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Therapeutic Potential of Intra-Articular Injection of Bone Marrow Mesenchymal Stem Cells on Tempromandibular Joints' Induced Osteoarthritis. An Experimental Study

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Abstract

Background: The advancement of regenerative medicine and innovative stem cell technology offers a unique opportunity to treat Osteoarthritis (OA).

Objectives: To evaluate effect of Bone Marrow Mesenchymal Stem Cells (BMMSCs) on healing of Temporomandibular Joints' (TMJ) in rats with induced rheumatoid arthritis.

Methods: Thirty rats were randomly divided into three equal groups; ten for each. Group I: negative control. Group II: positive control and was subjected to induction of adjuvant arthritis CFA (Sigma Aldrich, St. Louis, Missouri, United States) bilaterally into the TMJ on the start of experiment. Groups III (n=10) was handled as those in group II, but after 10 days, TMJ was injected locally with 1×10^6 cell/ml BMMSCs. Animals of all groups were euthanized three weeks after the start of experiment.

Results: Group I revealed normal characters of the TMJ, Group II showed thickening of disc, thinning of cartilage, disordered bone trabeculae and decreased, disarranged collagen fibers after 2 weeks, while after 3 weeks it showed furthermore aggravated effects with adhesion between disc and condylar cartilage. Group III showed slight widening of marrow spaces, almost normal thickness of disc and condylar cartilage, near normal arrangement of bone trabeculae and regenerated collagen fibers.

Conclusion: These results suggest that BMMSCs may represent a novel and effective therapy for treatment of rheumatoid arthritis.

Keywords: Arthritis; Bone marrow stem cells; Osteoarthritis; TMJ

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Introduction

Osteoarthritis (OA) is a degenerative disease of joints with destruction of articular cartilage associated with subchondral bone hypertrophy and inflammation [1]. Any synovial joint can develop OA, but knees, hips and small hand joints are the most commonly affected sites [2]. A significant percentage of patients suffering from OA have signs and symptoms of Temporomandibular Joint (TMJ) involvement [3-5]. Epidemiologic studies on TMJ osteoarthritis found a prevalence of 25% in 20-49 years age group [6] and 70% in 73-75 years age group [7]. Furthermore, clinical evidence of TMJ osteoarthritis occurs in 8% to 16% of population [8].

The Complete Freund's Adjuvant (CFA) induced arthritis model in rats is the most common model used by several researchers to evaluate the possible therapeutic effects of new drugs. This model closely replicates clinical arthritis and has been used for screening purposes. It is induced in rats by injection of CFA into certain dermal and tissue sites [9].

In contrast to traditional treatments based on drugs, proteins, or antibodies, stem cells are poised to revolutionize medicine as they possess the capacity to replace and repair tissues and organs such as osteoarthritic joints [10]. Mesenchymal Stem Cells (MSC) are found in multiple human adult tissues including bone marrow, synovial tissues, and adipose tissues. Since they are derived from the mesoderm, they have been shown to differentiate into bone, cartilage, muscle, and adipose tissue [11] and can regulate inflammatory responses [12]. Because of their multi-potent capabilities, MSC

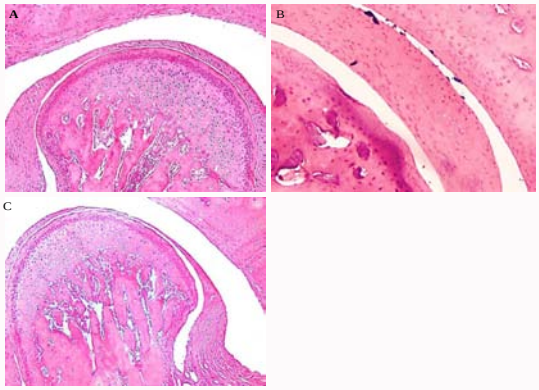

Figure 1: (A) The disc appeared biconcave, being less thick in the central area and is made up of fibrous connective tissue. The condyle comprises a thick layer of hyaline cartilage with layers of superimposed chondrocytes. It showed a complete structure of the subchondral bone which was clearly observable in the four condylar cartilaginous zones. Also, the articulating cortical condyle and temporal bone surfaces were covered with a layer of hyaline cartilage. There is a fibrous layer that increases in thickness from an anterior to a posterior position on the articular surface of the temporal bone. (B) Some chondrocytes were observed inside the TMJ disc. (C) The posterior ridge of the disc blends with highly vascularized, loose connective tissue located in the retrodiscal space.

lineages have been used successfully in animal models to regenerate articular cartilage and in human models to regenerate bone [13]. Thus, they raise the hope for them being used to treat diseases such as OA [10]. MSCs doesn't express major histocompatibility complex class II, so, they are not recognized by immune surveillance and can be utilized without immunosupression [14].

Transforming growth factor-beta (TGF-beta) is a multifunctional peptide that plays fundamental roles in the regulation of basic biological processes such as growth, development, tissue homeostasis and regulation of the immune system [15]. TGF-β induces the migration and mobilization of bone marrow-derived mesenchymal stem cells (BM-MSCs) to maintain bone homeostasis during bone remodeling and facilitate the repair of peripheral tissues [16]. Extensive efforts have been spent to search for alternative strategies to promote cartilage repair. The goal of the present study was to evaluate the therapeutic efficacy of MSCs based therapy for TMJ osteoarthritis.

Materials and Methods

Thirty male pathogen-free albino rats, weighing 150 g to 200 g, were selected. They were housed in Medical Experimental Research Center (MERC) in Faculty of Medicine, Mansoura University. All experimental procedures were performed under protocol of ethical committee of Faculty of Dentistry, Mansoura University, Egypt. The rats received water ad libitum and a standard pelleted diet and were kept in a 12 h light/dark cycle.

Study design

The rats were randomly divided into three equal groups; ten for each. Group I: rats were used as negative control; they were fed and kept in the housing conditions as the test groups. Group II: rats served as the positive control and were subjected to induction of arthritis by intra-articular injection of CFA (Sigma Aldrich, St. Louis, Missouri, United States) bilaterally into the TMJ on the start of experiment. Groups III: rats were handled as those in group II; however, rats were subjected to TMJ local injection of BMMSCs ($1 \times 10^{(6)}$ cells) suspended in 0.1 ml PBS bilaterally 10 days after the start of the experiment [17,18]. Animals of all groups were euthanized three weeks after the start of experiment. All surgical steps were performed in the Surgical Unit of MERC, Mansoura University, Egypt.

Isolation and culture of BMMSCs

All in vitro steps were performed in the Stem cell Unit of MERC, Mansoura University, Egypt. BMMSCs were isolated following the protocol described by Smajilagić et al. [19]. Briefly, Healthy 3-4 weeks white albino rats were euthanized with overdose chloroform anesthesia and cervical dislocation according to the guidelines laid down by the National Institute of Health (NIH) in the USA. Both, femora and tibia were aseptically removed. Bone heads at the ends of the diaphysis were cut from the femur and tibia, then a disposable aseptic syringe was used to flush out bone marrow using modified eagles' culture medium (α-MEM), supplemented with 10% Fetal Bovine Serum (FBS), 50 IU L^{-1} penicillin, freshly prepared ascorbic acid (AA) (50 µg/ml), , 2mM L-glutamine, , 0.3 µg mL^{-1} fungizone, 50 µg mL^{-1}streptomycin, 50 µg mL^{-1} gentamycin sulphate, 10^{-8} M dexamethasone, 10 mM β Glycerophosphate (β-GP). Cells were allowed to adhere for 5-6 days, then the non-adherent cell population was discarded and the culture medium was replaced with fresh culture α-MEM medium twice a week [19]. Confluence was reached 80% on days 14-21 of cell cultures, after which the cells were trypsinized and subcultured.

Characterization

The flow cytometric immunophenotype was determined using a BD Accuri C6 flow cytometer and program software. Digested passage 3 (P3) BMMSCs that reached an optimal growth state were rinsed in PBS, then resuspended in 0.5 mL PBS. Rabbit polyclonal anti-CD45 antibody, Anti-CD44 antibody and Mouse monoclonal Anti-CD105 antibody (Abcam, Cambridge, United Kingdom) were added separately, then incubated for 30 min in the dark at 4°C. Labeled BMMSCs were rinsed in PBS, then centrifuged at 200×*g* for 5 min and resuspended in PBS.

Preparation of BMMSCs for injection

When the cell confluence reached the required number, plastic syringes of 100 IU were loaded with stem cell suspension. Each syringe containing 0.1 ml of PBS carrying 1×10^{6} BMMSCs [18].

Technique of intra-articular injection

A pilot study was conducted to insure accurate point of injection using a blue dye. The joint was palpated 5 mm to 10 mm posterior to the lateral can thus of the eye while mandible was manipulated to move the condyle and positively identify the Joint. The injection area of the animal was disinfected using a sterile cotton pellet wet with 70% ethyl alcohol, followed by Betadine (7.5% Povidine-Iodine). The needle was injected from a posterior superior direction until the mandibular condyle was felt. One volume 50 µL of CFA was slowly injected into the joint over a time span of 2 mins [20].

Histological and immunohistochemical evaluation

The animals of each group were euthanized by overdose of diethyl ether, then decapitated and their heads were fixed in 10% neutral buffered formaline and demineralized by Ethylene Diamine Tetra Acetic acid (EDTA). After complete demineralization, TMJs of each rat were taken, processed for paraffin blocks in Pathology department, Faculty of Medicine, Mansoura University and prepared for histological examination by Hematoxylin and Eosin (H&E) stain as routine stain and immunohistochemical analysis by TGF-β.

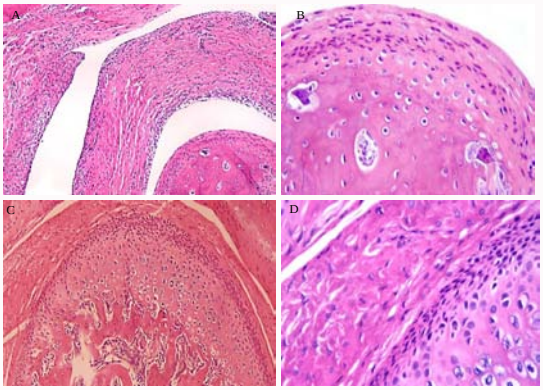

Figure 2: (A) CFA induced OA shows showed thickening of articular disc, thinning of articular cartilage, and irregular, disordered trabecular bone. Chondrocytes were almost lost in the cartilage of the disc and the temporal fossa. (B&C) CFA induced OA shows condyle with regional loss of chondrocytes, peripheral clustering and proliferation of chondrocytes, horizontal clefts, and subchondral bone resorption with adjacent bone marrow filled with fibroblast-like cells. (D) Group II shows adhesion between the disc and the condylar cartilage.

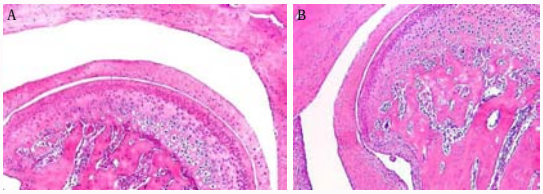

Figure 3 (A & B): BMMSCs treated group shows almost normal thickness of articular disc and condylar cartilage. Normal arrangement of bone trabeculae and the distinctive zones in condylar cartilage were recognized.

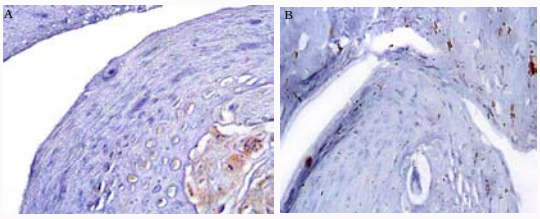

Figure 4: (A) Chondrocytes of group II animals were stained intensely with TGF-β (B) Group III showed moderate positive reaction with TGF-β.

Computer assisted digital image analysis (Digital morphometric study)

Slides were photographed using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using 40 X objective. The images were analyzed on Intel® Core I3® based computer using Video Test Morphology® software (Russia) with a specific built-in routine for distance measurement and stain quantification. The analysis provided an estimated quantification of the articular disc thickness and the immunohistochemical stain. Slides from each rat were prepared, 5 random fields from each slide were analyzed.

Measurement of cartridge thickness: Cartridge thickness was measured by free line tool after calibration against a micrometer side, to obtain results in um.

Stain quantification: Immune stain was quantified by preset routine after manual extraction of target area. The software routine of quantification includes:

Step 1: Image acquiring form the camera using a u-tech® frame grabber.

Step 2: Enhancing color tones of the images to reveal target stain color.

Step 2: Thresholding of the image at the level of the desired hue range to form a binary mask that represent target area.

Step 3: Define binary mask as Region of Interest (ROI).

Step 4: Apply % area calculation routine to obtain the % area of ROI in relation to total field area.

Step 5: All results were exported as. XLS as % area of positively stained area.

Statistical analysis

Data was analyzed using Statistical Package for Social Science software computer program version 23 (SPSS, Inc., Chicago, IL, USA). Data were presented as mean and standard deviation. One way Analysis of Variance (ANOVA) and post-hoc Tukey tests were used for comparing different groups in the study. P value less than 0.05 was considered statistically significant.

Results

Histologically, the sagittal view of the control group TMJ (normal untreated rats) was divided into three parts: glenoid fossa, fibro-cartilaginous disc, and TMJ condyle. The disc appeared biconcave, being less thick in the central area and is made up of fibrous connective tissue (Figure 1A). Some chondrocytes were observed inside the TMJ disc (Figure 1B). The condyle comprises a thick layer of hyaline cartilage with multilayers of regularly aligned chondrocytes. It showed a complete structure of the subchondral bone which was clearly observable in the four condylar cartilaginous zones. Also, the articulating cortical condyle and temporal bone surfaces were covered with a layer of hyaline cartilage (Figure 1A). The posterior ridge of the disc blends with highly vascularized, loose connective tissue located in the retrodiscal space (Figure 1C). Moreover, there is a fibrous layer that increases in thickness from an anterior to a posterior position on the articular surface of the temporal bone (Figure 1A). The CFA group rats (Group II) showed thickening of articular disc, thinning of articular cartilage, and irregular disordered trabecular bone. Chondrocytes were almost lost in the cartilage of the disc and the temporal fossa (Figure 2A). With regard to the condyle, regional loss of chondrocytes, peripheral proliferation and clustering of chondrocytes, horizontal clefts, and subchondral bone resorption with adjacent bone marrow filled with fibroblast-like cells (Figure 2B & 2C). Sometimes, adhesion between the disc and the condylar cartilage occurred (Figure 2D). Group III showed almost normal thickness of articular disc and condylar cartilage. Normal arrangement of bone trabeculae and the distinctive zones in condylar cartilage were recognized (Figure 3A & 3B). Immunohistochemically, group I animals were negative for TGF-β. Chondrocytes of group II animals were stained intensely (Figure 4A). Group III animals had very mild positive reaction (Figure 4B).

Group II had the highest disc thickness and the highest % area of TGF-β reaction. Statistically, an overall significant difference was found among the studied groups regarding disc thickness and TGF-β expression (Table 1). Multiple comparisons revealed significant difference between groups I and II as well as between groups II and III in relation to disc thickness. Meanwhile, groups I and III showed a non-

Table 1: Comparison of articular disc thickness (um) and immunohistochemical reaction (% area of reaction) between studied groups using one way ANOVA. Data are expressed as mean ± SD.

Comparisons	Articular disc thickness	(% area of reaction)
	(Mean ± SD)	(Mean ± SD)
Group I	259.3 ± 13.41	0.0000165 ± 0.000008127
Group II	675.1 ± 14.16	3.167 ± 0.2408
Group III	271.4 ± 24.24	0.4894 ±0.06289
P value	<0.001	<0.001

SD: Standard Deviation P: Probability P<0.05 was significant

Table 2: Multiple comparisons between studied groups using post-hoc Tukey's test.

Comparisons	Articular disc thickness	(% Area of reaction)
	(P value)	(P value)
Group I versus group II	< 0.001	< 0.001
Group I versus group III	0.17	< 0.001
Group II versus group III	< 0.001	< 0.001

P: Probability P<0.05 was significant

significant difference regarding the same parameter. Considering TGF- β expression, post-hoc Tukey test showed significant differences between each two groups (Table 2).

Discussion

Innumerous diseases, such as neoplasias, trauma, ankylosis and degenerative diseases may affect the TMJ and lead to the loss of its structures. For all these diseases there are specific treatments, each of which has a wide range of success. As a result there is always a need for progress in the treatment of some of these diseases. Furthermore much of the research in this area cannot be done on humans for ethical reasons. Studies in vitro and Comparison between human and rat TMJ: anatomic and histopathologic features in animal models may be used for this purpose [21]. Cell-based therapy and its associated safety and effectiveness should be carefully evaluated before clinical translation [10].

Rats are used extensively in biomedical research due to its accessibility. In particular, rats may be used since they are easy to handle and inexpensive to maintain, making them a convenient animal for use in experimental studies, giving rise to new ideas in the quest for new treatments for TMJ diseases. Morphologically and histologically, the articular structure of rats is, on the whole, similar to that of humans. This is very important for the validation of these studies to be extrapolated to humans [21].

In the present study, histological features of group I were consistent with Porto et al. [22] who demonstrated biconcave disc, being less thick in the central area and is made up of fibrous connective tissue. The condyle comprises a thick layer of hyliane cartilage with layers of superimposed chondrocytes. Also, they reported that the posterior ridge of the disc blends with highly vascularized, loose connective tissue located in the retrodiscal space. In accordance with the current work, Wanga et al. [23] mentioned that rat's TMJ showed a complete structure of the subchondral bone which was clearly observable in the four condylar cartilaginous zones. Moreover, they found a fibrous layer that increases in thickness from an anterior to a posterior position on the articular surface of the temporal bone. In addition, they observed chondrocytes inside the TMJ disc. Similar to our findings, Schek et al. [21] revealed that the articulating cortical condyle and temporal bone surfaces were covered with a layer of hyaline cartilage.

Several previous studies coincide with our observations reported irregular disordered bone trabeculae and subchondral bone resorption [24,25]. Kapilla et al. [24] explained that severe destruction of cortical, then subcortical bone that ultimately caused almost complete damage of the condyle might lead to the exposure of subchondral bone due to bone resorption by osteoclasts. In agreement with our findings, Wang et al. [26] reported significant thickening of all the three bands of articular disc in combination with other changes. Also, Xu et al. [27] demonstrated gradual decrease in chondrocytes number and condylar cartilage thickness with time, widened bone marrow cavities and disorganized trabecular bone structures. They attributed their results to the increased Receptor Activator of Nuclear Factor kb Ligand (RANKL)/Osteoprotegerin (OPG) ratio of subchondral bone indicating up-regulated osteoclastic activity and causing bone loss. In addition, Sanchez et al. [28] stated that OPG deficiency reduced cartilage thickness and enhanced chondrocyte apoptosis.

Group II of the present study showed adhesion between articular disc and condyle. This is convenient with Shinohara et al. [29] who observed fibrous adhesions in mice's TMJ after excessive mouth opening. They suggested that Tenascin-C (TNC) is involved in TMJ disorders. TNC is highly expressed during embryonic development, tissue repair, and in pathological conditions such as chronic inflammation. TNC interacts with several other extracellular matrix molecules and cell-surface receptors, thus affecting tissue architecture, tissue resilience, and cell responses [30].

The histological findings of stem cell group of the present study were in agreement with the studies conducted by Kehoe et al. [31] who demonstrated that the inflammation and cartilage destruction appeared less marked in MSC-treated mice compared to arthritic non-treated group. They explained this by the possibility that the anti-inflammatory factors produced by MSCs reduced leukocyte accumulation in the inflamed joint fluid or perhaps because MSCs differentiated into chondrocytes decreasing cartilage destruction which also explained the increase in collagen fibers in this group in comparison to the arthritic non-treated group. Moreover, they attributed the ability of MSCs in inducing tissue repair in OA to their multipotent differentiation potential and their ability to modify immune responses. Moreover and based on the International Society for Cellular Therapy (ISCT) criteria, Domnici et al. [32] owed the repair capacity of MSCs to their multi-potent capabilities to differentiate into osteoblasts, chondroblasts, adipocytes, and chondrocytes. Also, Murphy et al. [33] reported that the intra-articular injection of MSCs inhibited the progressive destructive effect of arthritis and that the stem cell treated group had less apoptotic chondrocytes than the arthritic group.

Transforming growth factor-beta (TGF-β) induces the migration and mobilization of bone marrow-derived mesenchymal stem cells (BM-MSCs) to maintain bone homeostasis during bone remodeling and facilitate the repair of peripheral tissues [34].

The present study reported that the group of induced OA had the highest % area of TGF-β reaction with statistical significant differences between each two groups. This agrees with Qian et al. [34] who found significant increase of TGF-β levels that were able to counteract the deleterious effects of MMP-3, in the synovial fluids at the early stage of TMJ OA combined displaced disc. Moreover, Boumediene et al.

[35] mentioned that at the initial stage of TMJ OA, TGF-β and MMP-3 levels in synovial fluids were increased while the destruction of bone and cartilage was not evident, which manifested as a reparative response at an early stage. In addition, previous studies have shown TGF-β to be an important anabolic with proven beneficial effects on cartilage repair in the initiation of OA. In their study, van der Kraan et al. [36] have demonstrated that an upregulated expression of TGF-β in early OA is found to be accompanied by increased synthetic activity. Also, Blaney et al. [37], Takahashi et al. [38] and Baugé et al. [39] have shown that TGF-β is an important inducer of cartilage Extracellular Matrix (ECM) production and is suggested to be a potential tool to enhance cartilage repair upon damage in OA. The present work revealed down regulation of TGF-β at the BMMSCs treated group than OA induced group. This is consistent with Lim et al. [40] who stated that TGF-β has been associated with articular regeneration and with improvement of articular pathology. Similarly, Freitag et al. [41] concluded that MSC enhanced repair via secretion of various anabolic factors, such as transforming growth factor beta (TGF-β), Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF) in addition to other bioactive molecules that modified the reparative responses. Also, Bobick et al. [42] demonstrated that TGF-β induced chondrogenesis by enhancing cell adhesion due to elevated N-cadherin levels [43]. In addition, Pelton et al. [44] found that TGF-β mRNA was expressed throughout the variable steps of chondrogenesis.

Conclusion

Within the limitations of the present study, our results suggest that TGF-β strongly induces chondrogenesis and local injection of BMMSCs may represent a novel and effective therapeutic strategy in the treatment of rheumatoid arthritis.

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