THE EFFECT OF MESENCHYMAL STEM CELL DERIVED MICROVESICLES IN REPAIR OF FEMORAL CHONDRAL DEFECTS IN DOGS

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ABSTRACT
Mesenchymal stem cells (MSCs) releases in culture extracellular vesicles called microvesicles (MV). MVs have beneficial cytokines that prevent progression of the disease and help in the regeneration process. This study is aimed to evaluate the effect of MSCs derived MVs in repair of induced chondral defect in a dog model. Methods: Chondral defects were created surgically (3 mm × 1 mm) in both femoral condyles of nine dogs, autologous MSCs were isolated and MVs were prepared and injected.

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intraarticularly in the right joint. The left joint was injected with normal saline as control negative. Evaluation of the treatment after first injection was carried out by physical examination and histopathology at different time periods (1½, 3 and 6 months). **Results:** Treated joints showed marked degree of cartilage regeneration and restoration of chondral histomorphological picture on the contrary of the control joints that showed deterioration over time and defect filling with only fibrous tissue forming a fibrocartilage at the end of six months period. **Conclusion:** We demonstrated in this study that administration of MVs was effective on the functional and morphological recovery of the injured cartilage and could be exploited as a cell free therapeutic approach in regenerative medicine.

**Keywords:** Microvesicles; Mesenchymal; Experimental dogs; Cartilage regeneration.

**INTRODUCTION**

The major approach of regenerative medicine is to replace damaged or diseased tissues with new cellular material by means of stem cell transplantation. Mesenchymal stem cells (MSCs) have been the focus of great interest in regenerative medicine for their ability to migrate to the site of injury, as well as for their multilineage differentiation potential and their straightforward *in vitro* expansion. Recent studies have suggested that the beneficial effect of MSCs is not attributed to their differentiation, but rather to the activation of a protective mechanism and stimulation of endogenous regeneration. This contention is supported by the production of bioactive soluble factors known to inhibit apoptosis and fibrosis, enhance angiogenesis, stimulate mitosis and/or differentiation of tissue-intrinsic progenitor cells and modulate the immune response.

MSC-secreted bioactive molecules acting as paracrine or endocrine mediators that directly activate target cells and/or cause neighboring cells to secrete functionally active agents. It has recently been demonstrated that extracellular vesicles or microvesicles (MVs) released from cells are an integral component of the cell-to-cell communication network involved in tissue regeneration, and therefore may contribute to the paracrine action of MSCs.

MVs play an important role in intercellular communications via their content molecules, and mimic, at least in part, the role that is played by their originating cells. Consistent with this notion, an increasing number of reports have suggested that MVs derived from MSCs, which are therapeutically beneficial as a treatment, can serve as a cell free regenerative option for multiple diseases. This is due to the fact that MVs contain a variety of molecules, including proteins, microRNAs, and mRNAs, and are associated with biological processes in a content molecule-dependent manner. Exosomes contain major histocompatibility complex (MHC) class I (as well as MHC class II when derived from antigen presenting cells), co-stimulatory molecules (CD86) 12, tetraspanins (CD9, CD63, CD82) 13-15, Fas and Fas ligand 16, several cytosolic (heat shock protein 73, annexin II, Gi2, gag) and membrane-bound proteins (Mac-1, MFG-E8).

While the role of MSC-derived MVs is still under investigation for treatment of osteoarthritis, it was found that they exert their effect via anti-fibrotic, anti-apoptotic, anti-inflammatory and pro-regenerative properties. They are a main source of an anti-inflammatory protein called Annexin-A1 that can start the healing process within the cartilage, and could prevent cartilage cell death during arthritis and could stimulate these cells to produce more cartilage, regenerating the tissue. MVs containing this protein are found naturally in our bodies, but it is figured that in arthritis, there are not enough to overcome the inflammation.
This study is aimed to evaluate the effect of direct intraarticular injection of MSCs derived MVs on the regeneration of experimentally induced femoral chondral defect.

MATERIALS AND METHODS

Work was divided into four steps; induction of experimental cartilage defect, acquisition and preparation of MSCs and MVs, intraarticular injection of the MVs and then the evaluation process.

Study Design

All animal’s experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

Nine mongrel dogs (2–5 years of age of both sexes) were used in this study. Dogs were housed individually in kennels and handled by trained personnel. All dogs went through a pre-study evaluation that included routine physical examination, lameness inspection, joint mobility and notation of pain on manipulation, range of motion and functional disability. Dogs having any signs of joint affections were excluded from the study.

Animals were equally and randomly allocated into two groups. Study group: right joints defects (n = 9) treated with 100 μg protein exosomes suspended in 100 μL phosphate buffer saline (PBS) and contralateral (n = 9) control group defects treated with 100 μL PBS. Intra-articular injection of exosomes was performed and follows up after first injection was assessed at three experimental times (1½, 3 and 6 months); thus resulting in three animals for each experimental time.

Induction of Chondral Defect

Under general anesthesia, lateral para-patellar stifle arthrotomy was applied. Using a rounded trephine with a 3 mm diameter and 1 mm depth, a partial thickness of the weight bearing articular surface was removed from the lateral femoral condyle without damaging the subchondral bone.

Isolation and Culture of BM-MSCs

Bone marrow was collected from the iliac crest under general anesthesia using a Rothensal bone marrow biopsy needle (stylet and needle, 15G, 1.8 mm). Bone marrow samples (20 mL/sample) were transferred to the lab and were processed within 4 h from the aspiration procedure. Over 15 mL Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY), 35 mL of the diluted sample was carefully layered, centrifuged for 35 min at 400 × g and the upper layer was aspirated leaving undisturbed mononuclear cell (MNC) layer at the interphase. This MNC layer was aspirated, washed twice in PBS containing 2 mM EDTA and centrifuged for 10 min at 200 × g rpm at 10°C. The cell pellet was re-suspended in a final volume of 300 μL of buffer. Isolated MSCs were cultured on 25 mL culture flasks in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and incubated for 2 h at 37°C and 5% CO₂. Adherent MSCs were cultured in MEM supplemented with 10% FBS, 0.5% penicillin, streptomycin and at 37°C in 5% CO₂ in air. Cytofluorimetric analysis was applied to characterize MSCs by using the following FITC-antibodies: CD29, CD105 and CD34 (Miltenyi-Biotec, Germany).

Isolation and FACS Analysis of MVs

MV were obtained from supernatants of third passage of MSCs (5 × 10⁶ cells/mL) cultured in RPMI deprived of FBS and supplemented with 0.5% of bovine serum albumin (BSA) (Sigma). After centrifugation at 2000 g for 20 min to remove debris, cell-free supernatant was centrifuged...
at 100,000 g (Beckman Coulter Optima L 90 K ultracentrifuge) for 1 h at 4°C, washed in serum-free medium 199 containing HEPES 25 mM (Sigma) and submitted to a second ultracentrifuga-

tion in the same conditions. The protein content was quantified by the Bradford method (BioRad, Hercules, CA). Electron microscopy analyses of MVs were performed. Purified MVs cultured overnight in the medium was used for collection of MVs. Images were obtained by secondary electron at a working distance of 15–25 mm and an accelerating voltage of 20 kV and 30 kV. Digital acquisition and analysis were performed using the Jeol T300 system. Cytofluorometric analysis was be performed using the following FITC- or PE-conjugated antibodies: CD63, CD44 (MiltenyiBiotec, Germany) and CD73 (Becton Dickinson, USA). FITC or PE mouse nonimmune isotypic IgG (DakoCytomation, Denmark) was used as the control.

**MVs Effect on Articular Chondrocyte Culture**

Articular chondrocytes were harvested from knee joint cartilage slices of two years dog by enzymatic digestion. In brief, cartilage slices from dog were dissociated enzymatically with collagenase type II (2.5 mg/mL) (clostridial collagenase, Gibco #17100-017) in α-modified Eagle’s medium (α-MEM; Gibco-BRL) for 5 h with continuous agitation. Following centrifugation at 1000 rpm×

5 min, the chondrocytes were resuspended. The cells were cultured with α-MEM containing 20% (v/v) FBS (Gibco-BRL) and 1% (v/v) penicillin/
streptomycin (Beijing Solarbio Science and Technology Co., Ltd.) in a 5% CO2 humidified incubator at 37°C with the culture medium replaced every other day after plating. Articular chondrocytes at passage 2, with a cell density of 2 × 10^4/mL, were used for further study about the effect of MVs on their culture proliferation. Cells were treated with MVs at a final concentration of 200 μg protein/mL for 24 h, and a group without MVs-treatment served as a control. Cells viability and proliferation were assessed by MTT assay (TACS™ TREVIGEN® 8405 Hegerman Ct. Gaithersburg) according to manufac-

ture instructions. Cells were cultivated in three 96-well tissue culture plates containing 2 × 10^4 cells/mL per well. The MTT reagent was added (10 μL per well) and the plate was incubated for 2–4 h. The plate cover was removed and the color absorbance in each well was measured at a range from 490 to 630 nm using an enzyme-linked immunosorbent assay plate reader (Dynatech MRX 5000; Dynex, Chantilly, VA).

**Physical Examination Evaluation**

The evaluation process was comprised physical and histopathological assessment. Lameness examination was done including joint mobility and notation of pain on manipulation, range of motion and functional disability according to the following score.30

**Histopathological Evaluation**

The collected samples were evaluated macroscopically according to the macroscopic evaluation scoring system of Rudert.8 All samples were fixed in 10% neutral buffer formalin. De-calci-

fication of specimens was done by using 8% formic acid (methanoic acid, Sigma) decalci-

fying solution in distilled water. Decalci-

fying solution was renewed every 48 h until softening of the specimens. The decalcified spec-

imens were trimmed, washed and dehydrated in ascending grades of alcohol. Additionally, they were cleared in xylene, embedded in paraffin, sectioned at 4–6 μm in thickness and stained with Haematoxyline and Eosin (H&E) as well as Masson’s Trichrome (MT) stain for detection of
collagen fibers and degree of matrix staining. Finally, evaluation was assessed according to score system of Mainil-Varlet.

**RESULTS**

**Characterization of MVs**

Cultured MSCs were confirmed by their morphology using inverted microscope (fibroblast like cells) (Fig. 1(a)). MVs were analyzed by electron microscopy that represented micrographs for spheroids MVs varying from 75.5 nm to 75.7 nm (Fig. 1(b)). For further characterization fluorescent analysis cell sorting (FACS) analysis showed CD90⁺, CD105⁺ and CD34⁻ for MSCs (Fig. 2(a)) and CD44⁺, CD63⁺ and CD73⁺ for MVs (Fig. 2(b)).

**MVs Effects on Chondrocytes Viability Assay in vitro**

Effect of MVs on viability of chondrocytes in culture was assessed. There was a significant increase in proliferation of cultured chondrocytes exposed to MVs compared to non-exposed chondrocytes (Table 1). There was less proliferative non-exposed chondrocytes compared to high proliferation capacity of exposed chondrocytes to MVs (Fig. 3).

**Clinical Assessment**

All animals tolerated the surgery well and remained healthy throughout the whole study period, lameness on both limbs occurred for three days after the induction of the chondral defects. After 1½ months, lameness was evident during...
trotting, mild pain on manipulation with normal range of motion and normal activity at the left limbs and no signs of pain or lameness in the right limbs. The later groups showed no lameness on both limbs and no detectable pain on manipulation.

HISTOPATHOLOGICAL EVALUATION

Macroscopically

At the time of euthanasia, joints showed no signs of infection or osteoarthritis. The cartilage samples were collected and evaluated according to the corresponding scoring system. Results were shown in Table 2.

At 1½ months, the defect site of the right joint exhibited a noticeable contour that was filled with whitish tissue and a depressed center (Fig. 4(a)). The left joint at the same period appeared brownish in coloration with more depressed center (Fig. 4(b)).

At three months the defect at the right joint obtained the same color and appearance of the normal adjacent cartilage and the outlines began to disappear (Fig. 4(c)). While the left joint at the same period showed the defect site filled with a whitish tissue layer and cracks in the adjacent normal cartilage were noticed (Fig. 4(d)).

At six months, the defect filling at the right joint showed the same color and appearance of

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<th>Average</th>
<th>(1½ Months)</th>
<th>(3 Months)</th>
<th>(6 Months)</th>
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<td>Right</td>
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<td>Color of the defect filling</td>
<td>2 1</td>
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Notes: Filling score: 1 = underneath the surrounding cartilage, 2 = up to surrounding cartilage with central depression, 3 = flush with surrounding cartilage.
Color score: 1 = brown to yellow, 2 = white, 3 = same as surrounding cartilage.
Surface score: 1 = rough, 2 = smooth.
the normal healthy cartilage and mounted up to the same level of the adjacent articular surface (Fig. 4(e)). While the left joint at the same period showed increase in both the defect depth and width with more new cracks at the adjacent normal cartilage (Fig. 4(f)).

**Microscopically**

The average values in microscopic changes of the cartilage were summarized in Table 3. The left joints showed minor changes throughout the whole study period, after 1½ months the defect was filled with fibrous connective tissue and the underlying subchondral bone showed thickening and hypercellularity. While after three months, the defect site showed maturation and organization of the fibrous tissue and formation of new blood vessels. After six months the defect site showed the formation of a fibrocartilage where singly allocated chondrocytes were found dispersed between the fibrous tissue. The right joints showed the defect filling with a large number of chondroblasts and dividing chondrocytes with irregular fibrous surface after 1½ months. While after three months the defect was filling by hyaline-like matrix containing single chondrocytes surrounded by lacunae with homogenous surface. At six months the defect site showed development of territorial and interterritorial collagen like matrix. Figures 5(a)–5(f)

| Table 3 Showing the Average Values of the Microscopic Changes of the Cartilage. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | 1½ Months (Right) | 3 Months (Left) | 6 Months (Right) | 3 Months (Left) | 6 Months (Right) | 6 Months (Left) |
| Percentage of defect filling   | 3               | 3               | 4               | 3               | 4               | 3               |
| Articular surface continuity   | 1               | 0               | 1               | 1               | 2               | 2               |
| Restoration of osteochondral architecture | 2           | 2               | 3               | 2               | 4               | 2               |
| Cellular morphology of regeneration tissue | 3          | 0               | 3               | 1               | 4               | 2               |
| Matrix staining                | 2               | 2               | 2               | 2               | 3               | 2               |

**Notes:**
- Percentage of defect filling score: 1 = 25%, 2 = 50%, 3 = 75%, 4 = 100%.
- Articular surface continuity score: 0 = discontinuous, 1 = continuous but rough, 2 = continuous and smooth.
- Restoration of architecture score: 1 = poor, 2 = unclear (heterologous), 3 = clearly differentiable.
- Cellular morphology score: 0 = fibrous tissue, 1 = fibrocartilage, 2 = hyaline fibrous hybrid, 3 = hyaline without zonal architecture, 4 = hyaline with zonal architecture.
- Matrix staining score: 1 = markedly reduced, 2 = slightly reduced, 3 = normal as adjacent cartilage.
DISCUSSION

Our follow-up six months study demonstrates that intraarticular administration of bone marrow MSC exosomes promotes therapeutic reparative of critical-sized osteochondral defects in dog model. MVs are a heterogeneous population of naturally occurring secreted small vesicles, with distinct biophysical properties and different functions both in physiology and under pathological conditions.\(^2\) In recent years, a number of studies have demonstrated that MVs might hold remarkable potential in regenerative medicine by acting as therapeutically promising nanodrugs.\(^24\) MVs from MSCs promoted angiogenesis by increasing endothelial cell proliferation and capillary network formation.\(^15\) The anti-fibrotic action of MSC-derived exosomes was also shown in liver by the reduction of collagen I and III deposit as well as TGF-\(\beta1\) expression and Smad2 phosphorylation leading to the inhibition of
epithelial-to-mesenchymal transition and protection of hepatocytes. Finally, MVs were shown to inhibit auto-reactive lymphocyte proliferation and promote secretion of the anti-inflammatory cytokines IL-10 and TGF-β.3

The rounded trephine with a predetermined diameter and depth provided two main advantages; the first was that the trephine-shape made it easier to locate the area of interest for histosampling at the end of the experiment period. The second was that the selected depth (1 mm) was very successful in preventing the penetration of the subchondral bone in all operated joints to avoid liberation of progenitor cells from the bone marrow. So avoiding any influence or contribution in the reparative process ensured that any chondral repair is entirely due to the injected Mvs. The size of the chondral defect was similar to that induced by Wang et al.30 to detect the histopathological progression of the articular cartilage healing process.

Clinical evaluation revealed minor changes while the macroscopic results showed marked regeneration signs in the treated joints which

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**Fig. 6 (a)–(f) Histopathological analysis using MT stain (cross-section of the middle part of the defect). Right column represents the study group and the left column represents the control group:**

- **(a)** Photomicrograph showing the defect site filling with irregular and fibrillated surface (blue arrow). MT 100X.
- **(b)** Photomicrograph showing the defect site filling with irregular and fibrillated surface (red arrow). MT 100X.
- **(c)** Photomicrograph showing the defect filling which is homogenous and regular surface. MT 100X.
- **(d)** Photomicrograph showing the defect site surface filled with fibrous tissue bundles shown by the red arrow. MT 200X.
- **(e)** Photomicrograph showing homogenous hyaline like matrix with no evidence of fibrous tissue formation. H&E 200X.
- **(f)** Photomicrograph showing the defect site filled with fibrous tissue in which are dispersed chondrocytes as shown by the red arrow. MT 200X.
began from 1½ months where the defect filling was whitish with a depressed center and reached the same level of the adjacent articular cartilage after three months from treatment to take the same architecture and glistening appearance of the adjacent healthy cartilage. These findings agreed with previous publications. On the contrary, the control joints where the gross examination revealed increase in depth of the defect after 1½ months and the defect site appeared brownish in color and rough in texture and increase in width after three and six months with increased cracks and more damage to the adjacent cartilage which confirmed the progressive cartilage damage that was not completely prevented by the repair process.14

The histopathological evaluation showed that the control joints ended by formation of fibrocartilage after six months which agrees with previous studies. The formed fibrocartilage is unable to withstand the mechanical action of the joint and the weight bearing activity which confirms that the degeneration of the articular cartilage is a progressive disease as fully discussed in Ref. 5. The Treated joints showed marked progression of healing over time where after 1½ months the large number of dividing chondroblasts was detected which is a marked sign of cartilage regeneration as agreed in Ref. 9. These chondrocytes lead to the formation of mature chondrocytes surrounded by lacunae after three months in a homogenous hyaline like matrix. After six months, the chondrocytes became allocated in clusters accompanied by the development of the territorial and interterritorial collagen fibers which followed the same healing procedure of the mesenchymal stem cell therapy in previous studies.

This study demonstrated that the administration of MVs derived from MSCs has a therapeutic effect on the functional and morphological recovery of the injured cartilage and was able to rebuild the articular surface. This work could confirm hypothesis of immunomodulatory paracrine action of MSCs rather than differentiation. Additionally this might be attributed to the effect of CD44 which is a receptor for hyaluronic acid and can also interact with other ligands, such as osteopontin, collagens and matrix metalloproteinases. And the Integrin beta-1 CD29 plays an important role in tissue regeneration. It has been established that xenotransplantation of human embryonic MSCs in immunocompetent animals for cartilage repair induces immediate adverse tissue reaction, resulting in poor reparative outcomes. MVs shows some advantages with respect to the MSCs as they have minimal immunogenicity allowing an allogenic use and, being naturally occurring component of biological fluids, have a low inherent toxicity. Moreover, due to their small size, they may easily diffuse across the biological barriers reaching target cells. MicroRNAs (miRNAs) are a class of ~22 nucleotide noncoding RNAs with essential roles in regulating cell fate and functions. Other studies previously showed that MSC exosomes contain many regulatory components including microRNAs (miRNAs), mRNAs, and proteins that are likely to be mediators in cell–cell communications to induce changes in cell functions and processes. Our study demonstrates significant increase in chondrocytes proliferation exposed to MVs in culture compared to non-treated chondrocytes. This finding coincided with other recent research as regard exosomes enhanced proliferation and migration of chondrocytes. The same researchers founded that synovial mesenchymal stem cells SMSC microRNA-140-exosomes in preventing osteoartheritis (OA). We recommended future work analysis for microRNA within BM-MVs that could enhance cartilage repair after transplantation in the experimental model.
CONCLUSION

In conclusion, the MVs derived from MSCs can regenerate injured cartilage with great efficacy as regard functional and morphological recovery. MVs could be exploited as a new cell-free therapeutic approach for regenerative medicine.

We represent that this submission is original work, and is not under consideration for publication with any other journal.

COMPETING INTERESTS

All authors declare that no conflict of interest.

References


