered in red then measured. The parameter used was area percentage.
3. The CD44 positive chondrocytes was counted in the articular cartilage using the software.

RESULTS

3.1 Morphological identification of BM-MSCs (Group II) by phase contrast inverted microscope and immunohistochemicstry

The present study showed that on 12th day of the primary culture, there were spindle and star shaped adherent cells with multiple processes interdigitate with each other and central vesicular nuclei with multiple nucleoli. Giemsa stain revealed the same features with granular cytoplasm and bluish vesicular rounded nuclei with apparent nucleoli. Nearly most of the cells were CD 44 positive cells and all of them were CD34 negative (Figure 1).

3.2 In vivo studies (Light microscopic examination)

All animals survived all the procedure of the experiment & mortality rate was zero. All the presented coming figures will carry the femur on the left side and the tibia on the right side

3.2.1 Haematoxylin and eosin stained sections

The articular cartilage of control and Hyaluronic acid injected groups (Groups1 & IV) revealed the same microscopic picture. They displayed regular surface without covering perichondrium. Superficially inward, four poorly demarcated zones can be identified. The most superficial tangential zone contained flattened chondrocytes parallel to the surface of the cartilage followed by transitional zone of dispersed, spherical rounded larger chondrocytes. The radial zone comprised rows of individually located chondrocytes situated inside their lacunae perpendicular to the surface. A zone of calcified cartilage was seen between the radial zone and the underlying subchondral bone, separating them. A basophilic mark (tidemark line) was viewed as a clear well-defined boundary demarcating the uncalcified from calcified cartilage (Figure 2). Osteoarthritis induced group (Group III) demonstrated massive affection of the articular cartilage of the knee joint with special predilection to weight-bearing areas of the tibia. These were in the form of wide scope surface irregularity ranging from focal localized superficial erosion (fraying) up to diffuse wide fibrillation and replacement of subchondral bone marrow by loosely arranged spindle cells contained within a fine stroma. The chondrocytes displayed disorganization, degeneration, depletion and cluster formation with pyknotic or karyolytic nuclei. (Figure 3). Subgroup V a of stem cell treated group (Group V) sacrificed after 4 weeks revealed incomplete recovery of the articular cartilage showing decreased staining affinity of its matrix and an apparent reduction in the chondrocytes ‘number with focal degenerated or absent chondrocytes in association with multiple transverse fissures in the radial zone and discontinuous tidemark line. Subgroup V b of stem cell treated group sacrificed after 8 weeks depicted well evidenced repair of the articular cartilage. This was evidenced by restoration of the thickness, structure, and regular surface of the articular cartilage that became as in control group. Furthermore, normalization of the organization and structure of the chondrocytes into four zones was a prominent feature (Figure 4).

3.2.2 Mallory trichrome stained sections

Control and Hyaluronic acid injected groups (Groups1 and IV) showed normal appearance, arrangement and distribution of the collagen fibers in the different zones of the articular cartilage evidenced by the prominent homogenous diffuse bluish staining of the matrix. Osteoarthritis induced group displayed marked reduction of the collagen fibers manifested by absence of blue staining of collagen in fissured areas and faint heterogeneous staining with variable intensities in other areas. MSCs-HA treated group showed moderately and faintly stained matrix in those sacrificed after 4 weeks and deeply and uniformly stained intercellular matrix in those sacrificed after 8 weeks to be more or less similar to the control group (Figure 5).

3.2.3 Safranin O/ fast green stained articular sections

Control group (I) and Hyaluronic acid injected group (Group IV) showed uniformly red stained intercellular matrix mainly in the non-calcified part and for less extent in the calcified region. Osteoarthritis induced group (Group III) displayed markedly reduced staining of their matrix. * MSCs-HA treated group (Group V) showed moderately and faintly stained matrix in those sacrificed after 4 weeks and deeply and uniformly stained intercellular matrix in those sacrificed after 8 weeks (Figure 6).

3.2.4 Immunohistochemical results using CD44

*Articular cartilage of control (I) and Hyaluronic acid injected group (Group IV) showed many negative CD44 chondrocytes with few CD 44 positive ones in between. Similarly, osteoarthritis group (Group III) demonstrated the same result. Stem cell treated group (Group V) revealed large number of CD44 positive chondrocytes in the articular cartilage of knee joint of MSCs-HA treated subgroup scarified after four weeks and some CD 44 positive cells in those sacrificed after 8 weeks. (Figure 7).

3.3 Statistical Results

3.3.1 Histological score of the articular cartilage damage (Modified Mankin’s grading scale)

There was a non-significant difference (p>0.05) in the mean of the histological score of Hyaluronic acid injected group in normal joint with its subgroups IV a & IV b (0±0&0±0) and MSCs- HA treated group (subgroup V b, sacrificed after 8 week) (0.2±0.447) compared with the control group(0±0), On the other hand, the mean of the histological score of the osteoarthritis group (Group III) and MSCs-HA treated group (subgroup V a, sacrificed after 4 weeks) (1.6±0.894) depicted a significant increase with p< 0.05 in comparison with the control group(0±0).

As regards to the mean of the histological score of MSCs -HA treated group (sacrificed after 4 weeks) (1.6±0.894) there was a significant articular cartilage damage (*P value*=0.002) in comparison to MSCs-HA treated group (sacrificed after 8 weeks) (0.2±0.447) (Table 2 and Graph 1).

3.3.2 Evaluation of collagen fiber content in Mallory's trichrome stained sections

There was a non-significant difference with $p>0.05$, in the mean of collagen fiber content of the articular cartilage of group IV; Hyaluronic acid injected in normal joint group with its subgroups (Iv a and IV b) (119.82±3.89 and 119.76±4.88) and in comparison to the control group with mean of (121.43±5.54). While there was a significant decrease with $P< 0.05$, in the mean of collagen fiber content in the articular cartilage of the osteoarthritis group (Group III) (73.20±3.94), MSCs–HA treated group (sacrificed after 4 weeks) (112.39±4.43) in comparison to the control group with mean of (121.43±5.54).

As regards to the mean of collagen fiber content of the articular cartilage of subgroup V a; (MSCs-HA treated group sacrificed after 4weeks) (112.39±4.43), there was a significant reduction (P 0.0176&0.0203) in comparison with the control animals (121.43±5.54) and MSCs-HA treated group (sacrificed after 8 weeks) (118.32±5.89) respectively (Table 3 and Graph 2).

III) The number of CD44 positive chondrocytes in the articular cartilage.

There was a significant increase ($p < 0.001$) in the mean of number of positive CD44 chondrocytes in the articular cartilage of MSCs-HA treated group (subgroup V a, sacrificed after 4 weeks) (18.8 ±4.11) and MSCs -HA treated group (subgroup V b, sacrificed after 8 weeks) (10.8±1.16) when compared to the control group (2.6±1.95). There was a non-significant difference with $P>0.05$, in the mean of number of CD44 positive chondrocytes in the articular cartilage of group IV (Hyaluronic acid injected in normal joint with its subgroups, sacrificed after 4 weeks and 8 weeks) (2.45±1.09 and 2.49±1.49) and osteoarthritis group (3.4±2.41) when compared to the control group (2.6±1.95).

There was also a significant increase ($P< 0.001$) in the mean number of CD44 positive chondrocytes in the articular cartilage of MSCs-HA treated group (subgroup V a, sacrificed after 4 weeks) (18.8 ±4.11) as compared to the mean of MSCs-HA treated group (subgroup V a, sacrificed after 8weeks) (10.8±1.16) (Table 4 and Graph 3).

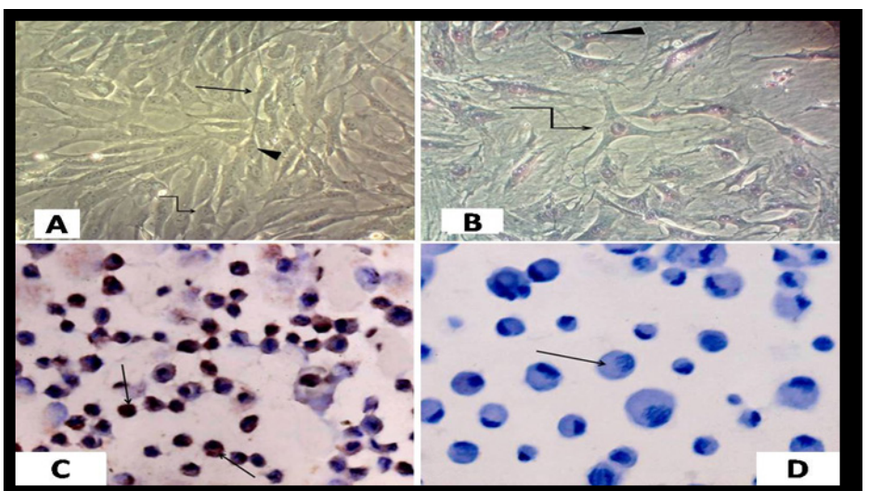


Fig. 1: A primary culture of rat BM-MSCs 12 days post seeding shows spindle shaped (→) and star shaped cells ( ) with multiple interdigitating processes with central vesicular nuclei and multiple nucleoli (▲). B Giemsa staining of the primary culture of rat BM-MSCs displays star shaped colonies of adherent cells ( ) interconnected together with cytoplasmic processes with granular cytoplasm and bluish vesicular nuclei with prominent nucleoli (▲). C Immunostained primary culture of rat BM-MSCs, shows many CD44 positive cells (→). D Immunostained primary culture of rat BM-MSCs, shows negatively immune stained reaction for CD34 in all cultured cells (→). (Inverted microscope, A X20, B X 400, C and D Avidine-biotin Peroxidase X200)

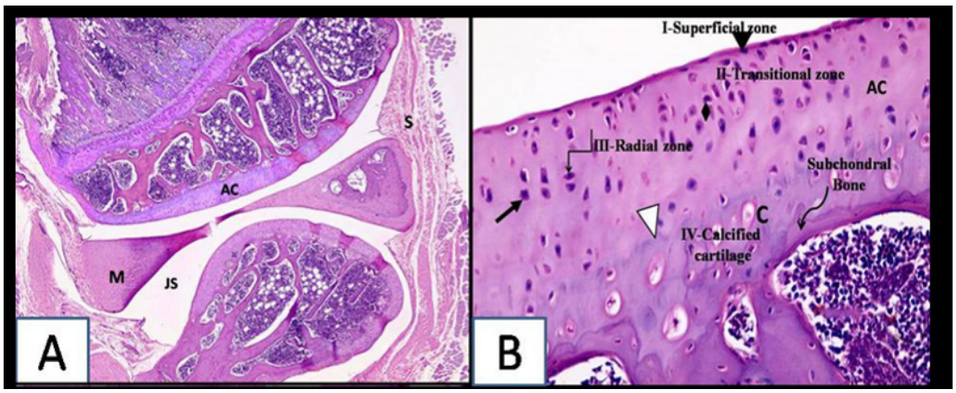


Fig. 2: Knee joint section of the control group (group I); A- shows normal joint space (JS), regular hyaline articular cartilage surface with no covering perichondrium (AC) and wedges of fibrocartilage in between the 2 articular surfaces [menisci (M)] and the synovial membrane (S) are seen. B-shows regular hyaline articular cartilage surface with its four zones: superficial tangential zone (▲), the transitional zone (♦) and the radial zones (→) with individually located chondrocytes inside their lacunae ( ). The calcified cartilage zone is (C) bounded by tidemark line (Δ) and underlying subchondral bone ( ). (H&E, AX 40 and BX400

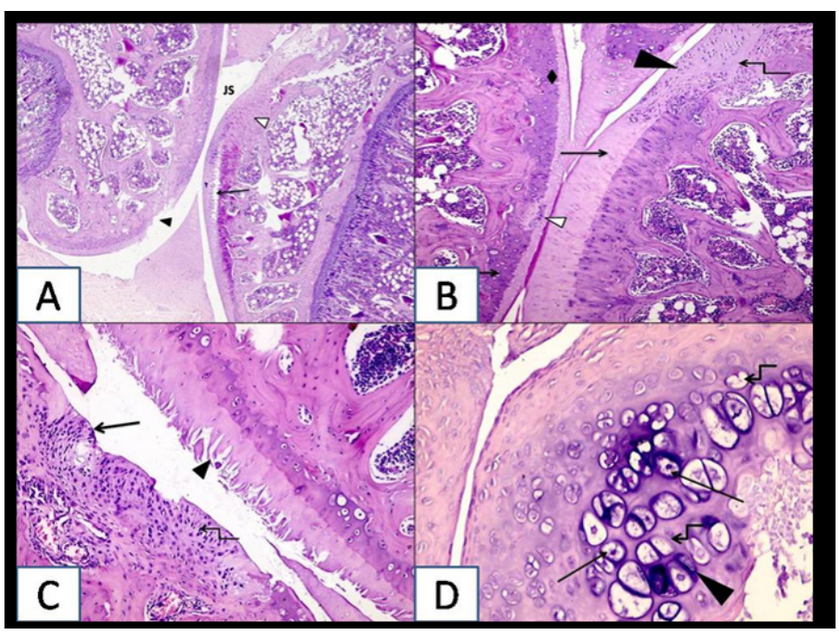


Fig. 3: knee joint sections of osteoarthritis induced group (groupIII); A shows small superficial erosion (fraying) (▲) transverse fissuring of the radial zone (↑) and disappearance of the calcified cartilage (Δ). B shows narrowing and obliteration of the joint space (JS), thinning of the articular cartilage (♦), loss of the chondrocyte zonal arrangement (↑). The tidemark line can be seen interrupted in focal areas with disappearance of the calcified cartilage in these areas (Δ) and replacement of the articular cartilage (▲) with loosely arranged spindle cells contained within a fine stroma reaching the trabecular bone ( ). C shows fibrillation of the tibial articular cartilage reaching the middle zone (▲) with depletion and loss of the zonal organization of its chondrocytes. The other femoral articular surface shows complete absence of the articular cartilage (↑) with replacement of the underlying subchondral bone marrow by loosely arranged spindle cells contained within a fine stroma( ). D shows variably sized clusters of chondrocytes (cell cloning or chondrones) at the joint margins (▲) with pyknotic (↑) and karyolitic nuclei ( ). (H&E, A& BX 40, CX200 & DX400)

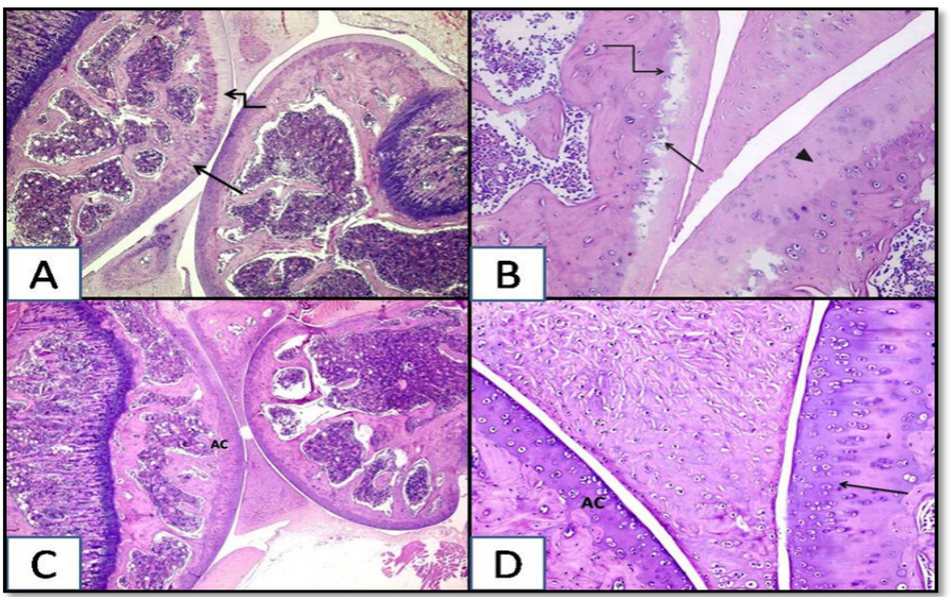


Fig. 4: A &B are sections in the knee joint of a rat of subgroup V a depicting restoration of the structure of the articular cartilage (↑) and decreased staining affinity () in some areas. B shows areas with apparent decrease in the number or absence of the chondrocytes (▲) and transverse fissure in the radial zone (↑) with focal absence of the tidemark line in the area of the fissure ( ). C & D are sections in the knee joint of a rat of subgroup Vb. C shows normal thickness and regular surface of the articular cartilage (A C). D shows regular surface of the articular cartilage with normal staining affinity (AC) and normal structure and zonal organization of the chondrocytes (↑). (H&E, A X40, BX100, C X40, DX200)

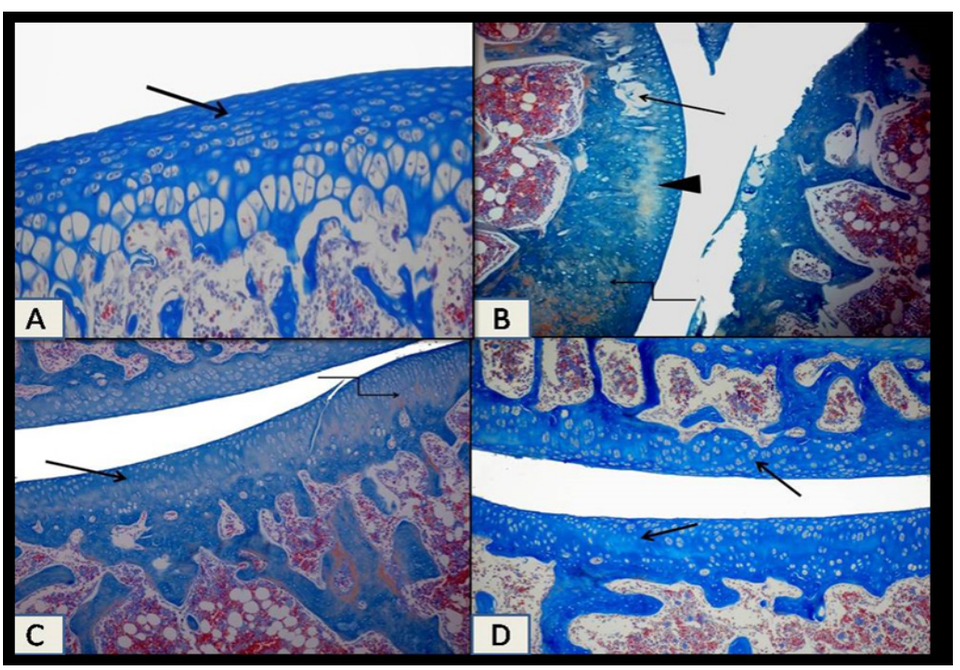


Fig. 5: A- Articular cartilage of group I shows prominent blue staining of the collagen fibers in different zones (↑). B knee joint of a rat of the osteoarthritis group (group III) shows apparently decreased collagen fiber content ranging from complete loss of blue color (↑) to faint (▲) and heterogonous staining ( ) in nearby areas. C knee joint of a rat of subgroup V a shows homogenous blue colored areas (↑) near to patchy lightly stained matrix ( ). D knee joint of a rat of subgroup V b shows prominent homogenous blue stained matrix (↑). (Mallory trichrome Group A, ×200, B, C and D ×100)

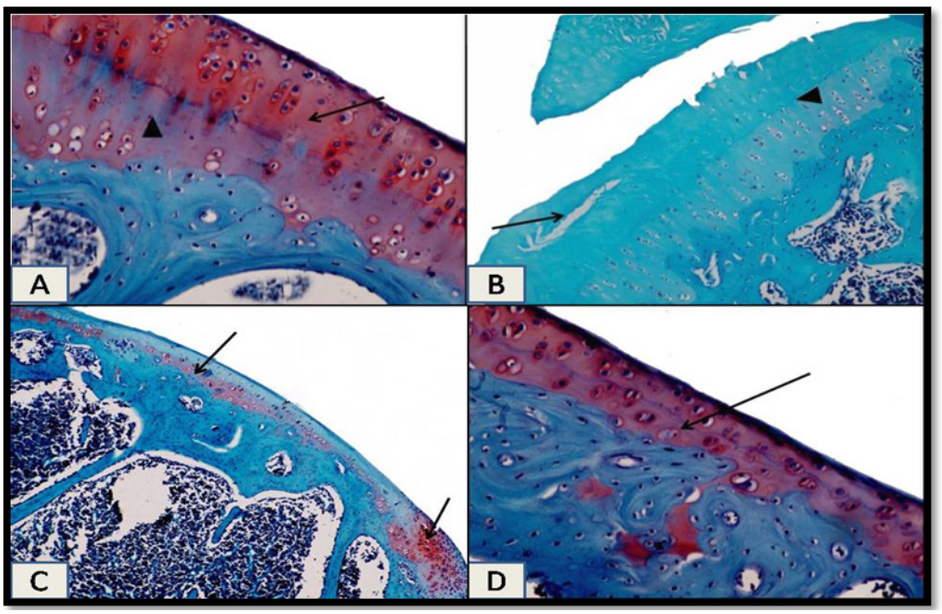


Fig. 6: Articular cartilage of knee joint of the studied groups; A Group I shows deeply and uniformly red stained matrix mainly in the non-calcified part (↑) and for a lesser extent in the calcified region (▲). B osteoarthritis group (group III) shows fissuring of the cartilage (↑) and unstained matrix (▲). C subgroup V a shows faintly stained matrix (↑). D subgroup V b depicts strongly, deeply and uniformly stained matrix (↑). (Safranin O /Fast green AX 400, BX 200, C X100, D X200)

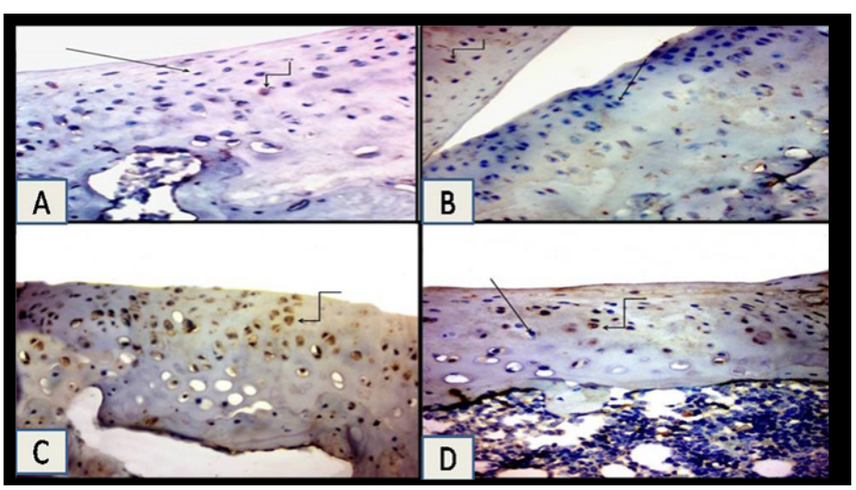


Fig. 7: Immunostained sections of the articular cartilage of rat tibia for CD44; A control group and B osteoarthritic group show negative CD44 chondrocytes (↑) in-between few CD44 positive ones ( ). C subgroup Va shows several CD44 positive chondrocytes ( ). D subgroup V b shows some CD44 positive chondrocytes ( ) in-between CD44 negative ones (↑). (CD44 immunostaining counterstained with Hx A, B, C and D × 400).

Table 2: Comparison between groups as regard means ± SD of the score of the articular cartilage damage in different groups.

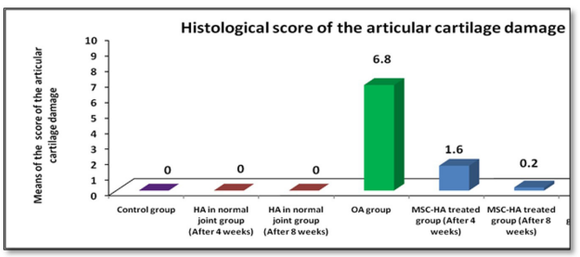
	Control group (GI)	HA in normal Joint group (G IV)		OA Group (G III)	Treated group (G V) MSCs-HA group		*P-value*
		After 4w	After 8w		After 4w	After 8w	
Mean	0	0	0	6.8	1.6	0.2	<0.0001**
SD	0	0	0	2.049	0.894	0.447	
Post – test (Tukey – Kramer)							
Control versus HA injected in normal joint				No difference	Control versus MSCs – HA (4WKs)		0.002*
Control versus OA group				<0.001**	Control versus MSCs – HA (8WKs)		0.173

Table 3: Comparison between groups as regard means ± SD of the collagen fiber content in the articular of the knee joint of different groups,.

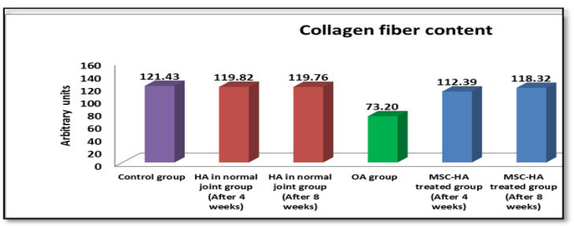
	Control group (GI)	HA in normal joint group (G IV)		OA Group (GIII)	Treated group (G V) MSCs-HA group		*P-value*
		After 4 w	After 8w		After 4 w	After 8w	
Mean	0	0	0	6.8	1.6	0.2	<0.001**
SD	0	0	0	2.049	0.894	0.447	
Post–test (Tukey-Kramer)							
Control versus HA injected in normal joint.		No difference		Control versus MSCs- HA (4WKs)			0.002*
Control versus OA group		<0.001**		Control versus MSCs -HA (8WKs)			0.173

Table 4: Comparison between groups as regard means ± SD of the number of CD44 positive chondrocytes in the articular cartilage damage in different groups.

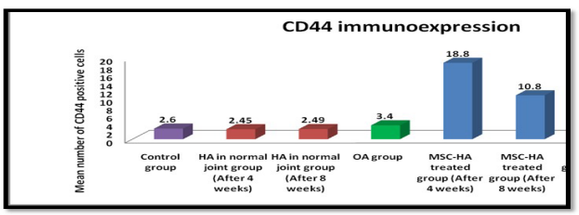
	Control group (GI)	HA in normal joint group (G IV)		OA Group (GIII)	Treated group (G V) MSCs-HA group		*P-value*
		After 4 w	After 8w		After 4 w	After 8w	
Mean	2.6	2.45	2.49	3.4	18.8	10.8	<0.001**
SD	1.95	1.09	1.49	2.41	4.11	1.16	
Post–test (Tukey-Kramer)							
Control versus HA injected in normal joint.		0.831		Control versus MSCs- HA (4WKs)			<0.001**
Control versus OA group		0.3105		Control versus MSCs -HA (8WKs)			



Graph (1): Comparison between groups as regard means of the score of the articular cartilage damage in different groups.



Graph (2): Comparison between groups as regard means of the collagen fiber content in the articular cartilage of knee joint of different groups.



Graph (3): Comparison between groups as regard means of the number of CD44 positive chondrocytes in the articular cartilage of knee joint of different groups.

DISCUSSION

The present study highlighted the role of mesenchymal stem cell that are derived from bone marrow and suspended in HA in alleviating the experimentally induced osteoarthritis in the knee joint of adult male albino rats.

Noteworthy, intra-articular injection of MIA into the rats' knee joint induced wide range of osteoarthritic changes after 2 weeks (group III) in all treated animals of the present study. This was coincided with that reported by -AL-Saffar *et al*., (2009) Mapp *et al*., (2013); Sangeetha *et al*., (2014) and Soliman, (2012)[21,22,23,24].

The mechanism of MIA-induced knee joint OA was adopted by several studies such as Janusz *et al*., (2001); Combe *et al*., (2004); Fernihough *et al*., (2004)[12,25,26]. MIA is a metabolic inhibitor that is induced glycolysis` disruption via inhibiting glyceraldehydes3-phosphate dehydrogenase enzyme activity in chondrocytes. As the chondrocytes normally live in a hypoxic environment, they produce energy via anaerobic glycolysis, therefore, glycolysis` disruption of by MIA might ultimately lead to cell death.

Furthermore, intra articular injection of MIA increased the activity of matrix metalloproteinases (MMP) which is involved in the cartilage destruction and disruption of metabolism of their cells leading to cell death[12]. In addition, accumulation of inflammatory cells with releasing of many cytokines as TNF-α and IL-1β set up a vicious circle of inflammation and damage to the knee joint[27].

Hypercellularity of the chondrocytes and cluster formation observed in the present research could be explained by (Hashimoto *et al*., 1998)[28] who recorded that OA increases expression of gene of transforming growth factor-β (TGF-β), which upregulates the 'Meltrin-α' expression. Meltrin-α is a new gene family of multifunctional proteins that subsequently lead to chondrocyte multiplication through improved IGF-1` bioavailability in the OA cartilage. The cloning phenomenon could indicate that the chondrocytes make efforts to refill and repopulate the cartilage tissue and improve matrix synthesis. Also, it might signify return to an earlier developmental stage of cartilage differentiation[29]. Abir *et al*., (2011)[30] added that injury to the cartilage` components may lead to death of their chondrocytes and initiated a compensatory repair response with subsequent chondrocyte proliferation.

Interestingly, chondrocyte depletion observed in the articular cartilage of OA induced group agreed with Aigner *et al*., (2001)[31] who reported very low proliferative activity of chondrocytes during OA. Soliman, (2012)[24] observed decrease in the population of chondrocytes in the osteoarthritis group especially in the weight-bearing areas. They added that, in the advanced cases of OA, the matrix fissuring which seen above the calcified zone may be due to a decrease in the number of living chondrocytes.

One of the prominent findings in the knee joint of osteoarthritis induced group was matrix depletion evidenced by decreased basophilia in H&E stained sections, faint reaction for Mallory trichrome and Safranin O Fast green stains. Guzman *et al*., (2003)[32]; Soliman (2012)[33] observed the same findings. This might be due to decreased concentrations of glycosaminoglycan secondary to the major chondrocytes cell death in OA with subsequent failure of cartilage matrix turnover[31,34].

Regarding HA-MSC treated group sacrificed after 4 weeks from HA-MSCs injection, the present study showed incomplete recovery of the articular cartilage..In agreement with the current result, Oshima *et al*., (2005)[35] noticed irregularly aligned chondrocytes 4 weeks after MSC transplantation with osteochondral defect. This was also matched with the results of Shalabi *et al*., (2012)[36] who observed hyaline-like cartilage filling the defect along with disorganized chondrocytes and cluster formation.

Interestingly, moderate staining for Mallory Trichrome and Safranin O/Fast Green stains of the articular cartilage matrix of MSC-HA treated groups sacrificed after 4 weeks was attributed to a delay in laying intercellular matrix by the newly chondrocytes[37].

Well evidenced repair of the articular cartilage of MSC-HA treated rats sacrificed after 8 weeks was a prominent finding in this study. These data confirmed the observation of Lee *et al*., (2007)[11] and Xie *et al*., (2010)[38] who reported that BM-MSCs promoted regeneration of the osteochondral tissue by a hyaline-like tissue with formation of abundant sulfated GAG in the joint defect. They added that proper healing by hyaline-like tissue is suggested to occur as a result of differentiation of the injected BM-MSCs to chondrogenic cells. Similarly, Dashtdar *et al*., (2011)[39] concluded that BM-MSCs produced excellent healing comparing with the untreated cartilage defects. Kasemkijwattana *et al*., (2011)[40] as well showed that BM-MSC implantation had the potential for good filling and treatment of large traumatic cartilage defects of the knee with long-lasting hyaline-like cartilage with no postoperative complications.

Restoration of the normal architecture of the articular cartilage in MSC treated rats which were sacrificed after 8 weeks and not after 4 weeks was attributed to the time needed by MSCs to proliferate and differentiate to regularly arranged chondroctyes to form proper hyaline cartilaginous matrix. These results agreed with Lutianov *et al*., 2011[41] who recorded that stem cells firstly differentiated to chondrocytes, then formed the extracellular matrix resulting in new cartilage formation to treat chondral defects in the knee.

Chen and Abatangelo, (1999)[42] explained how intra-articular injection of BM-MSCs suspended in hyaluronic acid promote healing of the articular cartilage. It has been documented that hyaluronic acid stimulates the migration and mitotic division of mesenchymal stem cells and consequently the natural repair process of recruiting cells is accelerated and amplified. Furthermore, Lee *et al*., 2007[11] reported that intra-articularly injected MSCs suspended in HA could home into the injury site, adhere, multiply, and regenerate cartilage.

As regard to the mechanism of action of BM-MSCs in articular cartilage regeneration, Baksh *et al*., (2004)[43] mentioned that mesenchymal stem cells are considered multipotent cells that can be differentiated to many cell types, including chondrocytes and osteoblasts. Moreover, Caplan and Dennis, (2006)[44] added that MSCs secrete a diversity of growth factors and cytokines that have autocrine and paracrine effects, including inhibition of local immune system and apoptosis in addition to promoting neuroprotection, angio¬genesis, modulation of inflam¬matory response, with reduction of scar forma¬tion. Lee *et al*., (2007)[11] mentioned that combination between MSCs and hyaluronic acid will result in synergistic effect. Li *et al*., (2011)[45] reported that the differentiated BM-MSCs may be a hopeful approach for cartilage repair. They found that co-culture of chondrocytes with goat BM-MSCs in vitro had a positive effect in stimulating BM-MSCs' chondrogenic effects. They found an increase in GAG, rise in expression of chondrogenic genes and collagen II, as well as down regulated in expression of collagen I gene. In addition to they identified CD44 positive in the cultured BM-MSCs.

Additionally, Cselenyak *et al*., (2010)[46] and Vasconcelos, (2012)[47] reported that MSCs are passively captured in the micro vessels or capillaries including post-capillary venules and arterioles followed by immediate release of a wide variety of trophic cytokines and soluble growth factors.

Mcllwraith *et al*., (2011)[48] reported that cytokines, such as TNF-α, and TGF-β which induce many of the destructive processes of OA were decreased. These effects are referred to the trophic effects and are different from direct differentiation of MSCs into repair tissue. Fanglong *et al*., (2014)[49] as well demonstrated in their study that upregulation of Aggrecan, downregulation of MMP-13, and a sig¬nificant increase in GAG in therapy groups suggested that cells of bone marrow stimulate matrix synthesis of the cartilage with reduction of the inflammation of the chondrocytes.

Interestingly, CD44 positive chondrocytes were highly significantly elevated in the articular cartilage of MSCs-HA treated group sacrificed after 4 and 8 weeks from MSCs-HA injection compared to the control animals. Conversely no significant difference was observed in osteoarthritis (group III) compared to the control group.

Ishida *et al*., (1997)[50] mentioned that the CD44 on the surface of chondrocytes plays an important role in normal and abnormal cartilage functions. Its adhesion to HA induces many stimulatory signals that regulate proliferation of chondrocyte and matrix synthesis. CD44 is a cell receptor for hyaluronan which plays an important role in chondrocyte matrix interaction. Also, it is a transmembrane glycoprotein on the chondrocytes[51]. Additionally, Li *et al*., (2011)[11] mentioned that CD44 plays an important role in adhesions of aggrecan and link protein with chondrocyte surface and interacting with hyaluronic acid. It is essential for organizing pericellular matrix.

CD44 is localized to the lacuna wall, cell surface, and intracellular part of the chondrocytes in middle and deep region of the normal cartilage[52]. This explained the presence of few positive CD44 chondrocyts in the articular cartilage of the control joints.

Sun *et al*., (2003)[53] and Lee *et al*., (2007)[11] reported that the bone marrow contains progenitor cells that are capable of differentiating into cartilage cells and regularly expressing CD44. This explained the presence of few positive CD 44 chondrocytes in the articular cartilage of the osteoarthritis joints.

The presence of increased number of CD44 positive chondrocytes in articular cartilage of stem cell treated group sacrificed after 4 and 8 weeks form MSc injection could be attributed to increased proliferation of the already existing chondrocytes or to the newly transdifferentiated chondrocytes of intra-articularly injected mesenchymal cell with subsequent upregulation of CD44[54].

CONCLUSION

Based on the previous data, it could be concluded that intra-articular injection of in vitro expanded BM-MSCs suspended in hyaluronic acid promotes articular cartilage repair by hyaline-like cartilage in MIA induced OA in the knee joint of adult male albino rats.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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الملخص العربى

دراسة هستولوجية لتأثير الخلايا الجذعية الميزنشيمية المشتقة من نخاع العظم والمعلقة فى حمض الهيالورونيك على الغضروف المفصلى المصاب بالخشونة لمفصل الركبة فى ذكر الفأر الأبيض البالغ

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المقدمة: إن استراتيجيات العلاج الحالية تقتصر على تخفيف الأعراض على المدى القصير من خلال العلاج الطبي. هناك أدلة متزايدة على أن الخلايا الجذعية الوسيطة المستمدة من نخاع العظام تعتبر معالجة جديدة لتجديد الغضاريف بسبب قدرتها على التحول وسهولة الحصول عليها.

الهدف من البحث: دراسة الأثر العلاجي لحقن الخلايا الجذعية الوسيطة المشتقة من نخاع العظام فى إصلاح الالتهاب المفصلى العظمى المستحدث فى مفصل الركبة لذكر الجرذ الأبيض البالغ.

المادة وطرق البحث: استخدم55 من ذكور الجرذان البيضاء البالغة وقسمت إلى خمس مجموعات. المجموعة الأولى كانت بمثابة المجموعة الضابطة. المجموعة الثانية لجمع نخاع العظام من عظمتى الساق وعظم الفخذ. المجموعة الثالثة لإحداث الالتهاب المفصلى العظمى فى مفصل الركبة عن طريق حقن مونوصوديوم أيودوأسيتيت وتم أخذ عينة مفصل الركبة بعد إسبوعين من الحقن. المجموعة الرابعة: تم حقن حمض الهيالورونيك في هذه المجموعة في مفصلى الركبة السليم والتي يتعين بحثها بعد 4 و 8 أسابيع من حقن حمض الهيالورونيك. المجموعة الخامسة: تم إحداث الالتهاب المفصلى العظمى فى مفصل الركبة كما حدث فى المجموعة الثالثة وبعد أسبوعين تم حقن الخلايا الجذعية الوسيطة معلقه فى حمض الهياليورونيك فى مفصل الركبة اليمنى وحقن حمض الهياليورونيك فقط فى مفصل الركبة اليسرى. تم فحص عينات مفصل الركبة بعد 4 و 8 أسابيع من الحقن بالخلايا الجذعية وحقن حمض الهياليورونيك وعمل شرائح وصبغها بإستخدام صبغات الهيماتوكسيلين و الإيوسين, مالورى تراي كروم, سافرانيل أو وصبغة هستوكيميائية مناعية باستخدام الأجسام المضادة سى دى 44 ثم أجريت قياسات مورفومترية و تم إثباتها بالتحليل الإحصائى

النتائج: إن حقن الخلايا الجذعية الوسيطة المستخلصة من نخاع العظام معلقة في حمض الهيالورونيك داخل المفصل عزز إصلاح الغضروف المفصلي نتيجه الالتهاب المفصلى العظمى المستحدث بالغضروف المفصلي الطبيعى الزجاجي. بالإضافه إلى أن عدد الخلايا الغضروفية سى دى 44 الإيجابية في الغضروف المفصلي المعالج بالخلايا الجذعية زادت إحصائيا.

الخلاصة: إن حقن الخلايا الجذعية الوسيطة المستخلصة من نخاع العظام معلقة في حمض الهيالورونيك داخل المفصل يعزز إصلاح الغضروف المفصلي نتيجه الالتهاب المفصلى العظمى المستحدث بمادة مونوصوديوم أيودوأسيتيت في مفصل الركبة فى ذكور الجرذان البيضاء البالغة بغضروف يشبه الغضروف المفصلى الطبيعى الزجاجي.