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Impact of Bone Marrow Mesenchymal Stem Cells on the temporomandibular joint in rats joint osteoarthritis induced by complete Freund's adjuvant. Histological, Histochemical and Biochemical investigations

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ABSTRACT

Objective: The purpose of the current study was to evaluate the therapeutic impact of administering bone marrow-derived mesenchymal stem cells (BMSCs) on the recovery of Freund's adjuvant-induced TMJ osteoarthritis in rats. **Materials and Procedures:** Thirty Sprague-Dawley rats were divided into three groups of ten. Group I; negative control: rats with normal features of TMJ were fed ad libitum and kept in normal housing conditions. Group II; positive control: rats were given an intra-articular injection of complete Freund's adjuvant (CFA) into the right to induce unilateral arthritis. TMJ. Group III; experimental group the rats, 30 days after osteoarthritis induction with CFA, have been given a single injection into the right TMJ of 1×10^6 BMSCs suspended in 0.5 ml of phosphate buffer solution. To measure TNF-α and IL-10 cytokines before and after BMSCs were administered, blood samples were taken. All rats were sacrificed 30 days after BMSCs injection and TMJ harvested and microtechnically processed for histological and histochemical studies. **Result:** the group that received experimental stem cell treatment displayed histological, histochemical, and biochemical characteristics that were comparatively like those observed in the control animals. **Conclusion:** bone marrow derived stem cell therapy is relatively successful treatment for CFA induced temporomandibular joint osteoarthritis.

Key words: TMJ, CFA, osteoarthritis, stem cells therapy.

Introduction

There are many temporomandibular disorders (TMDs) as inflammatory, traumatic, and infective entities. By far, the most significant types of chronic arthritis are rheumatoid arthritis and osteoarthritis. The osteoarthritis (OA) is one of the most sever TMD type but it also affects the other different joints such as those of the wrist, knee, shoulder, and ankle. The TMJ is among the most commonly affected joints. affected by arthritis characterized mainly by movement limitation with a decrease in the ability of chewing.

Since there is great similarity between the human and rat TMJ from either the anatomical or histological structures, except with some structural differences, Therefore, the rat TMJ serves as a good model for similar research on TMJ disorders. Since the most common model used by researchers to evaluate the possible therapeutic effects of new

arthritis drugs, Complete Freund's Adjuvant (CFA) is known for its ability to trigger arthritis. In addition to being utilized for screening, this model may accurately mimic clinical arthritis. [1]

Tumor necrosis factor (TNF) simulates the prototype for diverse groups of cells signaling partners. The TNFs have cell signaling partners and lymphocytes can produce three isoforms of TNF that is trimeric lymphokines serving roles in inflammation providing defense against bacterial infections, eradicating tumor cells, and causing chronic illness wasting. While other TNF receptor ligands control cell division and death, TNF itself also plays a role in immune system development. Additionally, TNF contributes to inflammation in autoimmune conditions like rheumatoid arthritis. In these conditions, blocking TNF before it reaches its receptor effectively reduces inflammation. Monoclonal antibodies to TNF or constructs with extracellular domains of the TNF receptor are injected to achieve this. [2]

Interleukin-10 (IL-10), this cytokine synthesis inhibitory factor (CSIF) is another name for this cytokine that reduces inflammation. IL-10 is transmitted by a receptor complex made up of two IL-10 receptor-1 and two IL-10 receptor-2 proteins. which is encoded by the human IL-10 gene. As a result, the functional receptor is composed of IL-10 receptor molecules. Immune system cells are modulated by interleukins, therefore when function receptors are lost due to mutations, immune cell function is compromised. [3] Deficient immune cells are the result of loss-of-function receptor mutations because interleukins are cytokine receptors that influence immune system cells. TNF and interleukin-2 plasma membrane receptor activation Encourage sphingomyelin to generate lipid-soluble second messenger ceramide. [3]

In contrast to the traditional treatments of osteoarthritis, because stem cells can mend and repair tissues and organs, including those affected by TMJ osteoarthritis, they have the potential to completely transform medicine depending on the use of medications, proteins, or antibodies. [4] Mesenchymal stem cells (MSC) are found in many adult human tissues, including bone marrow, adipose, and synovial. Since they come from mesoderm, it has been shown that they can differentiate into bone, cartilage, muscle, and adipose tissue. [5] beside their capability to regulate the inflammatory responses. [6] Since bone marrow mesenchymal stem cells (BMMSCs), one of the adult tissue-derived MSCs, are presently experiencing several clinical applications, such as the knee joint and the articular cartilage repair in patients with osteoarthritis, they mimic the most employed stem cells. [7]

The preclinical research shows promise because BMMSCs have not yet been used clinically to treat TMJ issues. [8] Notably, recent investigation has demonstrated that (1) in vitro chondrogenic pre-induction of MSCs may considerably improve treatment outcomes, and (2) intra-articular injection of MSCs may slow the development of cartilage and subchondral bone lesions in rabbit TMJ-OA. Additionally, four weeks after transplantation, the cartilage, subchondral bone, and synovial membrane lining have shown that the transplanted MSCs support the cells' function in TMJ regeneration and repair. [9] These results have motivated the current investigation, which aims to evaluate the impact of BMMSCs on rat osteoarthritis repair.

The Osteoarthritis Cartilage Histopathology Assessment System (OCHAS), which was created by the Osteoarthritis Research Society International (OARSI), was used to evaluate the OA severity parameter in this investigation. By assessing the depth or severity along with the OA's horizontal (stage) and vertical (grade) progression through cartilage manifestations, the OARSI scoring system was created to evaluate the cartilage pathogenesis. The combined OA grade, which was 0–6 points, and the OA stage, which was 0–4 points, represented the combined evaluation of OA in terms of both severity and extent, ranging from 0–24 points. [10]

Osteoarthritis scoring system, presented by (OARSI), assessed the Osteoarthritis Cartilage Histopathology Assessment System (OOCHAS) using the following criteria, the histological features obtained from hematoxylin and eosin stain beside the findings recorded from toluidine blue stain.

I.Materials and Methods

II.1. Animals grouping and experimental design**:**

(a) Study setting: Thirty adult male Sprague-Dawley rats, ages 4-6 weeks, weighing an average of 160-180 grams, were used in this investigation, which was conducted at Cairo University's Faculty of Veterinary Medicine's animal house (Cairo, Egypt). The experimental animals were kept in normal cages with sawdust bedding in regulated circumstances, which included 20°C, 30–40% humidity, and 12 hours of light and 12 hours of darkness. Every experimental animal was fed a regular food and given unlimited access to water.

(b) Ethical consideration: the experiment was conducted in compliance with Cairo University's Faculty of Pharmacy's Ethical Research Committee Protocol. Following the protocols were developed in accordance with the

Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines were conducted.

(c) Sample size: based on Abou Elkhier et al, [11] sample size of current study's 80% power to detect a difference between means of 0.53 with a two-tailed significance level (alpha) of 0.05 at 95% confidence intervals is based on 10 rats per group. The P value in those experiments should be less than 0.05 (two-tailed) in order for the results to be considered statistically significant.

(d) Animal grouping: after a week of adjustment, thirty Sprague-Dawley rats were split into three major groups of ten each at random: Group I: negative control; rats with normal TMJ structure were fed and housed in the same conditions as the test group. Group II: positive control; rats were given complete Freund's adjuvant (CFA) intraarticularly into their right TMJ (Sigma Aldrich, St. Louis, Missouri, United States of America) to induce arthritis. Thirty days after an overdose of anesthetics (xylazine and ketamine) caused arthritis, they were sacrificed. Group III: experimental group; the rats were treated similarly to those in group II; 30 days after the trial began, $1 \times 10^{(6)}$ BMMSC cells were subsequently injected into the right TMJ while suspended in 0.1 ml of phosphate buffer saline (PBS). [12,13]

II.2. Osteoarthritis induction**:** prior to receiving a CFA injection, the animals were put into unconsciousness by injecting 10 mg/kg xylazine and 70 mg/kg ketamine solution intraperitoneally. [14] and the hair in the right TMJ region was cut out with a blade and hair cream. To improve the delineation of the area anatomical features, enable proper CFA injection, facilitate needle penetration, and prevent inflammation, trichotomy was carried out in the TMJ area. 50 µl of Complete Freund's Adjuvant (CFA) (Sigma-Aldrich, USA) was injected intraarticularly into a 1:1 dilution (oil/saline) using a 30-gauge needle attached to a 1-mL plastic syringe. [14] to cause arthritis in the right TMJ according to anterosuperior puncture technique into TMJ described by Zhou et al. [15] Needle was situated between the temporal fossa and condyle. Anterior superior puncture technique resulted in rat TMJ inflammation, severe swelling and chromodacryorrhea one day after CFA injection (Fig.1).

Fig.1: TMJ region showed sever swelling and chromodacryorrhea seen 1 day after CFA injection.

II.3.1: Biochemical investigations for TNF-α and IL-10: blood samples were withdrawn before, after induction of osteoarthritis and after stem cell treatment 12 hours after fasting, the blood samples were taken in EDTA-coated vials and centrifuged at 1500×g for 10 minutes at room temperature after being extracted from the retro-orbital plexus using a heparinized capillary tube that was placed into the eye's medial canthus. Cytokine estimation was performed on clear plasma that had been aliquoted. As directed by the supplier (R&D Systems, Minneapolis, Minnesota, USA), certain test using enzyme-linked immunosorbents (ELISA) [16] Tumor necrosis factor (TNF-α) and interleukin-10 (IL-10) were the pro-inflammatory cytokines found in serum and measured using kits. [17] On 30th day for both control and experimental groups of animals, the following procedures were performed:

(a) TNF-α ELISA Kit: Size: 96T, catalog number: MBS355371, 15.6 pg/ml–1000, pg/ml bodily fluids, tissue lysates, or cell culture super nates are the detection ranges. 7.8 pg/ml to 500 pg/ml (plasma, serum) It is less than 1 pg/ml. TNFα in rat blood serum was quantitatively detected after being stored at 8°C for six months.[18]

(b) Interleukin-10 (IL-10) ELISA Kit: Size: 48T/96T, catalog number: MBS764911 Rat's responsiveness Detection Range: pg/ml, 31.25–2000 Sensitivity: less than 18.75 pg/ml, Application: for the quantitative measurement of IL-10 in blood serum; six months of storage at 4°C. Anti-IL-10 sandwich enzyme-linked immune-sorbent assay technology served as the foundation for this kit.[16]

II.3.2: Statistical analysis**:** the data of biochemical measurement recorded by serum proinflammatory cytokines TNF-α and IL-10, as determined by osteoarthritis essays, were statistically examined. To compare the various groups, we utilized the one-way analysis of variance (ANOVA) test. The use of Tukey's post hoc analysis was employed when

the ANOVA test revealed a significant difference. The mean ± SEM was used to show all data, and statistical analysis measures were performed in a blind manner. A parametric analysis was made possible by the application of Bartlett's test, which revealed a Gaussian distribution for the data. A one- or two-way analysis of variance (ANOVA) and the Bonferroni post-hoc test were used to assess the results. Statistical significance was defined as P values below 0.05. GraphPad 6 software (San Diego, CA, USA) was used for all statistical tests and graph creation.

II.4. Preparation of bone marrow-derived MSC: The tibiae and femurs of six-week-old male Sprague-Dawley rats were flushed with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum to collect bone marrow. The nucleated cells were extracted using a density gradient (Ficoll-Paque, Pharmacia) and resuspended in complete culture media with 1% penicillin-streptomycin added. to produce large colonies, the cells were next incubated for 12–14 days at 37°C in 5% humidified CO_2 as the primary culture. [19]

Following the development of large colonies (80–90% confluence), the cultures were rinsed twice with PBS, and the adhering cells were trypsinized using 0.25% trypsin in 1 mM EDTA for five minutes at 37°C. After centrifuging, the cells were resuspended in serum-supplemented medium and given a Falcon 50-cm2 culture flask to incubate. The fusiform shape and adhesiveness of the MSCs in culture allowed for their identification, and these subsequent cultures were known as first-passage cultures. [19,20]

II.5. Microtechnical processing and tissue sections treatment: 30 days after BMSCs injection, the rats in all groups were anesthetized, sacrificed and TMJ was dissected out, prepared for microtechnical processioning and stained for histological and histochemical investigations. The experimental right side of the skull was exposed to 10% neutral buffered formalin for 72 hours after it was sagittal split in half. The right hemicrania was demineralized for three weeks using 14% ethylene diamine tetraacetic acid (EDTA) at pH 7.1. The specimens were sagittal sliced at a thickness of 5-7 µm parallel to the mandibular ramus's lateral surface after being immersed in blocs of paraffin. The tissue sections were treated as follow:

II.5.1. Histological stain: the tissue sections were stained using the following methods: (a) tolidine blue (TB) stain [21] to document cartilage pathogenesis; Additionally, the histological structure of the TMJ in the different animal experimental groups was evaluated using (b) hematoxylin and eosin (H & E) stain [21]. The final score was calculated by evaluating the combined OA grade and OA stage using the recommended OA score approach. The suggested score functions as an index for the combined grade and stage. Score = grade X stage is the straightforward formula.

The osteoarthritis scoring was determined using the following two parameters: (1) Grade, which functions similarly to a biological progression or severity indication of the osteoarthritic process, is defined as the depth at which OA progresses into cartilage. Regardless of its horizontal extent, the grade was qualitatively evaluated by recording the histological features in line with the Pritzker KPH et al, grade system [10]. (2) Regardless of the underlying grade, stage is the horizontal extent of cartilage involvement on one side of a joint compartment. The stage was evaluated using morphometric analysis in accordance with area criteria. (3) The evaluation of the combined OA grade and OA stage was used to determine the final score. As may be seen from the following formula, therefore, the score is a sum of the severity and extent assessments of OA: Grade X Stage = Score.

According to the highest grade and most comprehensive stage available, this approach produced an OA score between 0 and 24. Additionally, it assigned severity (grade) and extent (stage) equivalent ordinal number weights further biasing OA assessment in favor of the most advanced illness seen. Morphometric approaches were employed for staging based on the area or volume measurements. Two criteria were used to estimate the OARSI score includes the grade and stage scale suggested by Pritzker et al. (2006) [10].

II.5.2. Histochemical reaction : All animal groups' TMJ tissue sections were treated with (a) Periodic acid-Schiff (PAS): in order to document and compare the variations in the amount of glycoproteins in the cartilage matrices of the TMJ with those seen in the control group.[22] (b) AB-PAS (Alican blue-periodic acid Schiff): to document and compare the variations in the kinds of mucin in the cartilage matrices of the TMJ between the experimental groups and the control group. [22]

III. Results

III.1. TMJ in control rats (GI)

III.1.1 Histology of TMJ: (a) H&E stain: according to the OARSI standard histological grading system, which indicates the lack of OA symptoms, grade 0, The joint's constituent parts showed a normal and undamaged arrangement. From the articular surface inward, the mandibular condyle was made up of the usual structure, which

included the fibrous, proliferative, and cartilage layers as well as the subarticular bone, which contained bone marrow spaces with many trabeculae interlacing (Fig.2).

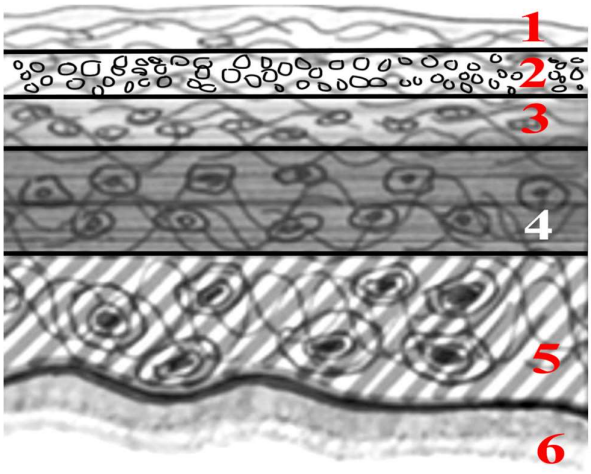
The opposing fibrocartilaginous layers covering the articulating surface of the glenoid fossa and the condylar head were gloved externally by fibrous tissue capsule. The inner fibrous capsule was lined by delicate synovial membrane, except the articular opposing bone surface composed of one or more ill-defined cellular layer widely spaced cuboidal or flattened cells supported by underlying connective subintima. (Fig.3). Numerous fibroblasts were dispersed over thick, parallel avascular layers of collagen fibers to produce the condylar head's covering of fibrous tissue. (Fig.4).

(b) TB stain: there was a prominent metachromatic stain in the matrix of the condylar fibrous, proliferative, and cartilaginous layers. There are three layers of chondrocytes and matrix: superficial, mid, and deep and the cartilage surface is smooth. Normal articular cartilage: histologic features, revealed intact, uninvolved cartilage (Fig.5). Grade 1 and stage 0: with OARSI score was (0) (Fig.16)

III.1.2-Histochemistry of TMJ

a- Periodic acid-Schiff (PAS) reaction: the TMJ of control rats revealed homogenous intense periodate reactive materials in most components of TMJ as evidenced by magenta reaction indicating neutral concentration of mucosubstances with gradual decreased activity toward the central part of condyle (Fig.6). Fibrocartilage layer of glenoid fossa and mandibular condylar head revealed intense periodate reactive materials in most components of TMJ as evidenced by the intense reaction of glycoproteins with gradual decreased activity toward the subarticular bone of condyle and temporal bone (Fig.6).

b- Alcian blue- Periodic acid Schiff reaction: the condylar cartilage demonstrated intense periodate reaction with violet color occurred in all component of TMJ structure alcianophilic reaction and in the matrix of the fibrocartilaginous layer, while the cartilage condylar layer and the condylar subarticular bone demonstrated alcianophilic reaction. The periodate reaction was seen in all layers of the mandibular condyle while the condylar subarticular bone showed intense periodate reaction with violet color (Fig.7). The temporal bone, articular disc and most of condylar subarticular bone marrow cells showed intense reaction.



1- Fibrous articular layer.

2-Proliferative layer cell rich zone.

3-Transitional zone.

4-Fibrocartilage layer.

5-calcified cartilage layer.

6-subcortical bone of condylar head.

Fig (2): Schematic drawing for the histological structure of condylar head in control rat.

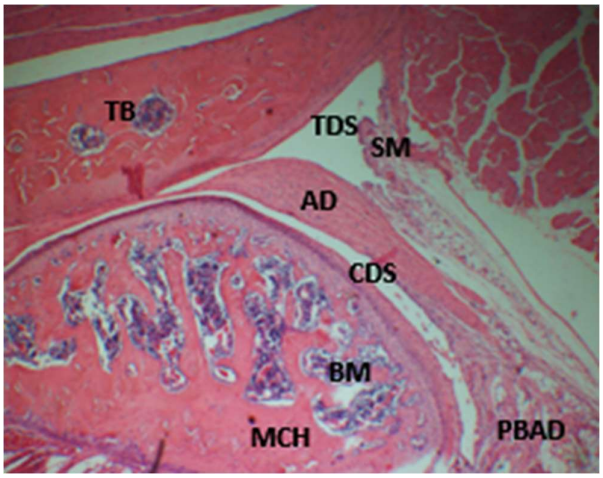


Fig (3): control group (G1): the positional relationships between the TMJ's histological structural elements. Articular disc (**AD**), condylodiscal space (**CDS**), temprodiscal space (**TDS**) mandibular condylar head (**MCH**), bone marrow spaces (**BM**) temporal bone (**TB**), synovial membrane (**SM**), and posterior band of articular disc (**PBAD**). (H& E stain, orig. mag, X40)

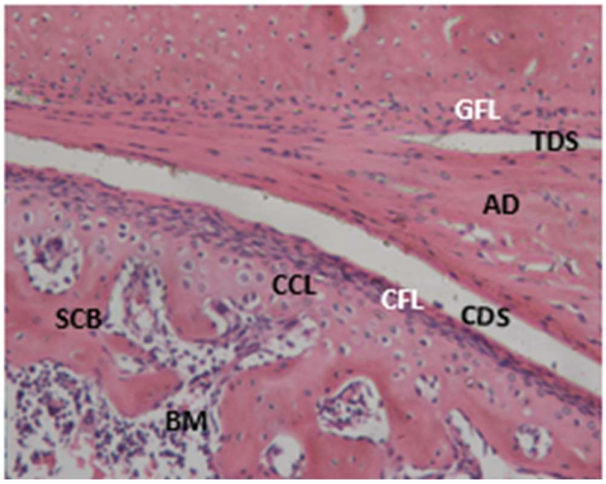


Fig (4): control group (GI): structural elements of the condyle's many levels, include the condylar fibrous layer (**CFL**) and condylar cartilaginous layer (**CCL**). Glenoid fibrous layer (**GFL**), articular disc (**AD**), condylodiscal space (**CDS**), temprodiscal space (**TDS**). Below the

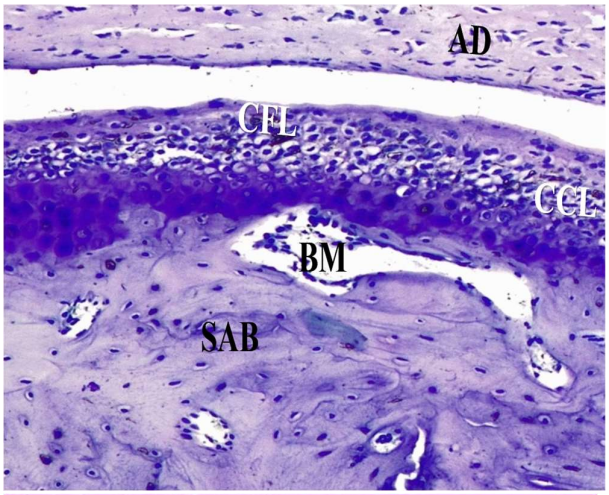


Fig (5): control group (GI): In particular, the cartilage matrix and the rims surrounding chondrocytes show strong staining of the condylar fibrocartilage layer (**CFL**) and condylar cartilage layer (**CCL**). intense response in the condylar cartilage matrix's hypertrophic layer. Condylar subarticular bone (**SAB**). (TB, stain, orig, Mag. X 100)

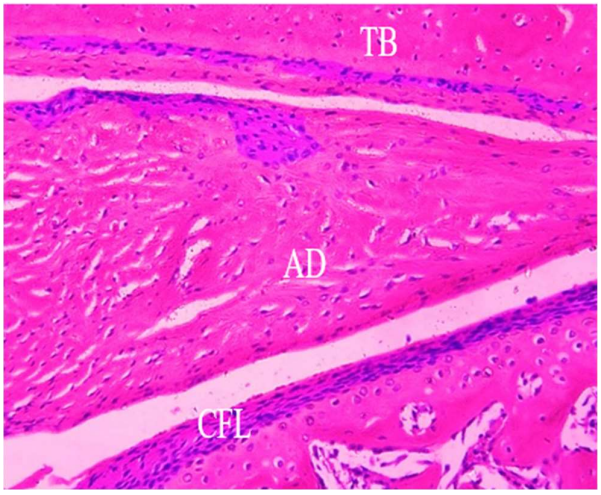


Fig (6): control group (GI): intense periodate magenta reaction throughout the matrix of condylar fibrocartilage layer (**CFL**), temporal bone (**TB**) and articular disc (**AD**) having a stronger response in the perilacunar rim surrounding the chondrocytes. (PAS reaction, orig. Mag. X 400)

III.2. TMJ after CFA (GII)

III.2.1 histology of TMJ:

(a) H&E stain as OA became worse, the involvement of cartilage getting deeper. In the end, the subjacent bone seemed exposed to the articular surface, and the cartilage began to partially erode. Regional chondrocyte loss, peripheral chondrocyte growth and clustering, and subchondral bone resorption were all observed in the condyle, with nearby bone marrow containing fibroblast-like cells. (Fig.8).

OA is typically represented by the area of resorption, which is made up of fluid that is primarily composed of proteoglycan. As OA worsened and cartilage got more deeply implicated, fibrocartilage was also seen in the affected area. In the end, the subjacent bone mimicked the articular surface due to incomplete cartilage erosion.

The condylar cartilage layer thinned, the articular disc thickened, the bone marrow gaps widened, the bone trabeculae were disordered, the articular disc defasculated, and the deformation cell enlarged layer of the condylar cartilage was worsened in TMJs (Fig8)

Histopathological alterations seen in rats given the CFA injection group (II) demonstrated clear regional variations and validated the existence of inflammation in the synovium. The outer layer of the condylar portion of the posterior attachment and the posterior bands of the disc were more prominently labeled than the intermediate zone. Particularly, there is fatty degeneration at the posterior junctions where the band and articular capsule attachment meet (Fig 8).

The subcortical bone appears denser than the surrounding bone, and the condylar head displays a mineralized cartilage layer with a fibrocartilaginous layer on the bone surface. Additionally, the articular bone plate is thicker beneath the surrounding cartilage as well as in the exposed portion. This bone plate is typically less mineralized than deeper trabecular bone and has a higher metabolic activity, while having a thicker bone/marrow ratio. Articular disc thickening, tearing and defasciculation, uneven articular cartilage, disorganized trabecular bone, and nearly absent chondrocytes in the disc and glenoid fossa cartilage (Fig 8).

In addition to appearing badly deteriorated and unclearly stratified in the hypertrophic zone, the TMJ condyle subchondral bone of CFA group (II) also had aberrant calcification and uneven cell distribution. These findings imply that the control group and the CFA-induced arthritis group underwent notably different histological alterations. localized chondrocyte loss and subchondral bone resorption with neighboring bone marrow in relation to the condyle. (Figure 8).

Adhesion between the articular disc and condylar cartilage, thickening of the articular disc, thinning of the condylar cartilage, uneven, disorganized bone trabecular, and the lack of marrow gaps were all more severe in the CFA TMJs (Fig 8). Degradation of cartilage is the main incremental feature of OA. A different mechanism that usually takes place at When highly fractured cartilage matrix domains are mechanically dislodged, matrix fibrils are released into the synovial fluid, which facilitates excavation at the fusion area between the articular capsule and disc in the posterior band.

Articular disc enlargement, articular cartilage defasculation, and irregular, disordered trabecular bone were all observed in the CFA-induced OA. Chondrocytes were nearly gone from the temporal fossa and disc cartilage. (Fig. 8) and revealed a thickened and hyperplastic synovial membrane along with indications of inflammation affecting the synovial membrane's intima and subintima. The articular upper chamber was loaded with inflammatory fluids and mononuclear inflammatory cells. Numerous mononuclear cells penetrated the synovial membrane, and the synovial cells seemed to be hyperplasic. The inflamed synovium also contained large lipid droplets (Fig 8). In the control group, no obvious inflammation was observed interestingly, in the posterior band, of CFA induced arthritis in rat TMJ.

Grade -4 The primary incremental characteristic of grade 4 OA is cartilage degradation. Two distinct processes are discernible: (a) Delamination is the loss of a superficial zone caused by the shear stresses of osteoarthritis resulting from CFA. When additional growth dislodges the piece, delamination takes place. Although the surface of the cartilage is relatively smooth, delamination is recognized because the surrounding tissues display the structure and role of mid-zone cartilage arrangement.

(b) Excavation represents cavity creation in a confined cartilage volume associated with matrix loss. Usually near the boundaries of erosion, physical displacement of deep cartilage matrix regions resulting in the release of matrix fibrils into the synovial fluid, chondrocyte regeneration, matrix breakdown, loss of matrix stain, and process contributes to excavation. Numerous parameters, including the pre-existing cartilage histological structure, the rate at which OA advances in cartilage, and the homogeneity of the reaction, are likely to influence the mechanisms of excavation and the size of the released matrix pieces.

These fibers are produced by either existing chondrocytes that have undergone metaplasia to become fibro chondrocytes or by chondrocytes that have relocated from the reparative cartilage present in the repair tissue within the articular layers. The mid zone, which is brought on by persistent oedema and ultimately leads to matrix loss, is another process that may be present in grade 4 OA, particularly in thick cartilage. If this process persists, it may result in delamination of superficial and some upper zones. Another process that leads to matrix loss is the loss of fibrillar matrix in a perichondral area without collagen condensation or further repair.

(b) TB stain demonstrated that all of the mandibular condyle's layers lacked metachromatic stain, with the exception of a few, weakly stained cartilage islands that were widely apart. excavation, delamination, and loss of matrix domains. There is some erosion of the unmineralized cartilage. Mineralized bone or cartilage makes up the articular surface. Reparative fibrocartilage may fill up surface voids caused by the bone plate (Fig 9). Grade (4) and stage (4). The OARSI score was (16) that is the highest score among the all studies groups (Fig 16).

III.2.2 Histochemistry of TMJ

a- Periodic acid Schiff (PAS) reaction: the articular disc attachment especially had the moderate periodate magenta reactive intense reaction and temporal bone in the glenoid fibrous layer and a mildly stained matrix are visible in the condylar cartilaginous layer. Also, showed weak reaction with faintly periodate reaction at condylar subarticular bone of the mandibular condylar head. The glenoid fibrous layer appeared intense reaction of magenta color. (Fig 10).

b. The Alcian blue and Periodic acid Schiff (AB-PAS) reaction: in the experimental group (CFA) (GII) displayed a decreased level of cartilage proteoglycans, showing a pronounced violet coloration in both the condylar fibrous layer and the condylar cartilaginous layer of the mandibular condyle, which suggested a diminished acidic response. While the reaction showed faint moderate and lighter reaction in area of subarticular bone of condyle and bone marrow

spaces was not showed reactive. The periodate reaction appeared moderate in both deep layers of condylar head and the condylar subarticular bone (Fig 11).

The mandibular condylar cartilage showed a moderate alcianophilic response observed throughout the matrix of the condylar cartilage layer and the condylar fibrous layer. The condylar subarticular bone demonstrated faint reaction with widely spaced islands that showed weak alcianophilic reaction at calcified and subcortical bone layer (Fig 11).

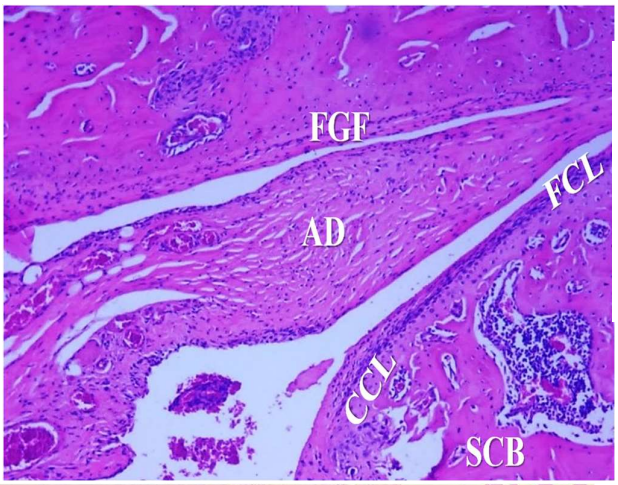


Fig (7): control group (GI): periodate reactive seen matching and homogenous in different anatomical components of temporomandibular joint. Intense periodate reaction with violet color occurs in fibrous condylar layer (**FCL**) and condylar cartilage layer (**CCL**), and fibrous layer of glenoid fossa (**FGF**), articular disc (**AD**) and subcortical condylar bone (**SCB**). (AB - PAS reaction, orig. mag. X200).

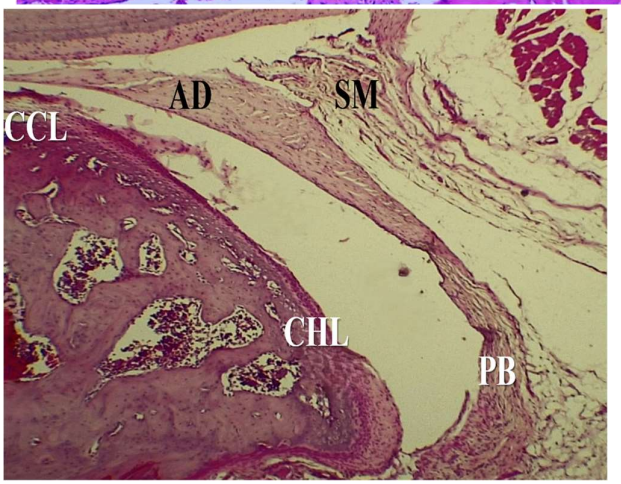


Fig (8): CFA rats of (GII): deformation of cell hyper trophic layer (**CHL**) involved the loss of a superficial zone fragment related to osteoarthritic features. Further extension dislodges the fragment resulting in defasculated and friable articular disc (**AD**) and delamination shoed in condylar cartilage layer (**CCL**) involved the subjacent tissues have the appearance of disorganization cartilage in mandibular condylar head. Fatty degeneration (**FD**) at posterior band of articular capsule. Synovitis with dilated blood vessel in synovial

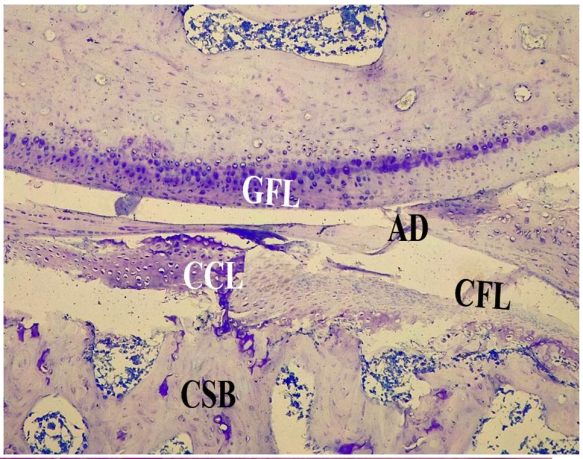


Fig (9): TMJ CFA rats of (GII): there are different reactions in the staining was present appeared intense in both glenoid fibrous layer (**GFL**) and condylar cartilage layer (**CCL**) while the condylar fibrous layer (**CFL**) demonstrated moderate staining with atrophic matrix and where **articular** disc (**AD**) was very

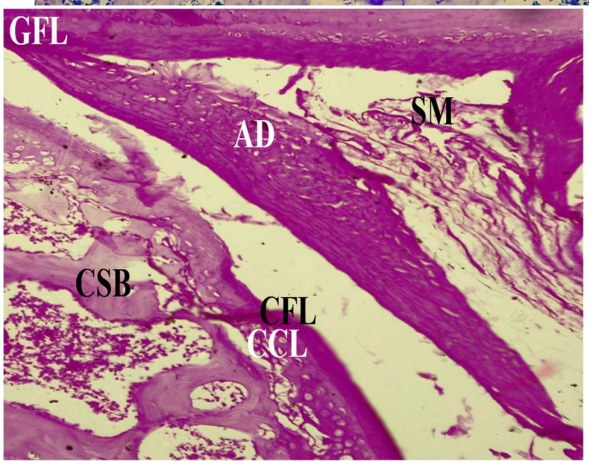


Fig (10): CFA rats of (GII): the articular disc (**AD**) had moderate periodate magenta reaction in the condylar fibrous layer **(CFL)** and cartilaginous layer (**CCL**) appears with moderately stained matrix and temporal bone in glenoid fibrous layer (**GFL**) and weak reaction of magenta stained color at condylar subarticular bone of the mandibular condylar head (**CSB**). (PAS reaction, orig, mag. X 100).

III.3.1 TMJ arthritis after stem cell (GIII): -
Histology of TMJ

(a) H&E stain the microscopic examination of TMJ of rats treated with stem cell revealed suppression of inflammatory responses, despite mild dilated blood vessels and synovial membrane cell hyperplasia. When compared to the CFA group, the histopathological characteristics considerably diminished following stem cell treatment.

Bony healing was indicated by histological evaluation of the H&E-stained samples in the stem cell group (III), which revealed regions of fibrous tissue, fibrocartilaginous tissues, fine trabecular bone, and bony ossifications. All the temporomandibular joint's components showed a relative enhancement in the fibrous covering layer, the cartilaginous region of the mandibular condyle, and the articular disc, which thinned without fraying or defasculating fibrous tissue and resembled the control group (I). (Fig 12)

A fully calcified formation of the subchondral bone was observable in the four layers of condylar cartilage that encase the condylar head and consist of multiple fibroblasts dispersed through densely paralleled avascular layers of collagen fibers. The condyle is composed of a thick layer of fibrous cartilage with multiple layers of regularly aligned chondrocytes. Additionally, a layer of fibrous cartilage coated the condyle surfaces of the articulating cortical bones. (Fig 12).

The articular disc and condylar cartilage thicknesses of the stem cell group (III) were nearly normal. The characteristic zones in condylar cartilage and the normal arrangement of bone trabeculae were identified. The TMJ displayed almost normal articular disc and condylar cartilage thickness, a minor enlargement of the marrow gaps, The mandibular condyle showed a near-normal arrangement of bone trabeculae, condensed subarticular bone filled with numerous bone marrow spaces throughout the condyle, and nearly normal TMJ characteristics. The dislodgement and cartilage erosion that were present in the condylar head's body also vanished (Fig.12).

The stem cell group (III)'s sagittal perspective seemed most likely because the TMJ of the control group (I), which consisted of normal, untreated rats, was separated into the TMJ condyle and the fibrocartilaginous disc. The disc,

which is composed of fibrous connective tissue, appeared biconcave, was thinner in the middle, and grew closer to the mandibular condylar head's typical fibrous layer as the chondylodiscal and temprodiscal spaces widened. A noticeable weakening of the mandibular condyle's fibrous layer occurred. Changes in the fibrocartilaginous layer of the condyle were seen, together with uneven condylar cartilage thickness and noticeable chondrocyte proliferation (Fig 12).

Grade (2): discontinuity in the surface and focal fibrillation penetrates the superficial zone and reaches the mid-zone section. In contrast to the CFA group (II), this could be associated with cell proliferation, enhanced matrix staining, and/or cell mortality in the central region, as well as the disappearance of cartilaginous layer erosion and delamination.

(b) TB stain finding revealed a strong metachromatic staining observed in the cartilaginous layer of the condyle matrix, with occasional or absent staining in the superficial zone (Fig 13). Groups of cells multiplying and a hypotrophic reaction that extends past superficial fibrillation. Enhanced surface discontinuity and fibrillation in the superficial zone rather than surface wear were observed in grade 2 and stage 2, along with decreased matrix loss within the superficial zone discontinuity. Depletion of cationic stain matrix (Toluidine Blue) in the middle third of the cartilage. The OARSI score of (4), which was lower than the CFA G II score, concurred with these findings. (Fig 16).

III.3.2 Histochemistry of TMJ

a- Periodic acid-Schiff (PAS) reaction: the TMJ of rats treated with stem cells revealed that heavy intense amount of periodate reactive materials in the component of TMJ as evidenced by the appearance of magenta reaction indicating concentration of glycoprotein as evidenced by magenta coloration in articular disc, interference with fibrocartilaginous layer of roof of glenoid fossa and condylar head at central part. We showed decreased activity toward the subarticular bone of condyle. (Fig 14).

b- Alcian blue and PAS reaction: the microscopic examination of TMJ of rats treated with stem cell revealed that well moderate violet reactive materials in the most component of TMJ closely to normal evidenced by the appearance of moderate violet color reaction with weak acidity in all structure area of TMJ section (Fig 15).

It showed well intensely violet color reaction at the fibrocartilaginous and condylar cartilage layers of mandibular condyle. However, the reaction was weak in the condyle's subarticular bone. (Fig 15) that was observed in the CFA group (II) as opposed to the stem cell group (III).

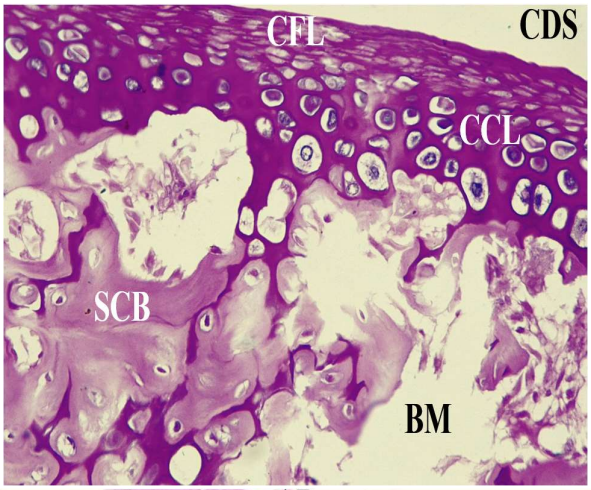


Fig (11): CFA rats of (GII): the reaction appeared well intensely violet color reaction at the condylar fibrous layer (**CFL**) and condylar cartilaginous layer (**CCL**) of mandibular condyle and blue or purple nuclei of chondrocytes which indicated high acidity reaction of tissue. The reaction became faint moderate and lighter reaction at area of subarticular bone of condyle (**CSB**) and bone marrow (**BM**) in deep layer of mandibular condylar head. (AB - PAS rection, orig, mag. X 400)

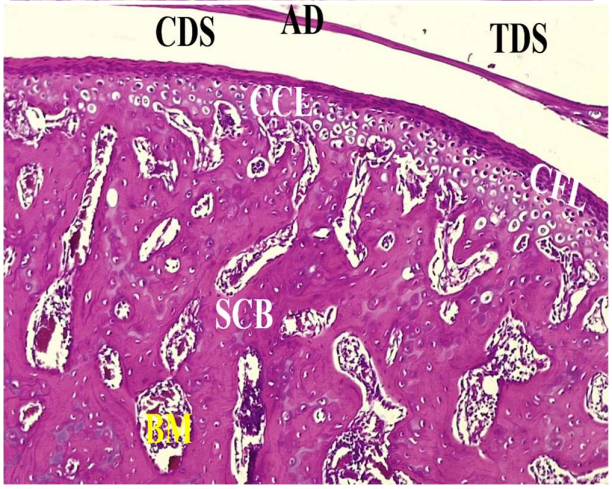


Fig (12): stem cell treated rats of (GIII): this section seemed most likely because the mandibular condylar head's normal fibrous cartilage layer (**CFL**) and condylar cartilage layer (**CCL**) were separated in the control group's (I) TMJ normal, untreated rats. The four condylar cartilaginous zones were clearly visible, displaying the full anatomy of the subchondral bone (**CSB**). Articulate cortical bone of condyle with proper gaps between bone marrow (**BM**). (H & E stain, orig, mag. X 100)

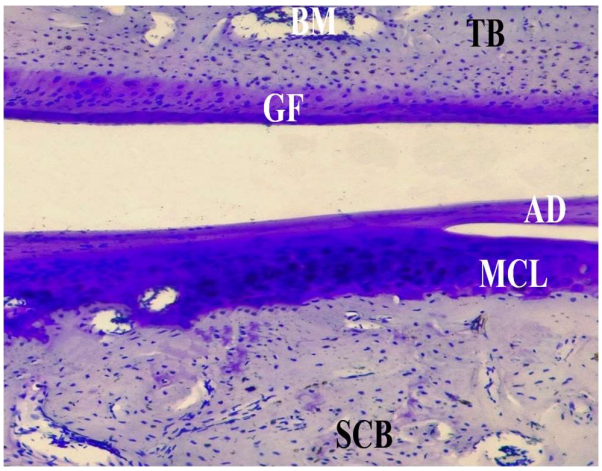


Fig (13): stem cell treated rats of (GIII): intense stain at fusion area of articular disc (**AD**), fibrocartilgenous layer of roof of glenoid fossa (**GF**), and mandibular condyle cartilaginous layer (M**CL**) with heavy metachromatic stain in the condylar cartilage. Articular disc (**AD**) showed moderate metachromatic stain in its matrix. While the reaction of stain showed faint moderate and lighter stained in area of subarticular bone of condyle (**CSB**) and temporal bone (**TB**). (TB stain, orig, mag. X 100)

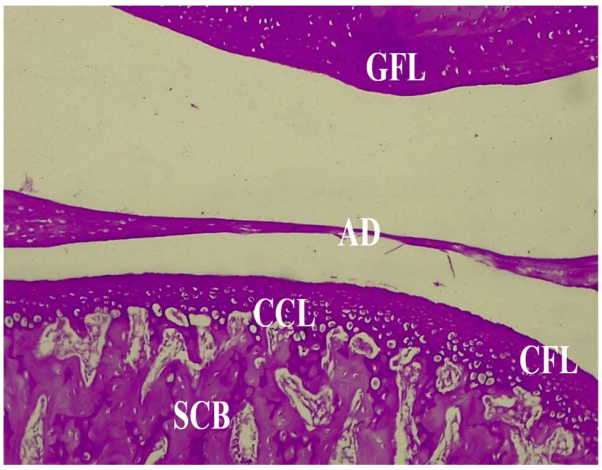


Fig (14): stem cell treated rats of (GIII): heavy intense amount of periodate reactive materials in most component of TMJ evidenced by the appearance of deeply magenta reaction indicating intensely concentration of mucosubstances as evidenced by heavy coloration in articular disc (**AD**), fibrocartilaginous layer of roof of glenoid fossa (**GFL**), fibrocartilaginous layer (**CFL**) and condylar cartilaginous layer (**CCL**) of condylar head. Gradual lighter than and decreased activity toward the sub articular bone (**CSB**) of condyle. (PAS reaction, orig, mag. X 100)

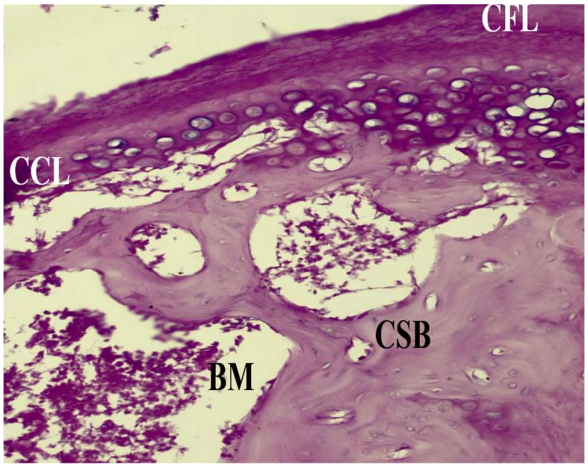


Fig (15): stem cell treated rats of (GIII): section displayed a modest acidity reaction of mucosubstances at the mandibular condyle's condylar cartilaginous layer (**CCL**) and condylar fibrous layer (**CFL**), resulting in a violet tint. However, the subarticular bone of the condyle (**CSB**) displayed a weaker and fainter reactivity. and bone marrow (**BM**) spaces. (Ab - PAS reaction, orig, mag. X 200)

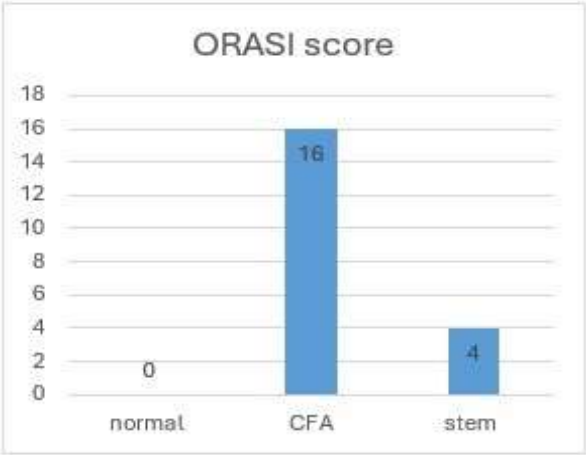


Fig (16): diagram showing differences in osteoarthritic scores between the control and experimental groups using the OARSI scoring system (Pritzker KP et al)

The mean value between the control group, CFA group and stem cell group relative to the first mediator (TNF-α) and second mediator (Interleukin-10).

Table (1): mean and standard deviation (SD) values of TNF- α and interleukin-10 level in the different animal groups.

Variables	TNF-Alpha level		Interleukin-10 level	
	Mean	**SD**	**Mean**	**SD**
Group I (Control)	90.77	0.60	185.36	2.18
Group II (CFA)	117.86	1.84	151.60	1.04
Group III (stem cell)	97.77	1.62	177.43	1.68
p-value	**< 0.001***		**< 0.001***	

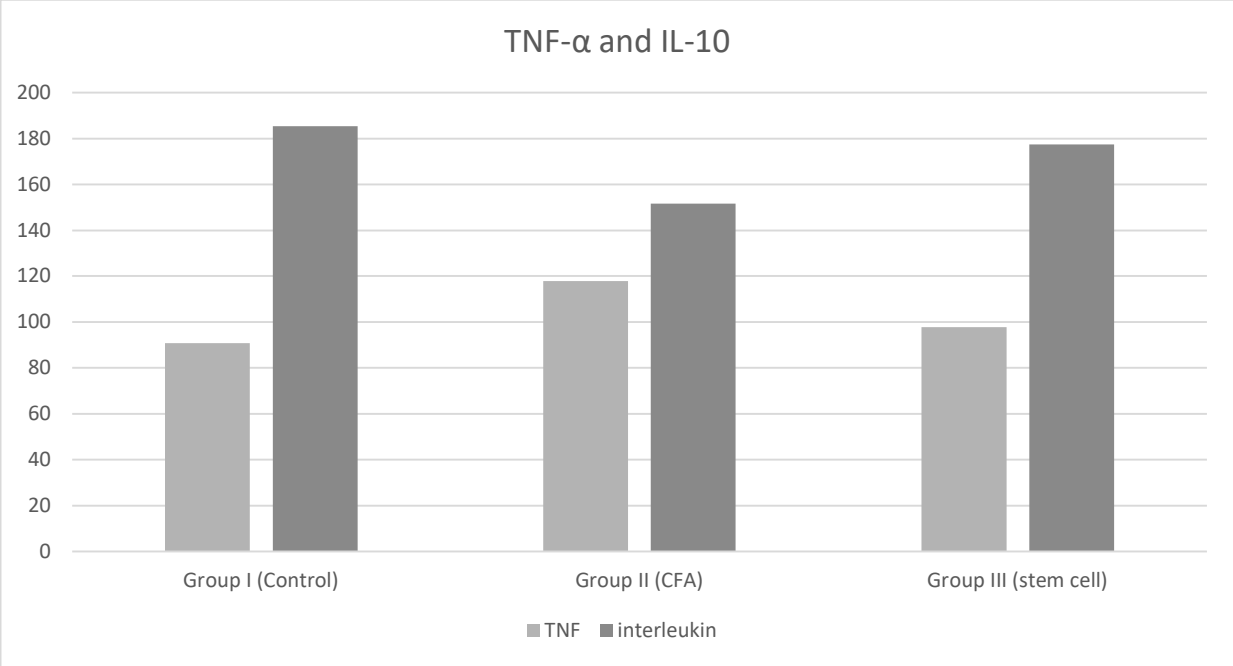


Diagram (1): bar chart reflecting the average levels of interleukin 10 and TNF-α in the various animal groups.

Discussion

Following morphometric approaches, the scores of the samples in the control and experimental groups obtained from stage X grade were compatible with the OARSI system score, which simulates the most basic The OARSI system is applied and assessed using either grade (with six levels) or stage (with four levels). When evaluating the OA stage morphometrically, a cartilage A more thorough evaluation of cartilage histology is made possible by the OA score. [10]

Rat TMJ arthritis caused by intraarticular injection of CFA has demonstrated that injecting CFA into the rat TMJ's superior joint space causes a significant inflammatory reaction that may persist for a duration of up to four weeks. [23, 24] However, due to the overwhelming extent of this inflammation, it was challenging to discern a therapeutic benefit of treatment with drugs. It would be more suitable to use an animal model that can be modified with respect to the inflammatory process to examine the effectiveness of the different treatment options, including the pharmaceutical drugs. Regarding the extent of inflammation generated in their models, other research employing different animal models remains silent. [25,26] Therefore, Whether the rat TMJ could induce submaximal levels of inflammation is the topic this study attempts to answer. It was shown in this work that the OARSI system and biochemical analysis could be used to create the CFA-triggered inflammatory reaction in the rat's temporomandibular joint.

According to another study, the rat's Harderian gland's overproduction of porphyrins was a sign of chromodacryorrhea. [27] It has been injected into the inflammation of the TMJ as a major determinant of OA; nevertheless, within an hour, discomfort, edema, and the detailed degeneration process inside the intra-articular space of the TMJ are created. Following that, bone erosion may continue for a few weeks due to the destruction of articular cartilage. [28] Furthermore, it has been proposed that chromodacryorrhea may be a sign of a chronic underlying illness. [27] The CFA-induced inflammatory response had clinically produced an enlargement of the temporomandibular joint and the adjacent soft tissues, which was consistent with the current investigation. Rats given the 50µg dose of CFA showed a marked increase in edema in comparison to the control group.

Numerous clinical disorders, including osteoarthritis, rheumatoid arthritis, and inflammatory illness, have been linked to the cytokines IL-10 and TNF-α as important inflammatory mediators [29] These findings are consistent with the current study, which measured arthritic inflammation by biochemically examining TNF-α and IL-10. They also indicate that stress linked to CFA-induced TMJ inflammation can also result in edema and the presence of colored tears, which were both caused by CFA.

Regarding the blood cytokine levels of the proinflammatory mediators TNF-α and IL-10, we observed that the CFA group (II) had higher levels of TNF-α and lower or suppressed levels of IL-10 compared to the group under supervision. These results, which were characterized by the generation of inflammatory mediators including interleukin (IL-10) and tumor necrosis factor alpha (TNF-α), which resulted in joint injury from CFA, are in line with other research that mainly concentrates on joints. Additionally, they showed the recruitment of inflammatory cells, synovial hyperplasia and the progressive degradation of cartilage and bone processes such as mononuclear cell infiltration. [30]

In this study, we examined the effect of stem cells on adjuvant-induced TNF-α and IL-10 inflammatory parameters in experimental animals. In CFA (GII), we discovered that all samples had reduced levels of IL-10 but higher amounts of the proinflammatory cytokines TNF-α. This is in line with recent research that discovered that synovial fluids had greater concentrations of the proinflammatory cytokines TNF-a, IL-1, IL-6, IL-17, and oncostatin M (OSM), which enhanced the release of collagen type II from cartilage through matrix metalloproteinase (MMP). [31]

According to previous studies, the favorable inflammatory response in CFA-induced arthritis likewise mimicked adaptive changes in TMJ tissue. [32] In addition, it has been demonstrated that the rat TMJ disc exhibited degenerative alterations for 30 days following CFA injection, including thickness, distortion, and increased articular disc tearing, in addition to the synovium's abundant adipose tissue and mononucleated cell infiltration. Further investigation showed a hypertrophic response in the TMJ and phenotypic changes in the condylar cartilage. [33] These results are consistent with the CFA-induced arthritis data from our investigation. Day 30 showed the subchondral bone to be extensively deteriorated, sclerotic, and unclearly stratified, with aberrant calcification and uneven cell distribution. Notably, even the alterations in condylar fibrocartilage and condylar cartilage layer deformation with incomplete erosion, thickening of the articular disc, widening of bone marrow spaces, disordered bone trabeculae, and aggravated changes with defasculated articular disc and deformation cell hypertrophic layer of condylar cartilage can be seen in the mandibular condyle.

The effect of subchondral bone on TMJ arthritis has been the subject of an increasing number of TMJ research recently. It has been suggested that the development or progression of TMJ arthritis may be influenced by the greater turnover of subchondral bone. [32–34] These findings might help to explain why, in contrast to the control group, the TMJ remained visible but had articular disc defasculation, severe TMJ degeneration or deformation, and histopathological changes in every condylar cartilage layer of the mandibular head. These features are in good agreement with the findings of our investigation.

The current study on CFA-induced arthritis showed that the synovial membrane plays a significant role in joint inflammation and destruction, as evidenced by changes in synovial histology, including thickening of the synovial lining due to inflammatory cell infiltration, proliferation, and apoptosis of synoviocytes and lymphoid follicles. We observed fatty deterioration and macrophage infiltration in the articular capsule's posterior band. These characteristics differed from those of other earlier studies that discovered alterations in the adhesion layer of the synovial membrane along with many modifications to the histologic systems used to grade synovial inflammation in TMJs. [35]

Significant morphological changes have emerged because of the intra-articular injection of CFA in the right TMJ of the CFA group in this study. These alterations incorporate the breakdown of bone in the mandibular fossa, infiltration of mononuclear inflammatory cells, hyperplasia of the synovial membrane, and an excessive thickness of the articular disc. Similar outcomes were noted in earlier research, proving the effectiveness of the suggested induced arthritis model. [36]

When BMMSCs are directly injected intraarticularly into the TMJ, they have been found to decrease the proinflammatory mediator interleukin (IL)-10 and elevate TNF-alpha. This contrasts with the effect of stem cell therapy on rat TMJ arthritis caused by CFA intraarticular injection. This is consistent with other research by GONZALEZ [37] that demonstrated that MSCs can, in accordance with MURPHY et al, directly decrease immune cell functions by releasing interleukin (IL)-10 and transforming growth factor beta (TGF-b), or indirectly by triggering regulatory T (Treg) cells both in vitro and in vivo. [38] Researchers discovered that the intra-articular injection of BMMSCs stops the rising destructive effect of arthritis and that the stem cell-treated group displayed less apoptotic chondrocytes than the CFA arthritic group.

The results of the intraarticular injection of CFA arthritis into the TMJ rat in the stem cell-treated group (III) 30 days after BMMSCs were very similar to those of the control group (I) and better than those of the CFA group (II). This is in line with earlier research that found chondrocytes frequently display a hypertrophic phenotype. [39]

The grade of cartilage defect, the quantity of inflammatory cells, and the number of cartilaginous cell layers have all improved after 30 days of BMMSC injection into a CFA-induced arthritic joint. Our results suggest that BMMSCs injected intraarticularly into the TMJ may help treat osteoarthritis in rats whose arthritis was caused by CFA injection. In certain animals, the fibrous layer of the condyle might offset the thinning of the condylar cartilage layer, thereby helping to lessen the stress on the subarticular bone of the condyle and the articular disc may occasionally narrow noticeably before returning to normal without friability. The condylar subarticular bone, which had a considerable reduction in sclerosis, may also reflect this compensatory response. When combined, administered BMMSCs improved and provided some relief from the TMJOA progression indicated by the OARSI score, but they did not return the joint to its natural state.

Our research showed that the BMMSC-treated rats showed reduced defasculation, articular disc fraying, and cartilage layer degradation of the mandibular condyle than the CFA group. These findings for the BMMSCs treated group in this study are consistent with those of other KEHOE et al. studies. [40]

The current study showed that all the TMJ's component structures improved after receiving CFA injections following stem cell treatment. This could potentially strengthen the impact of BMMSCs on chondrogenic differentiation and anchoring stem cells for TMJ treatment. This clarifies and supports the results of prior research. In addition to the inflammatory mediators present in the arthritic environment and in human arthritic patients, BMMSCs have demonstrated the ability to develop into osteoblasts, chondroblasts, adipocytes, and chondrocytes (CENTENO et al.). [41,42]

Conclusion

The rat may be the best animal model for demonstrating osteoarthritis (OA) if an intra-articular injection of CFA causes osteoarthritis in the temporomandibular joint. The fact that the osteoarthritic group displayed more degenerative than inflammatory characteristics is consistent with the idea that OA is a degenerative disease.

In a rat with TMJ arthritis, the intraarticular injection of CFA by BMMSCs improved and relieved several osteoarthritic symptoms and changed the arrangement of some connected elements. A compensatory mechanism could be seen in this kind of modulation. to deal with the ongoing symptoms of osteoarthritis.

The BMMSCs may successfully recover and treat many of the arthritic features induced in rats by CFA and may revert and improve the TMJs into a nearly normal condition.

The TMJ osteoarthritis and degenerative changes of the TMJ linked to osteoarthritis, the BMSCs can reach the TMJ and improve the proliferative and healing capability of the structural components of the TMJ.

Additionally, to move toward preclinical and clinical research like that for the treatment of osteoarthritis in the TMJ, ongoing studies are needed to determine the target efficiency and biocompatibility in the therapeutic stem cell intraarticular injection of CFA.

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