Homing and efficacy of intra-articular injection of autologous mesenchymal stem cells in experimental chondral defects in dogs

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Abstract

Objective

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.

Methods

Partial thickness chondral defects were created on the lateral femoral condyle of stifle joint in domestic mongrel dogs. MSCs were harvested in a separate procedure, labelled with green fluorescent protein (GFP) using monster GFP vector and suspended in buffer phosphate solution for intra-articular (IA) injection. Dogs were divided into three groups. Group I, served as the control. The dogs in the two cell-treated groups received a single IA injection of MSCs one day (Group II) and one month (Group III) after creating the defect. Sacrifice was scheduled at 2 and 8 weeks post-surgery for group I, and 2 and 8 weeks post-treatment, for the cell-treated groups. Morphological, histological, and fluorescence analysis was performed.

Results

Recovery was significant both clinically and histologically in the two cell-treated groups (Group II and III) compared to the control (Group I), ($p<0.001$). In the meantime, Group-II showed better results at 8 weeks than Group III ($p=0.01$). Homing was confirmed by the incorporation of injected GFP-labelled MSCs within the newly formed cartilage.

Conclusion

The obtained results prove that the use of IA injection of autologous MSCs is a viable option for treating partial cartilage defects. Cell labelling gave evidence to the certainty of cell homing within the neocartilage of all treated cases and the participation in the reparative process.

Key words

cartilage repair, mesenchymal stem cell, intra-articular, chondral defect, homing

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Introduction

Mesenchymal stem cells (MSCs) are the stromal component of bone marrow (1, 2) and have long been regarded as undifferentiated cells capable of proliferation, self-renewal, production of a large number of differentiated progeny, and regeneration of tissues (3). At the moment, MSCs are considered to be the most promising prospect of tissue regeneration and repair (1, 2). As cartilage lacks blood supply and as it is composed of a unique type of cell, *i.e.* the chondrocyte which do not divide, it lacks regenerative power and subsequent wound healing. The end stage of any cartilage damage frequently leads to osteoarthritis (OA) with the significant decrease in quality of life of many living beings (4). Adult MSCs; which have the ability to differentiate into cells of the chondrogenic lineage, have emerged as a candidate cell type with great potential for cell-based cartilage repair technologies (5).

The use of MSCs for cell therapies relies on the capacity of these cells to home and engraft long-term into the appropriate target tissue (2). Although, understanding the capacity of direct migration ('homing') of stem cells to a predetermined anatomic location is vital to stem-cell-based cellular therapies (6); little information is available about the time-course of homing and differentiation and the mechanisms by which MSCs are recruited to tissues (1). During the past decade many theories of MSC homing have been reported. Researchers have stated that chemokines (released by damaged tissue) and their receptors are involved, as they are important factors known to control cell migration (2). Some authors suggested that the injured tissue might express specific receptors or ligands to facilitate trafficking, adhesion, and infiltration of MSCs to the site of injury (7).

In clinical settings, the optimal route for administration of stem cells depends on the anatomy and the extent of damage of the involved tissue or organ, offering a choice between two approaches: direct local or intra-lesional implantation versus systemic intravascular administration. Site-directed delivery of MSCs has shown their engraftment in several tissues, particularly after injury. Several research work have discussed the use of bone marrow cells to repair infarcted myocardium (8, 9), repair spinal cords of rats rendered paraplegic (10) and in treatment of large cartilage defects (11). As a result, cartilage repair with direct intra-articular (IA) injection of MSCs has been proposed as a potential cell therapy in a model of OA (12).

This study aimed to verify the likelihood of homing and the efficacy of intra-articularily injected MSCs in an experimentally induced, acute and chronic, partial chondral defects in dogs.

Materials and method

Dogs were randomised into three groups. The control group was not different from the test groups with respect to age and weight, but the test animals underwent a bone marrow aspiration for MSCs preparation 2 weeks prior to cell-treatment. Dogs were randomly divided into three groups. Group I, represented the control group (8 cases). The animals of this group were operated on but did not receive any IA injection. Group II, animals were injected with labelled MSCs IA one day after creating the chondral defect (12 cases). Group III, animals were injected with labelled MSCs IA one month after creating the chondral defect (12 cases). The sacrifice time was scheduled to be at 2 and 8 weeks post-induction for the controls and at the same intervals post-treatment for the cell-treated groups (Fig. 1).

Materials and methods have been divided into three main categories; laboratory, surgical and histopathological.

1- Laboratory work

The lab work consisted of four major steps: Acquisition, Isolation, Characterisation, and labelling of MSCs.

1.1-Acquisition MSCs: Under general anaesthesia, a 13-gauge needle was used to penetrate the cortex of the iliac crest of each dog, and about 10 ml of bone marrow was drawn in a syringe containing 1500 IU of heparin. Acquisition of MSCs was always done 2 weeks prior to cell-treatment.

1.2-Isolation of MSCs: Under complete aseptic technique; the isolation

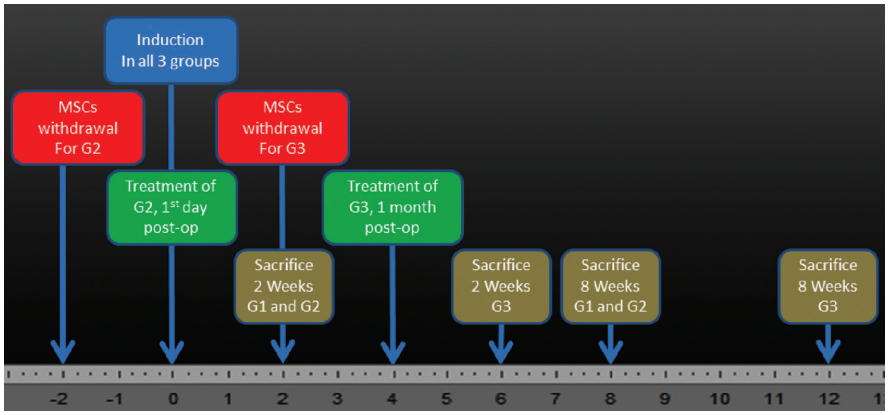

Fig. 1. Study design. Plan used to evaluate the effect of acute and chronic intra-articular delivery of autologous MSCs to the stifle joint of experimental dogs following surgically induced partial chondral defect at the lateral femoral condoyle of the stifle joint. Aspiration of MSCs was always done 2 weeks prior to cell treatment.

of MSCs was performed (13, 14). The bone marrow aspirate was layered onto Histopaque-1077 (Sigma, St Louis, MO, USA) and centrifuged at 400 g for 30 min. The collected buffy coat was mixed with 20 ml of Dulbecco's phosphate-buffered saline (DPBS) and centrifuged at 300 g for 5 min. The supernatant was discarded and the cells pellet was washed two more times with DPBS. After determination of cell viability and the number of viable cells by trypan blue staining, the cells were re-suspended in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (FBS; USDA, Gibco, Grand Island, NY, USA) and antibiotics (penicillin 10 000 U⁄ml, streptomycin 10 000 U⁄ml,) amphotericin B 25 U⁄ml). The nucleated cells were plated in tissue culture flask at a concentration of 2.5 x 10^5⁄cm^3 and incubated at 37°C in a humidified atmosphere containing 5% CO_2. On day 4 of culture, the non-adherent cells were removed along with the change of medium every 2 days. Undifferentiated MSCs reached 70–80% confluence after 14 days. The cells were counted with a haemocytometer and resuspended in 5 ml of phosphate buffer saline at a final density of 1.4–1.6 x10^6 cells/ml prior to intra-articular transplantation.

1.3- In vitro Characterisation of MSCs: On day 14, cells were identified as being MSCs by their morphology; the adherent colonies of spindle fibroblast-like cells were trypsinised, and counted. They were also characterised by their power to *in vitro* differentiate to chondrocytes. Differentiation into chondrocyte was achieved by adding 500 ng/mL bone morphogenetic protein-2 (BMP-2; R&D Systems, USA) and 10 ng/ml transforming growth factor β3 (TGFβ3) (Peprotech, London) for 3 weeks (15). The chondrogenic differentiation medium [DMEM with 10% FBS and BMP-2 and TGFβ3] was replaced every 3 days with fresh medium. *In vitro* differentiation into chondrocytes was confirmed by RT-PCR of Collagen II gene expression in cell homogenate. Total RNA was isolated from the differentiated MSCs using Trizol (Invitrogen, USA). RNA concentrations were measured by absorbance at 260 nm with a spectrophotometer, and 2 µg total RNA was used for reverse transcription using Superscript II reverse transcriptase (Invitrogen, USA). The cDNA was amplified using Taq Platinum (Invitrogen, USA). Collagen (II) primers used were designed according to the following oligonucleotide sequence: (sense, 5'-CAGGGGTGAACGAGGTTTC -3'; antisense, 5'-AATACCAGCAGCTCCCCTCT -3'), and canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (sense, 5'-AACATCATCCCTGCTTCCAC-3'; antisense, 5'-TCCTTGGAGGCCATGTAGAC-3') were used as internal control for polymerase chain reactions (PCRs) (15, 16). The RNA templates were amplified at 33 to 45 cycles of 94°C (30 sec), 58°C to 61°C (30 sec), 72°C (1 min), followed with 72°C for 10 min. PCR products were visualised with ethidium bromide on a 3% agarose gel.

1.4-Labelling of MSCs: Undifferentiated MSCs were harvested and were labelled with green fluorescent protein (GFP) using monster green fluorescent protein vector and lipofectamin trans-fast transfection reagent kit (Promega, Madison, WI, USA). Before transfection cells were seeded into individual wells of 6 well-plates. After 24h incubation in growth medium, the cells were exposed to 2µg GFP plasmid /well of cells. GFP plasmid was incubated with lipofectamin for 10–15 minutes before subjection to the cells. Following transfection the cells were incubated at 37°C in humidified air (5% CO_2) for 2h. The transfection medium was then removed and the cells were incubated for an additional 48h in complete medium (2 ml per well) (17). For imaging GFP autofluorescence of MSCs, unstained slides were directly analysed by confocal laser microscopy (LSM 510, Zeiss, Jena, Germany) incorporating two lasers (Ar and HeNe) equipped with an inverted Axiovert 100M microscope. (18).

2- Surgical work

2.1-Animals and groups: This animal experiment followed the guidelines developed by the American Psychological Association (APA) for the ethical conduct of care and use of animals (19) and approval was obtained from the faculty of Veterinary medicine, Cairo University. Work was done on 32 stifle joints of male domestic mongrel dogs, aging between 2-3 years and weighing between 15-20 kg. The animals were obtained, housed and supervised and taken care of at the kennels of the Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Cairo University. They were fed a standard diet and had water ad libitum.

2.2-Pre-surgical assessment: All the animals were healthy and had no joint problems and all survived the whole study period.

2.3-Surgical procedures: Partial chondral defect was induced in the stifle of experimental dog through performing a 3cm lateral incision close to the lat-

eral patellar ligament. Care was taken to limit bleeding and soft tissue damage. Using a circular hand-made drill of 3mm diameter and 1mm depth, a partial thickness of the weight-bearing articular surface was removed from the lateral femoral condyle without damaging the subchondral bone. Synovium and fasciae were sutured using 3/0 absorbable suture material. Skin was closed using 1/0 non-absorbable suture material.

2.4- In vivo transplantation of undifferenitated GFP-labelled MSCs in chondral defect: Autologous undifferentiated MSCs were injected intra-articularily in the stifle joint of experimental dogs following surgically induced partial chondral defect at the lateral femoral condoyle in the both treated groups as mentioned in the study design.

2.5-Post-surgical follow-up: During the first 5 days after surgery, all animals were given a systemic course of antibiotics. Skin stitches were removed after 7 days. All the animals were exercised once daily by having them walk on a hard surface. All dogs were evaluated clinically for any abnormalities in gait according to "Evers" grading scale (20).

At the end of the experiment, and according to the sacrifice table, dogs were put to sleep through intravenous injection of thiopental sodium, the distal femoral parts were isolated and cartilage samples were collected for histological evaluation.

3- Histopathological work

3.1-Histopathologic and histochemical assessment: The pathologist was totally veiled from the sample numbers and groups of this study. All cases were fixed in 10% neutral buffer formalin. Decalcification of tissue cases were done by using 8% formic acid decalcifying solution in distilled water. The decalcifying solution was renewed every 48 hours until softening of the tissues. The decalcified specimens were then trimmed, washed and dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin, sectioned at 4–6μm thickness and stained with haematoxylene and eosin as well as Masson's trichrome (MT) stain for detection of collagen fibers. For assessment of homing of MSCs, unstained paraffin- embedded 4μm thick sections were examined by fluorescent microscope for detection of GFP-labelled stem cells in the newly formed cartilage. Paraffin embedded tissue sections were counterstained with DAPI as follows: Sections in xylene were dewaxed, rehydrated in ethanol series (absolute, 95% for 5 min, 70%, 30% ethanol, dH_2O for 3 min) and equilibrated in McIlvaine's buffer (5 min, freshly prepared). Slides were drained and put on a paper towel. DAPI staining solution on slides (~20μl) was applied and incubated 15 min in dark (cover with a box). Excess solution was drained. Cover slips were mounted with Gel Mount.

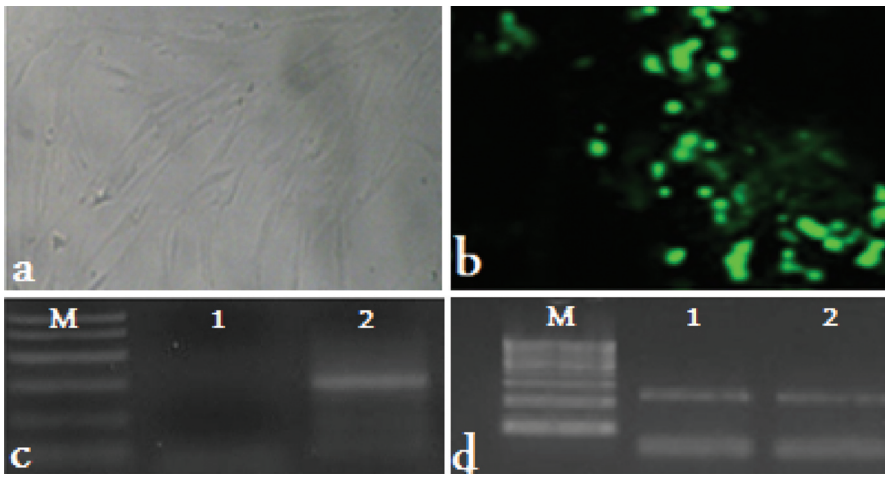

Fig. 2. Microscopic image of MSCs (**a**) after 14 days in culture with confluent 80–90% and (**b**) Fluorescent image of MSCs transfected with GFP. An agarose gel electrophoresis of PCR products, (**c**) PCR products of collagen (II) gene (190bp) and (**d**) PCR products of GAPDH gene (229 bp). Notice that Lane M: PCR marker (100, 200, 300, 500, 700, 900, 1000 bp), Lane 1: represents undifferentiated MSCs and Lane 2: represents differentiated MSCs.

3.2-Semiquantitative histological scoring: The Wakitani histological scoring system was used (21). This score has five categories including cell morphology, matrix-staining, surface regularity, thickness of cartilage, and integration with adjacent uninjured cartilage with a maximum score of 14 (poorest result). The cell morphology was graded from 0 (for tissue that was normal when compared with the adjacent, uninjured cartilage) to 4 points (when cartilage tissue was absent). Matrix staining, or the degree of metachromasia, was graded from 0 (for tissue that was normal when compared with the adjacent, uninjured cartilage) to 3 points (no metachromatic staining). Surface regularity, or the proportion of the surface of the defect that appears smooth when compared with the entire surface, was graded from 0 (when more than three quarters of the surface was smooth) to 3 points (when less than one quarter of the surface was smooth). The thickness of cartilage, or the average thickness of cartilage in the defect when compared with the surrounding cartilage, was graded from 0 (when the average thickness of the cartilage in the defect was more than two-thirds that of the surrounding cartilage) to 2 points (when the average thickness was less than one-third that of the surrounding cartilage). Integration of the neocartilage with the host cartilage was graded from 0 (no gap between the neocartilage and host cartilage) to 2 points (a complete lack of integration).

Statistical analysis

Statistical package for social science (SPSS) version 12 was used for data management and analysis. To test the difference between quantitative variables for more than 2 groups Kruskall-Wallis test was used. While to compare quantitative variables between two groups Mann-Whitney test was used, Bonferroni multiple comparison adjustment was done for pair wise comparison. Significance level was ≤ 0.05.

Results

MSCs culture, identification and labelling

Isolated and cultured MSCs reached 70–80% confluence at 14 days (Fig. 2a). The total number of MSCs injected was 1.4–1.6x10^6 cells/ml and some of them were labelled with GFP (Fig. 2b). Gene expression of collagen α1 (II) confirmed *in vitro* chondrogenic differentiation of MSCs (Fig. 2c) and gene expression of GAPDH, housekeeping gene, (Fig. 2d).

Post-surgical clinical assessment

All operated animals exhibited immediate postsurgical lameness that was graded as score three. In the control and chronic injection group, the severity of the lameness started to decrease after the 2nd week without the use of any medication. In the control, the 2nd degree lameness lasted for another 4 weeks. On week 6 after surgery, the dogs were able to walk, bear weight but with an apparent degree of lameness. From week 7, they continued to show a slight degree of lameness that was exaggerated by exercise. In the acute injection group, and after the injection of the MSCs on day one post-op, the severity obviously started to decrease between the 6th and 7th day without the use of any pain killer. By the end of the first week post-surgery, the lameness scale was evaluated as level two. This lasted for the following 4 weeks. Between week 5 to 6, the animals were able to bear weight. On week 7 the lameness started to decrease even on exercise until it completely vanished. In the late injection group, symptoms were similar to the control group for the first 4 weeks. After the injection of the MSCs at the end of the 4th week, the animals showed slight degree of lameness that was exaggerated by exercise, which lasted for the first 2 weeks post-injection. Between week 2 and 3 post-injection, the severity started to decrease, slowly but progressively, after the MSCs injection. From week 3–7 post-injection, the animals were able to do more exercise with less pain. From week 7 post-injection, the lameness started to further decrease until it disappeared (Fig. 3).

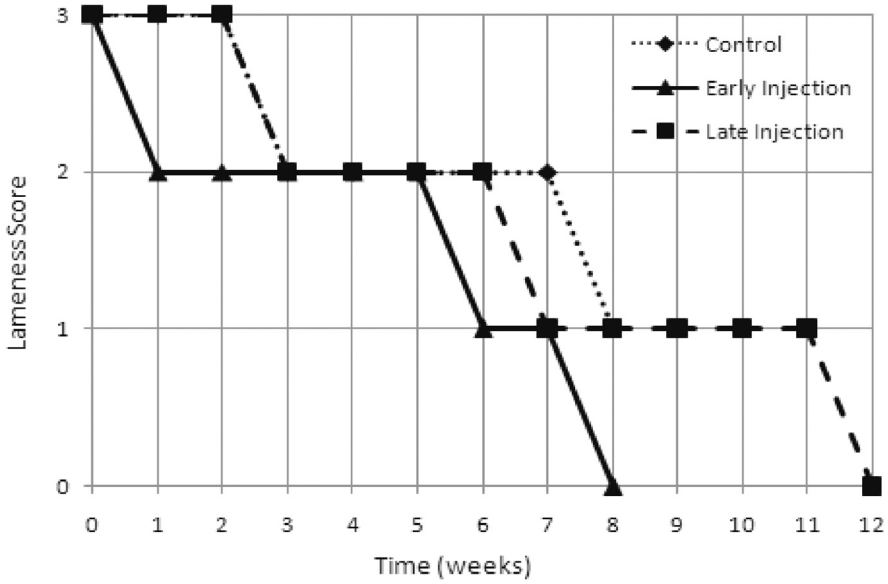

Fig. 3. Lameness scale throughout the whole study period. Note that the result of the early injection was better than both the control and the late injection.

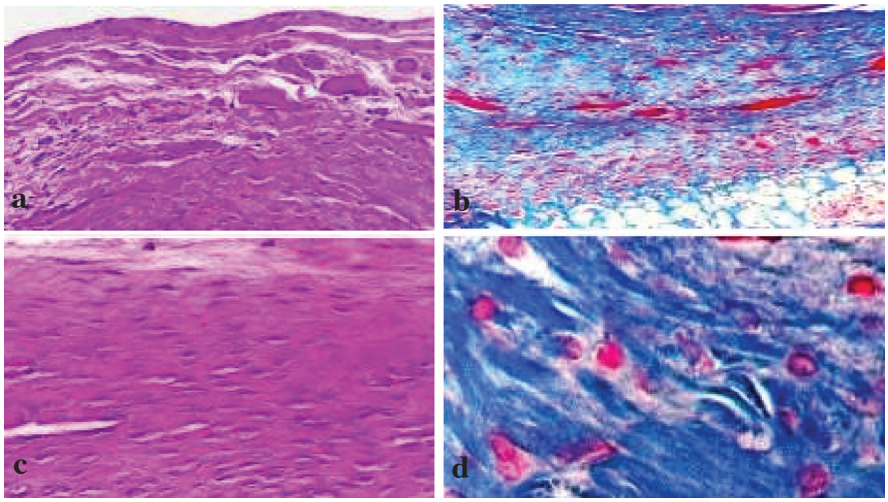

Fig. 4. Photomicrograph of control group at 2 weeks showing (**a**) deep coring defect in articular cartilage demonstrates the early exuberant response to the injury. The tissue initially is an undifferentiated connective tissue (H&E x100); (**b**) cluster of chondrocyte covered with thick layer of vascular granulation tissue (MT x100). At 8 weeks (**c**) chondrification of old granulation tissue with fibrillated surface (H&E x200) and (**d**) fibrocartilagenous mass in which the matrix is densely collagenous (MTx400).

Macroscopic findings

The articular surface showed the defect area occupied with a tough tissue that lacked the shiny appearance of the cartilaginous surface in the control group. It was filled with a smooth glistening tissue that took on a bluish colour resembling the surrounding intact cartilage in the early injection group, while the defect area was covered with a tough tissue that took on a pale colour but was clearly distinguishable from the surrounding intact cartilage in the late injection group.

Microscopic findings

Group I: After the 2nd week; the defect area was filled with fibrous tissue with severely irregular surface (Fig. 4a a/b). After the 8th week; the control group demonstrated poor healing and the reparative fibrous tissue was very thin, showing minimal metachromatic staining with an irregular surface (Fig. 4c/d).

Group II: After the 2nd week; the appearance of repair tissue was mostly fibrocartilaginous with markedly reduced metachromatic stain, irregular surface.

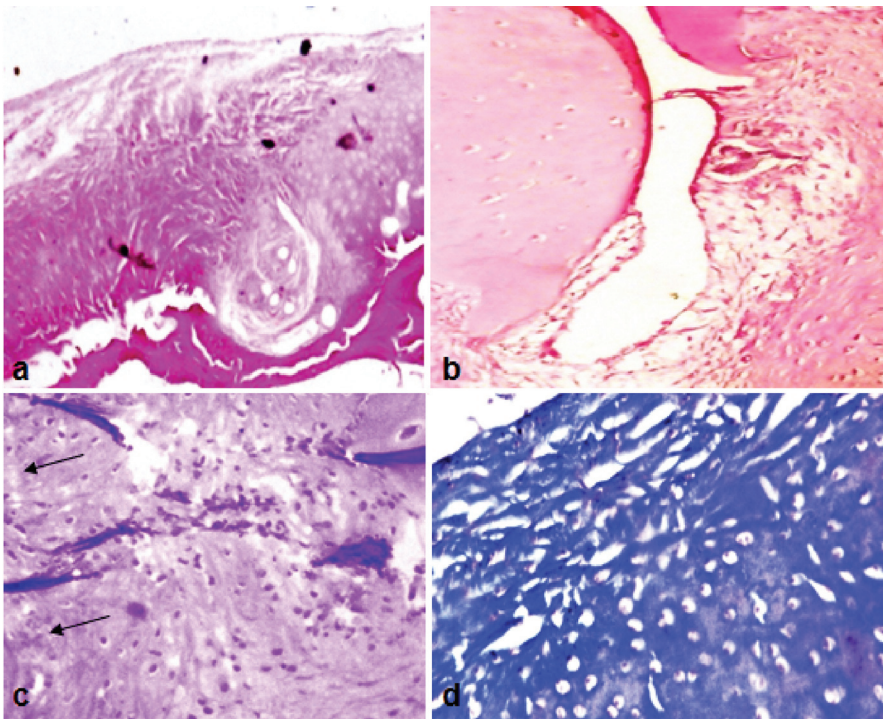

Fig. 5. Photomicrographs of the repaired chondral defect after 2 weeks of early injection of MSCs showing: (**a**) relatively poor integration with the normal cartilage, (**b**) irregular surface & overhanging edges (H&E x40), (**c**) Proliferating undifferentiated spindle cells with areas of fibrocartilage (arrows), (H&E x200), (**d**) Fibro cartilage and irregular surface & markedly reduced metachromasia (MT x100).

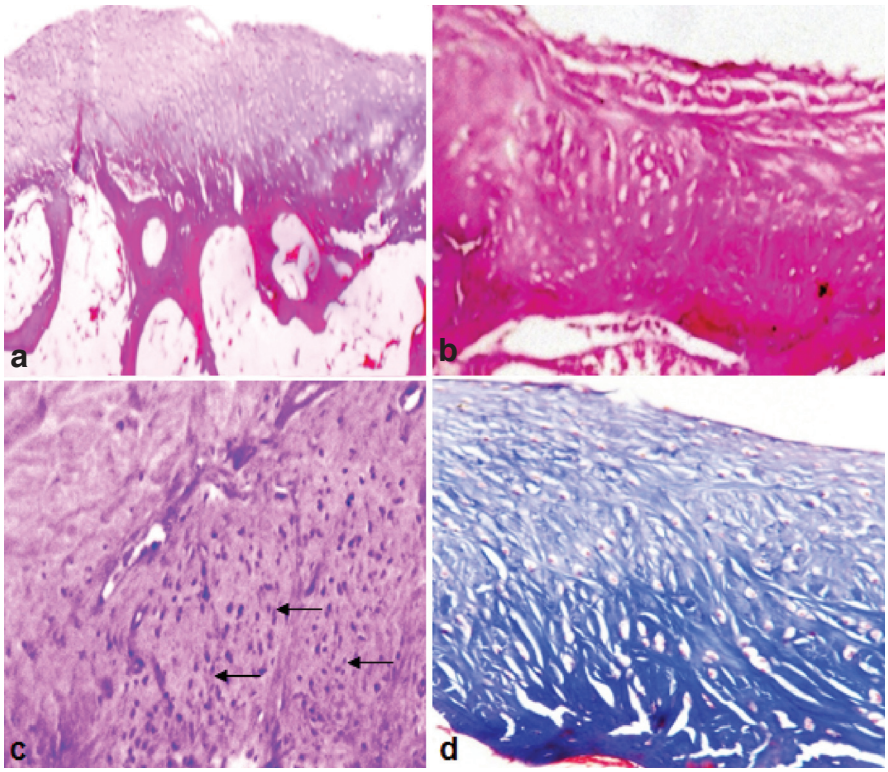

Fig. 6. Photomicrographs of the repaired chondral defect after 2 weeks of late injection of MSCs showing: (**a**) proliferating undifferentiated spindle(mesenchymal) cells, (**b**) less than 1/3 thickness fibrocartilage with fibrous superficial layer & irregular surface with relatively poor integration with the normal cartilage(H&E x40), (**c**) Repaired chondral defect with proliferating undifferentiated spindle (mesenchymal) cells (arrows), (H&E x200) and (**d**) Fibrocartilage and markedly reduced metachromasia (MT x100).

This repair tissue was thin, and there was relatively poor integration with the normal cartilage (Fig. 5). After the 8th week; the tissue was hyaline-like, with good integration, thickness, and moderate surface regularity. Intense metachromasia was also demonstrated in most cases and most of the chondrocytes had an appearance comparable with that of hyaline cartilage (Fig. 7).

Group III: After the 2nd week; the appearance of repair tissue was similar to that of early injection group. However, surface irregularity was marked (Fig. 6). After the 8th week; the appearance of the repair tissue was mostly fibrocartilage and hyaline formation but in some areas metachromatic staining was faint. This repair tissue ranged from 1/3–2/3 of cartilage thickness, and there was relatively good integration with the adjacent normal cartilage (Fig. 8).

The Wakitani scores for the three groups at 2 weeks were: 14 for all Group I cases; 10, 10, 10, 10, 12, 9 for Group II cases, and 11 for all Group-III cases. There was improvement in the quality of the repair tissue seen in the two MSCs-treated groups compared with the control group (p=0.003).

Comparing Group II and III together at 2 weeks, showed better results in Group II but with no statistical significance (p=0.06). However the results at 8 weeks showed statistical significance (p=0.01). [The Wakitani scores for the three groups at 8 weeks were 12, 13, 12, 11 for Group I; 5, 5, 5, 5, 7, 6 Group-II, and 7 for all Group III]. The repair tissue evaluated for the both groups (II, III) showed statistically significant better results at 8 weeks than 2 weeks (p= 0.002) which signify the improvement of the repair by time however longer follow up periods are needed.

Assessment of homing of MSCs

Injected undifferentiated GFP-labelled MSCs cells were detected under fluorescence microscopy in the neocartilage. Homing was proved in all injected specimens at 2 weeks and 8 weeks. Some GFP-labelled cells exhibited a chondrocyte-like phenotype (rounded form; surrounded by a lacuna) after 8 weeks indicating differentiation of injected MSCs while GFP-labelled

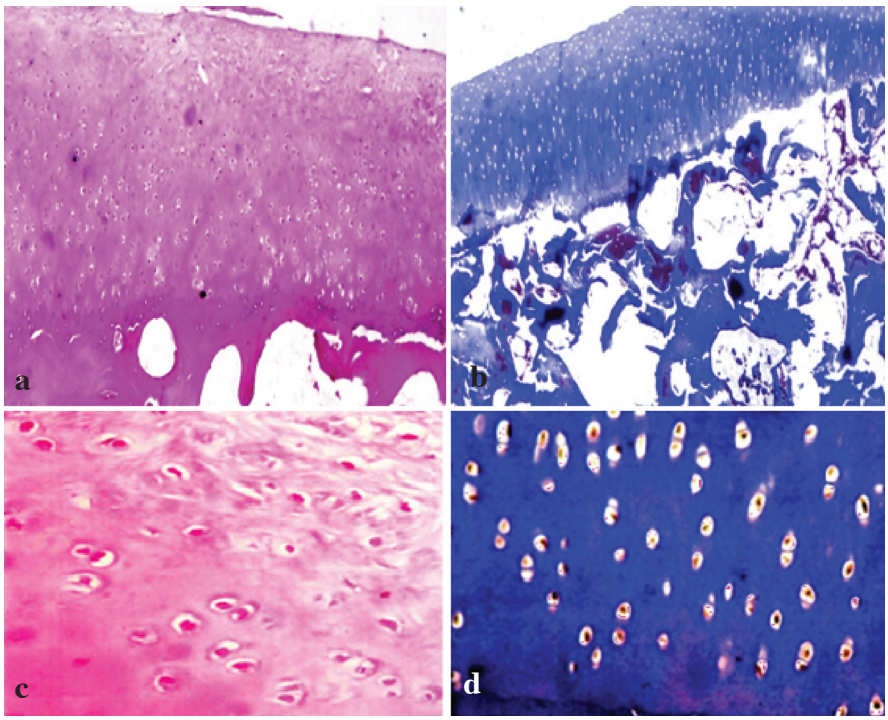

Fig. 7. Photomicrograph of the repaired chondral defect after 8 weeks of early injection of MSCs showing: (**a**) Mostly hyaline neocartilage more than 2/3 thickness with superficial fibrocartilage (H&E x40), (**b**) neocartilage of near normal morphology with slightly reduced metachromasia (MT x40), (**c**) Mostly hyaline neocartilage about 2/3 thickness with superficial fibrocartilage (H&E x400), (**d**) Hyaline neocartilage with slightly reduced metachromasia (MT x200).

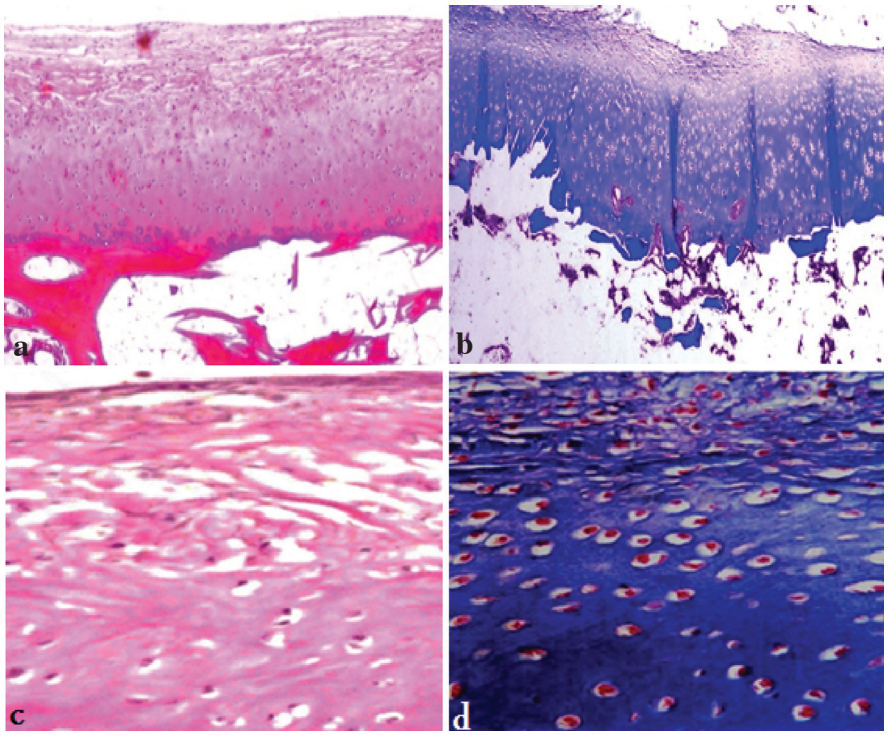

Fig. 8. Photomicrograph of the repaired chondral defect after 8 weeks of late injection of MSCs showing: (**a**) Neocartilage about half thickness with fibrous superficial layer (H&E x40), (**b**) Fibrocartilage with irregular surface and slightly reduced metachromasia (MT x40), (**c**) Neocartilage about half thickness with fibrous superficial layer (H&E x200), (**d**) neocartilage about 2/3 thickness with slightly reduced metachromasia (MT x400).

cells detected after 2 weeks remained spindle-like (undifferentiated mesenchymal) (Fig. 9). Paraffin embedded tissue sections were counterstained with DAPI and examined under confocal microscope to discriminate the false positive results (Fig. 10).

Discussion

Techniques that cause multipotent adult mesenchymal stem cells (MSCs) to differentiate into cells of chondrogenic lineage have led to a variety of experimental strategies to investigate whether MSCs can be used for the regeneration and maintenance of articular cartilage. Most of these techniques require an open arthrotomy or arthroscopy; methods that are invasive (11). The purpose of the current study was to evaluate the effect of a simpler technique which is the direct IA injection of autologus undifferentiated MSCs in healing of a partial thickness cartilage defect and testify the homing of the injected GFP-labelled cells to the damaged area. Also to study whether there is a difference in results for cases treated immediately after the induction of the lesions or one month later.

This study used dogs, as animal model, as they possess characteristics that are not found in traditional experimental animal models. They receive exceptional medical care; have comparable organ sizes (to humans) and generally cohabitate with their human owners, minimising different environmental effects (22, 23) which made them ideal for the study.

Using a circular trephine with a predetermined diameter (3mm) and depth (1mm) provided two major advantages. One, the rounded trephine made it easier to locate the area of interest for histosampling at the end of the experiment period. Two, the predetermined depth was very successful in preventing the penetration of the subchondral bone in all operated cases so no involvement of the vasculature would occur. Consequently, progenitor cells in blood and marrow cannot enter the damaged region to influence or contribute to the reparative process to ensure that any chondral repair is entirely from the injected cells.

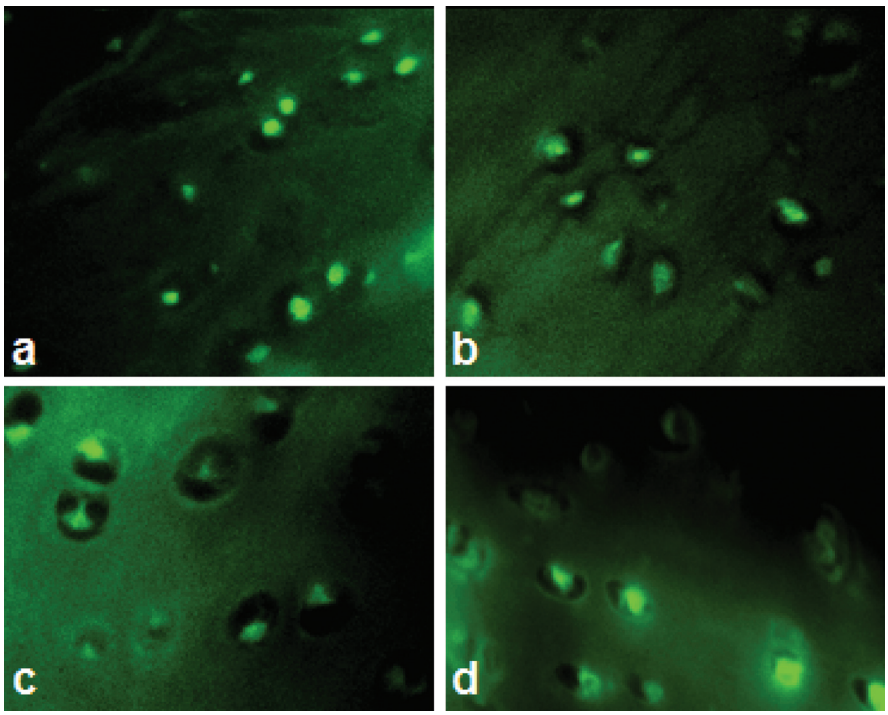

Fig. 9. GFP- labelled injected stem cells visualised by fluorescent microscope in formalin-fixed paraffin embedded tissue sections incorporated in the newly formed cartilage, indicating homing after injection: 2 weeks after (**a**) early and (**b**) late injection of MSCs 400x GFP-labelled cells exhibited spindle-like (undifferentiated mesenchymal) phenotype. 8 weeks after (**c**) early and (**d**) late injection of MSCs 400x GFP-labelled cells exhibited a chondrocyte-like phenotype (rounded form; surrounded by a lacuna.

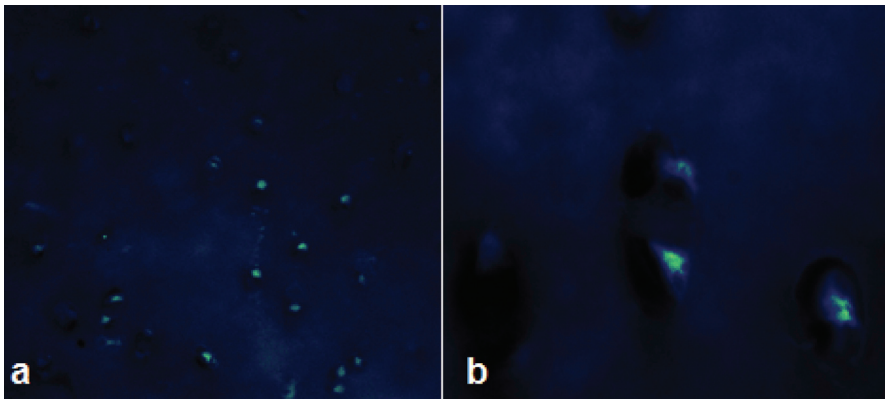

Fig. 10. Newly formed cartilage as seen by confocal laser microscopy using dual filter for green and blue for detection of GFP and DAPI showing: (**a**) intracellular green fluorescence of GFP (100x) and **b**) intracellular green fluorescent of GFP with chondrocyte-like morphology (400x); notice that the green signal here is not so bright for 2 reasons: 1-Merging of colours (green and blue) and 2- Long time from labelling of cells might lead to fading of fluorescence.

MSCs can be derived from several sources. MSCs that are derived from the bone marrow are relatively easy to obtain and will maintain their multilineage potential with passage, enabling considerable expansion in culture (24, 25, 26).

MSCs derived from bone marrow were used to initiate the process of cartilage defect repair similar to others who stated that the MSCs are the most promising cell source for cartilage repair (27).

In this study, IA injection was used. Similarly, the efficacy of the local delivery of adult mesenchymal stem cells to injured joints to stimulate regeneration of meniscal tissue and retards the progressive destruction normally seen in a model of OA were concluded (12).

The use of IA injections of mesenchymal stem cells suspended in HA was shown to be a viable option for treating large cartilage defects (11). Moreover, the necessity to reinforce the implanted cells against the unfavourable microenvironment by transduction of an anti-apoptotic protein, Bcl-xL, into the intra-articularily injected MSCs to prevent their death and improve their implantation efficiency into the cartilage was examined [28]. Both the homing of the MSC and the efficacy in healing were proved.

GFP is commonly used to monitor gene expression and protein trafficking within intact cells. GFP fusion proteins are easily visualised by standard fluorescence microscopy to track the real time subcellular localisation of a protein of interest.

GFP is used as a reporter gene, and GFP cDNA encodes for a 238 amino acid chromophore that produces strong green fluorescence when excited by blue light. It requires no exogenous substrates or co-factors for activity, thus cells expressing GFP can be easily scored by FACS scan or fluorescent microscopy (29).

In this study, the ability of GFP transfected mesenchymal stem cells to be easily detected and its longevity enhanced our hypothesis for successful homing.

Homing was proved in all injected specimens at 2 weeks and 8 weeks as GFP-labelled injected MSCs were detected in the neocartilage. Some cells exhibited a chondrocyte-like phenotype (rounded form; surrounded by a lacuna) after 8 weeks indicating differentiation of injected MSCs while in other areas cells remained spindle-like (mesenchymal). Thus we can propose that the local environment of the homing site can induce a chondrogenic phenotype in undifferentiated MSCs.

Others expressed nearly the same results as they transplanted PKH26- labelled mesenchymal stem cells in a large osteochondral defect in rabbits. Two weeks after transplantation, immature cartilage was formed. By 8 weeks, this cartilage had thinned. Most chondrocytes within the regenerated cartilage were PKH26-positive (30).

These results showed that undifferentiated MSC were able to help in repair of the osteochondral defects and differentiated according to the environment.

Longer follow up period is needed to detect the genotypic and phenotypic fate of the injected undifferentiated or differentiated MSCs. The biomechanical dynamic work is the goal of our coming research.

In this study, the control group; after 2 weeks the defect area was filled with fibrous tissue with severely irregular surface (mean Wakitani score 14). At 8 weeks the control group demonstrated poor healing and the reparative fibrous tissue was very thin, showing minimal metachromatic staining with an irregular surface (mean Wakitani score 12). These findings are comparable with that of other researchers (31, 32, 11) who have reported the same results in control group and stated that partial-thickness defects grow larger and deeper during the course of the disease and never repair spontaneously. The hypothesis that has been expressed most frequently is that, because there are no blood vessels in mature articular cartilage, cells from perivascular mesenchymal pools cannot enter this area. Another proposal has been that articular cartilage does not have access to stem cells in marrow, which have a high potential for inducing repair, because articular cartilage is walled off from the subchondral bone marrow by calcified tissue. Indeed, the lack of a source of cells for repair is usually the reason given for the absence of healing (33).

The effect of MSC injection in the two cell treated groups on healing compared with control group show high statistical significance ($p<0.05$) this is comparable with other similar researches who demonstrated the potential and efficacy of the use of autologous mesenchymal stem cells in cartilage repair (11, 21, 30, 34-37).

Both treated groups of early injection (one day post-operative) and late injection (one month post-operative) showed nearly the same results after 2 weeks of injection with no significant statistical difference. The appearance of repair tissue in both groups was mostly fibrocartilaginous with markedly reduced metachromatic stain, irregular surface. This repair tissue was thin, and there was relatively poor integration with the normal cartilage. This could be explained by the short period of 2 weeks to represent difference in the effect of injected mesenchymal stem cells.

The differences between the two groups became clearer after 8 weeks ($p=0.01$). There was marked improvement in the quality of the repair tissue seen in the early injected MSCs group compared with the late injected.

This could be attributed to the different defect status in the two groups at time of injection). Such lesions fail to heal spontaneously and consequently undergo inexorable enlargement with time (30, 38, 39).

It was reported that early infusion of MSCs (2 days compared with 14 after myocardial infarction) resulted in significantly higher uptake in the heart. This suggests that injured tissue might express specific receptors or ligands to facilitate trafficking, adhesion, and infiltration of MSCs to the site of injury, but these may be down-regulated a fairly short time after injury occurs. The local microenvironment of the defect and the released cytokines attract MSCs to the injured site and induce their proliferation and differentiation as early as lesion occurs. By time lesion fails to heal spontaneously and consequently undergo inexorable enlargement. This process is mediated by chemical mediators that may hinder the healing process and interfere with the proper effect of late-injected MSCs (40).

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