

## THE EFFECT OF SINGLE VERSUS MULTIPLE INTRA-ARTICULAR INJECTION OF SYNOVIAL FLUID MESENCHYMAL STEM CELLS ON RAT TEMPOROMANDIBULAR JOINT WITH INDUCED ARTHRITIS. BIOCHEMICAL AND HISTOLOGIC ANALYSIS

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### **ABSTRACT**

**Introduction:** Osteoarthritis (OA) is the most common chronic musculoskeletal disorder. Nowadays, the role of synovial fluid mesenchymal stem cells (SF MSCs) is increasingly attributed to the treatment of Temporomandibular joint osteoarthritis. This study aimed to compare single versus multiple intra-articular injection of SFMSCs in terms of macroscopic, biochemical, and histological analysis.

**Material & methods:** 18 young adult male 200–300 grams albino rats were used in the current study divided randomly into 3 equal groups. Group (A) comprised control in which OA was induced with no treatment, group (B) comprised MSCs single intra-articular injection group, and group (C) comprised MSCs multiple intra-articular injection group. Biochemical and histologic analysis were performed.

**Results:** histological findings showed that both groups B, C showed regeneration of meniscus and retrodiscal tissues, but group C was superior in regeneration compared to group B. Biochemical analysis showed that TNF- $\alpha$  and IL-1B had the highest value in the control group, followed by the single injection group, while the lowest value was found in multiple injections group respectively.

**Conclusion:** Multiple Intra-articular SF MSCs is a potential disease-modifying therapy for patients suffering from TMJ-OA.

**KEYWORDS:** SFMSCs, Osteoarthritis, TMJ, Meniscus, cytokines

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## INTRODUCTION

Osteoarthritis (OA) is a low-grade inflammatory disease characterized clinically by temporomandibular joint (TMJ) tenderness, stiffness, chewing difficulty, instability, joint sounds and decreased range of motion. <sup>(1)</sup> It is usually asymptomatic at early stage which is explained by the avascular and aneural cartilage and meniscus, but painful at late stage. Moreover, the subchondral bone shows sclerosis, osteophytes formation, and increased trabecular thickness as an inflammatory response in addition to the articular cartilage, meniscus, and synovium degeneration. <sup>(2-4)</sup> Furthermore, compressive stimuli causes Chondrocytes to increase the production of inflammatory cytokine and extracellular matrix components degradation. <sup>(5)</sup> It was reported that a complex inflammatory response is developed in OA, thus inflammatory Mediators, as cytokines, tumor necrosis factor-alpha (TNF- $\alpha$ ), and interleukin 1 (IL1), enhance the production of destructive enzymes which affect the TMJ meniscus, retrodisal tissues and causes destruction of cartilage structures. <sup>(6)</sup>

Management of Osteoarthritis is mainly symptomatic treatment to reduce pain and inflammation. It includes intra-articular injection of different pharmacological agents, surgical procedures, and recently tissue engineering therapy for repair and regeneration. <sup>(7)</sup>

Nowadays, the role of mesenchymal stem cells (MSCs) in the management of TMJ-OA is a challenging field. <sup>(8-10)</sup> These cells have the ability to differentiate into bone, cartilage, collagen fibers and adipose tissue. MSCs have been reported as a successful and efficient therapy in animal studies for TMJ disc repair and cartilaginous tissue regeneration. <sup>(11-13)</sup>

MSCs were isolated from synovial fluid in 2004, and since then they became of great interest for their easy accessibility during arthrocentesis, self-renewal capacity, and immunosuppressive properties. <sup>(14)</sup>

Based on the above, the objective of this study

was to compare the macroscopic, biochemical, and histological effect of single versus multiple intra-articular injection of synovial fluid MSCs for management of TMJ induced Osteoarthritis in rats.

## MATERIALS AND METHODS

18 young adult male 200–300 grams albino rats were used in the current study. They were housed in plastic cages with a 12 hours light/dark cycle at temperature of  $22\pm 2^{\circ}\text{C}$ . The rats were under veterinary supervision in an animal house, were supplied a regular diet and drinking tap water throughout the duration of the study.

The study animals were randomly divided into 3 groups 6 rats each; group (A) comprised control in which OA was induced and rats left without treatment, group (B) comprised MSCs single injection group after induction of OA, and group (C) comprised MSCs multiple injection group after induction of OA. All experiments were conducted according to the guidelines of the International Association for the Study of Pain (IASP) in conscious animals. <sup>(15)</sup>

### Induction of osteoarthritis

Intra-peritoneal injection of Ketamine (70mg/kg) and Xylazine (10mg/kg) solution was performed to anesthetize the study animals. Then Each animal received intra-articular injection of 50 $\mu\text{l}$  of Complete Freund's Adjuvant (CFA) (5881; Sigma-Aldrich, USA), diluted 1:1 (oil/saline) in the right TMJ with a 30-gauge needle joined to a plastic syringe (1 ml) to induce osteoarthritis. <sup>(16-18)</sup> The needle was inserted immediately below the posterior inferior border of the zygomatic arch till it touched the posterior-lateral edge of the mandibular condyle. <sup>(19)</sup> The left TMJ was left normal not to compromise animal feeding. Treatment protocol in group (B, C) started one week after CFA injection according to Kuroki et al. <sup>(20)</sup> Synovial fluid sample was taken from each induced arthritic joint one week after induction and sent for MSCs preparation.

### Isolation and expansion of MSCs

Synovial fluid samples were diluted in expansion culture medium 1:6, then plated in Petri dishes.<sup>(21)</sup> MSCs were expanded in proliferation medium containing Dulbecco's modified Eagle's medium with low glucose (DMEM-LG, Gibco) with addition of 10% fetal bovine serum (FBS, Sigma), 1 ng/ml basic fibroblast growth factor (bFGF, Miltenyi Biotec), 1% glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). Then they were cultured at 37°C with 5% humidified CO<sub>2</sub>. After the 1st 3 days, the medium was changed twice per week until reaching confluence. During changing the medium, the non-adherent cells were discarded. When reaching nearly 80% confluence of the adherent cells, the MSCs were trypsinized (trypsin-EDTA 0.05%, Gibco) and plated at a density of  $0.5 \times 10^6$  cells/dish, and the medium was changed every 2–3 days. Pre-differentiation medium then was used at the final passage (P3).<sup>(22, 23)</sup> A preparation of  $2.5 \times 10^6$  cells in a volume of 50  $\mu$ L were used for intra-articular injection.

### Intra-articular injection of synovial fluid MSCs

In Group B, single intra-articular injection with synovial fluid MSCs ( $2.5 \times 10^6$  cells in a volume of 50  $\mu$ L) was performed. While in group C, intra-articular injection of Synovial fluid MSCs ( $2.5 \times 10^6$  cells in a volume of 50  $\mu$ L) was performed 4 times (one injection every week). (Fig. 1)

The animals of groups B, C were sacrificed after anesthesia overdose (Ketamine and Xylazine) at day 28 after injection. The control group A animals were sacrificed at the same time with the other 2 groups.

### Biochemical Analysis

Samples of synovial fluid were taken at day 28 after injection and immediately before scarification. The concentration of the cytokines was measured by an ELISA (Quantikine, R&D Systems, Minneapolis,

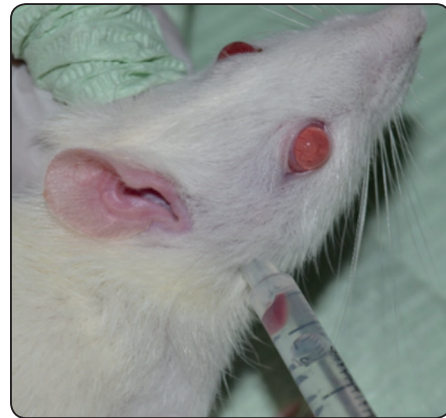


Fig. (1) A photo showing the intra-articular injection of synovial fluid MSCs in rat TMJ with induced osteoarthritis

MN). The detection concentrations of cytokines were IL-1B > 0.016, TNF- $\alpha$  > 0.008 pg/ml. The concentration of cytokines was calculated per 100 g of protein as previously reported.<sup>(24, 25)</sup>

### Histologic preparation

TMJs were dissected for morphological analysis. The meniscus, and retrodiscal tissue were harvested for histological analysis.

A histological sample was taken from each group, Samples were fixed with 10% neutral formalin, embedded in paraffin, and stained with Hematoxylin and Eosin (H&E) as a Routine stain for cellular details and detection of the formation of collagen fibers. All Stained specimens were inspected by the naked eye and under the microscope.

### Statistical analysis:

Data of biochemical analysis were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. Data were normally distributed so they were presented as mean, standard deviation (SD) values and were analyzed using one-way ANOVA test followed by Tukey's post hoc test. The significance level was set at  $p \leq 0.05$ . Statistical analysis was performed with IBM® SPSS® Statistics Version 26 for Windows.

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## RESULTS

Synovial fluid-derived MSCs were successfully isolated and expanded from all rats TMJ in the study groups B, C. All joint injections and follow-up procedures went well without adverse event that required study cessation. No rats had abnormalities identified during the daily physical examination in the follow up of intra-articular injection.

### Macroscopic findings:

During separating the menisci from the condyles of the three groups, by naked eyes, it was noticed the menisci of group A were incomplete with diminished thickness and length while the menisci of groups B & C were apparently complete with normal size. (Fig. 2)

### Biochemical analysis findings

For TNF- $\alpha$  and IL-1B concentrations, there was a significant difference between values of different groups ( $p < 0.001$ ). The highest value was found in the control group ( $0.145 \pm 0.010$ ) ( $0.148 \pm 0.015$ ), followed by the single injection group ( $0.025 \pm 0.014$ ) ( $0.028 \pm 0.008$ ), while the lowest value was found in multiple injections group ( $0.002 \pm 0.001$ ) ( $0.003 \pm 0.005$ ) respectively. Pairwise comparisons for both markers showed values of different groups to be significantly different from each other ( $p < 0.001$ ). Fig 3

### Histological findings

The meniscus of group A was incomplete and not intact with less dense collagen fibers, retrodiscal tissue was degenerated and infiltrated with inflammatory cells (Fig. 4 )

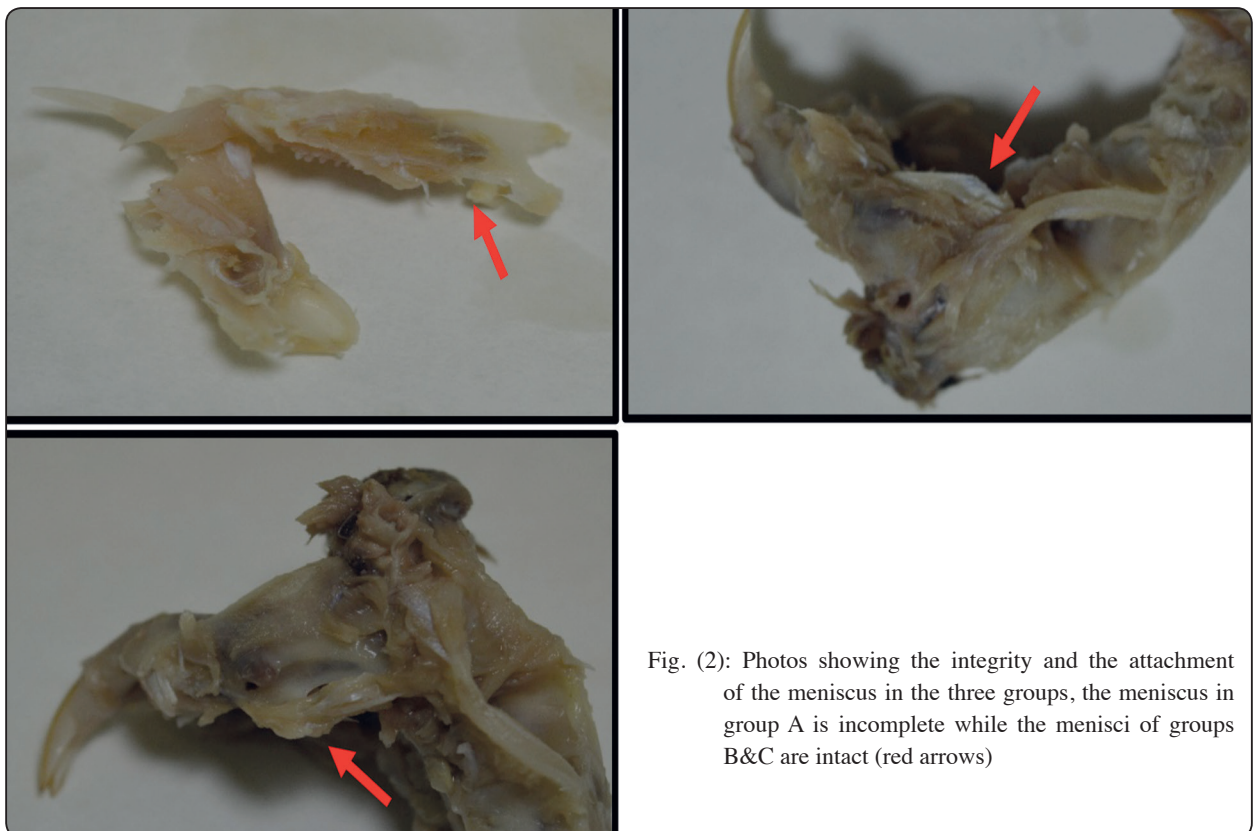


Fig. (2): Photos showing the integrity and the attachment of the meniscus in the three groups, the meniscus in group A is incomplete while the menisci of groups B&C are intact (red arrows)



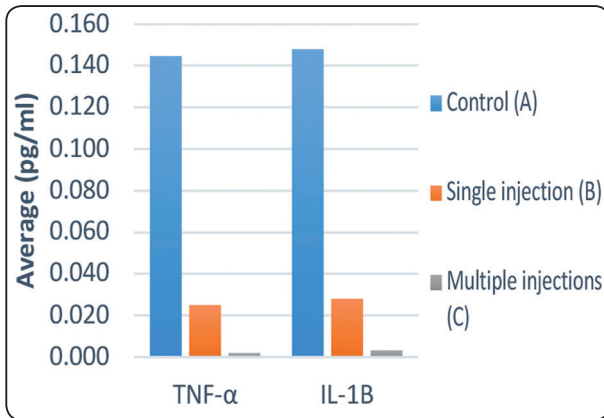


Fig. (3): Bar chart showing average TNF-α and IL-1B concentrations (pg/ml)

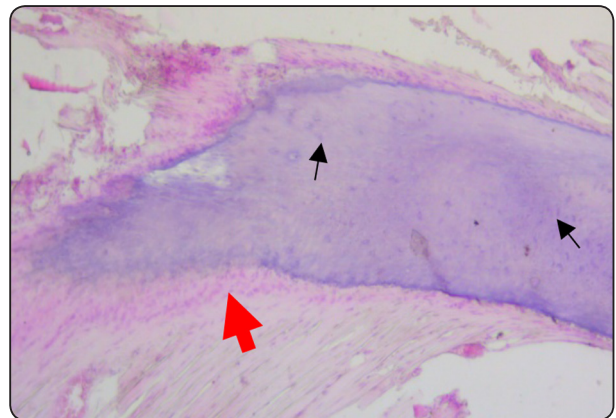


Fig. (5): Photomicrograph showing regenerated meniscus, the retrodiscal fibers are regenerated with proliferating fibroblasts (red arrow). The meniscus contains fibrocartilage cells (black arrows) (X 100)

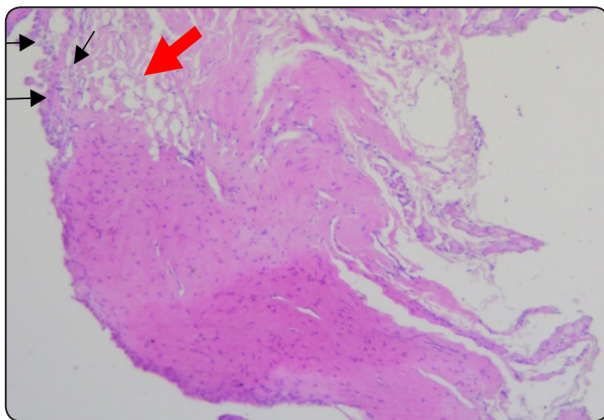


Fig. (4): Photomicrograph showing degenerated meniscus, the retrodiscal fibers are degenerated (red arrow) and infiltrated with inflammatory cells (black arrows) (X 100)

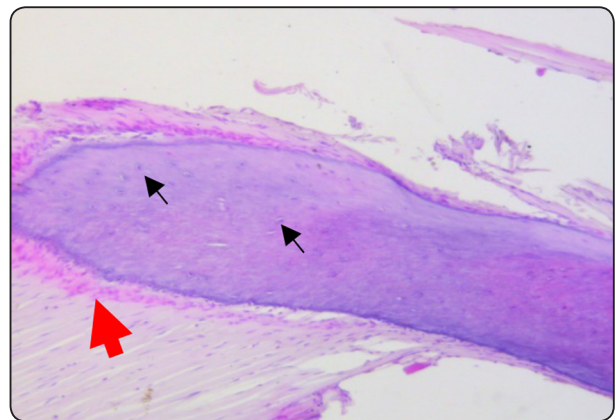


Fig. (6): Photomicrograph showing completely regenerated and intact meniscus, the retrodiscal fibers are regenerated and completely surround the meniscus with proliferating fibroblasts (red arrow). The meniscus contains fibrocartilage cells (black arrows) (X 100)

It was noticed that the meniscus of group B started to regenerate, fibrocartilage cells were noticed inside the meniscus, retrodiscal tissue started to regenerate and infiltrated with inflammatory cells but less than group A (Fig. 5)

The meniscus of group C completely regenerated, fibrocartilage cells were noticed inside the meniscus, retrodiscal tissue completely regenerated with proliferating fibroblasts surrounding the meniscus. (Fig. 6)

**DISCUSSION**

Currently, cell therapy is an essential tool and an important alternative for regenerative medicine. This study was conducted to compare biologic and histologic outcomes of two different treatment regimens of intra-articular injection of synovial MSCs in rats with induced OA. It was concluded by Prado et al, <sup>(26)</sup> that the mesenchymal nature of the synovial fluid derived stem cells is a new minimal invasive strategy for cell therapy that could treat joint pathologies, and repair cartilage defects.

In the current study, autologous synovial fluid MSCs were used to avoid the previously reported specific immune response against allogeneic MSCs.<sup>(27, 28)</sup>

Our study showed that both single and multiple intra-articular injection of synovial fluid MSCs are safe and feasible procedures and possess promising effects in reducing the degeneration and promoting regeneration of the joint meniscus and retrodiscal tissue based on macroscopic and Histologic Parameters compared to the untreated control group. This results corresponds to the results of Zayed et al,<sup>(29)</sup> who demonstrated that implantation of equine SFMSCs was successful, and advantageous where the cells can be ready and applied within a short time period, and less aggressive compared to the other techniques of articular cartilage regeneration.

Moreover, this corresponds to the previously reported studies who stated that synovial fluid Mesenchymal stem cells have a major role in meniscus repair due to high proliferative and chondrogenic potential.<sup>(30-33)</sup>

The current study demonstrated the superior effect of multiple intra-articular injection on the regeneration of TMJ meniscus and retrodiscal tissues as compared to the single intra-articular injection. This results corresponds to the results of Ozeki et al<sup>(34)</sup> who compared the chondroprotective effects of repeated injections of MSCs with a single injection of MSCs for tibial plateau and femoral condyle. They reported that weekly injection of MSCs has superior efficacy compared to a single injection in inhibiting OA progression. This also was supported by some studies which reported that after the single administration of MSCs into the knee joint, it is well known that the number of cells rapidly decreases which explains the need for multiple injections.<sup>(35,36)</sup> Furthermore, this result is in accordance with the result of Murphy et al.<sup>(37)</sup> who concluded that the chondroprotective effects of

stem cells in induced OA in goats were due to the regeneration of the meniscus.

Regarding the cytokine level, the current study reported significantly high levels of TNF- $\alpha$  and IL-1B in the control group. This corresponds to the previous studies which reported that IL-1B and TNF- $\alpha$  have a major role in the pathogenesis of the degenerative changes in TMJ OA.<sup>(38, 39)</sup> Moreover, the concentration of cytokines showed significant reduction in both groups B, C, although slightly higher cytokine concentration was noticed in group B than in group C. This was Consistent with the suppression of inflammation as supported by the current study histologic findings as the number inflammatory cells were decreased in group B and disappeared in Group C. these results corresponds to the results of Zhang et al<sup>(40)</sup> who demonstrated that MSC mediate the regeneration of TMJ OA through reduction in inflammation and tissue degeneration followed by increased proliferation and matrix synthesis.

From the results of the current study, we can conclude that multiple intra-articular injections of synovial fluid MSC is a minimally invasive conservative technique, and may be a promising therapeutic option in the management of Temporomandibular Joint osteoarthritis which promote TMJ repair and regeneration.

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