Original Article

Does Stem Cells Approach Ameliorate the Prospective Alterations of Mandibular Joint Histology and Acetylcholinesterase Expression in Experimentally Induced Alzheimer's Disease?

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ABSTRACT

Background: Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive degenerative brain disorder. This work aimed to illuminate the possible effects of Alzheimer's disease and subsequent bone marrow mesenchymal stem cell therapy (BM-MSCs) on rat temporomandibular joint (TMJ).

Methods: Thirty adult male albino rats were divided into 3 groups (ten rats each) and received a daily intraperitoneal injection of 0.9% saline (group I), 70mg/kg b.w. AlCl3.6H2O dissolved in distilled H2O (groups II and III). However, group III received a single dose of BM-MSCs after 6 weeks of daily AlCl3.6H2O. After another 6 weeks, dissected TMJ specimens were prepared for histopathological and VEGF immunohistochemical examination, AChE western blotting and detection of collagen gene expression (types I&II) by real-time PCR.

Results: Comparing to group I, H&E sections of group II presented retarded endochondral ossification, osteoarthritic and osteoporotic changes of TMJ components ascertained by significant VEGF overexpression. All were significantly alleviated in group III with the still significant increase of VEGF positivity compared to group I. The significantly upregulated AChE and reduced collagens I&II gene expression in group II compared to group I, were restored in group III with an increase compared to group I that was significant for collagen I gene and insignificant for both AChE plus collagen II gene expression.

Conclusions: The alleviated findings subsequent to stem cell therapy might exceed the normal in some respects like enhanced ossification. MSCs can most probably treat neural and aging-related diseases with the ensuing peripheral changes. Yet, we recommend conducting further studies for a therapeutic approach that has the potential to mediate self-renewal and differentiation of host stem cells to overcome all possible adverse effects of exogenous grafting.

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Key Words: Acetylcholinesterase; alzheimer's disease; BM-MSCs; osteoarthritis; temporomandibular joint.

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INTRODUCTION

Alzheimer's disease (AD), a common degenerative brain disorder, gives rises to an irreversible decline in both memory and cognitive functions. Two major varieties of this disease are known; familial AD which occurs in young population less than 65 years old with a current average of 2.3% and the other sporadic AD form which affects persons aged 65years or older with an average of 18.5% in the Middle East. The expectation of the World Health Organization of a possible increase by 125% in 2050 in the Middle East has been documented[1,2]. AD often causes peripheral complications such as immobility and trouble swallowing thus can lead to malnutrition, increased risk of pneumonia and death,[1] along with the main neuropathological changes such as β-amyloid plaque accumulation, neurofibrillary tangle formation and neuronal loss[3].

Osteoarthritis (OA), the chronic joint disease, can arise secondary to genetic defects, metabolic disorders, inflammation and trauma[4]. Likewise, osteoporosis is a chronic multifactorial metabolic bone remodeling disease characterized by bone fragility with fracture tendency. It occurs either idiopathic or secondary to medications and some diseases such as rheumatoid arthritis[5]. A positive relation has been found between both systemic osteoporosis as well as jawbones pathology. Furthermore, Osteoporosis could be one of the earliest indicators for AD in men and women. AD and osteoporosis shared common risk factors involving gender, old age, low calcium and vitamin D levels and hyperparathyroidism besides the ApoE genotype[6,7,8]. Thus, osteoporosis could be treated with anti-AD drugs, anti-catabolic anti-osteoporosis drugs such as sex steroids, calcium and vitamin D supplements[5,9]. However, these approaches have not reversed osteoporosis in addition to the serious adverse effects as hypercalcemia, osteonecrosis of jaws and cancer that could result from the long term use of these therapies[5,10]. For OA, only a symptomatic therapy has been approved like anti-cycloxygenase-2 and non-steroidal anti-inflammatory drugs[11] as well as anti-AD drugs like Memantine.

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The cholinergic system is composed of acetylcholinesterase (AChE), acetylcholine (ACh), choline acetyltransferase (ChAT), acetylcholine receptors (AChRs) and acetylcholine transporter[12]. AChE, a nuclear protein rapidly hydrolyzes the acetylcholine neurotransmitter to terminate the cholinergic transmission in central and peripheral synapses[13]. It is expressed as multiple splice variants that may serve non cholinergic functions in neuronal and non neuronal tissues[3]. In bone for example, AChE exists in a globular structure in osteoblasts[14]. At present, AChE inhibitors such as donepezil and tacrine are FDA approved drugs involved in the palliative therapeutic strategy for mild forms of AD[15]. Because of the complexity of AD, these approaches may reverse the symptoms but for a short time period instead of curing the disease. Hence, the Multi-Target Directed Ligands Strategy, a single compound, has been suggested to confront this disease[16].

Non hematopoietic stem cells can differentiate into somatic cells having a mesenchymal lineage and are collectively known as mesenchymal stem cells (MSCs) or marrow stromal cells. MSCs, the inherently multipotent cells are isolated from diverse postnatal adult tissues involving bone marrow, adipose tissue and dental pulp. Pluripotent stem cells may be found in bone marrow due to the presence of few embryonic-like stem cells. The advantageous aspects of MSCs applications involve their ease of obtainment in large quantities with much high proliferation capability, endurance of more passages with late senescence, renovation of their populations and differentiating into a wide range of cells, mesenchymal as well as non mesenchymal cell types including neurons. The adult MSCs particularly BM-MSCs are the most valuable to be utilized in advanced tissue engineering as well as cell therapy[17,18,19] since they possess high chromosomal stability reducing its potentiality for tumor formation[20]. Moreover, MSCs have an immunosuppressive function that discriminates them from other stem cells and reveals successful clinical relevance[17]. In the late 1960s, Friedenstein was the first to portray bone marrow stromal cells (BM-MSCs) as fibroblastic cells showing adhesion, tri-linear and plastic differentiation capacities giving rise to adipocytes, chondroblasts and osteoblasts[21]. In favor of cell therapy, stem cells can be systemically injected via the intravenous route or locally via direct injection into damaged tissues and organs[19]. It was thought that the peripheral intravenous injection of a small quantity of MSCs would be a safe, effective, practical and sufficient therapeutic approach to alleviate systemic inflammatory diseases[20].

To the best of our knowledge, researches concerning the possible joints alterations in association with Alzheimer's disease, particularly mandibular joint are still needed. Therefore, this study is to evaluate the potential influence of stem cells therapy on the cartilaginous and bone tissues as well as on the expression of AChE in the mandibular joint in the experimentally induced AD.

MATERIALS AND METHODS

Ethical clearance

With approval and following the guidelines of the Research Ethics Committee (FDASU-REC) [Approval: FDASU-REC- IR071802], -Faculty of Dentistry, Ain Shams University, Egypt. The rats were housed within (standard polycarbonate cages (Pretty industries, Model: CR5) (three / cage) under controlled relative humidity, a temperature of 24–26°C, 12/12 h dark-light cycle, proper ventilation, adequate diet and tap water. The welfare of the animals was evaluated under the supervision of a specialized veterinarian all over the experimental period.

Experimental design

Thirty adult male albino rats aged 6-7 months with 250-300 gm body weight[22] were randomly divided into three equal groups (ten rats each) after 7 days of acclimatization as follows: group I (control group) received a daily intraperitoneal injection of 0.9% saline under ether anaesthesia[23]. For induction of Alzheimer's disease, both group II (ALZ group) and group III (BM-MSCs treated group) were injected intraperitoneally with aluminium chloride, 70 mg/kg b.w. AlCl3.6H2O (Sigma, St. Louis, MO, USA) dissolved in distilled water at dose volume not exceeding 0.5ml/200 gm b.w.[1,23]. Group III (BM-MSCs treated group) received a single injection of BM-MSCs via tail vein under ether anesthesia[20] after 6 weeks of the daily intraperitoneal injection of AlCl3.6H2O[1,23].

Isolation, culture and transplantation of rat BM-MSCs

Isolation and culture of rat BM-MSCs: Rats were injected intraperitoneally with 10% hydral for anaesthetization. After removing the femurs and tibias of male white albino rats aged 6 weeks old, the two terminal parts of these bones were dissected then flushed with Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% fetal bovine serum (GIBCO/BRL) to harvest the bone marrow for cells collection. The nucleated BM-MSCs were isolated with a density gradient using [Ficoll / Paque (Pharmacia)] , resuspended and cultured in DMEM with the addition of 1% streptomycin- penicillin (GIBCO/BRL). Subsequently, cells incubation was established for 12–14 days in 5% humidified CO2 at 37 °C for a primary culture or upon production of large colonies. On the development of large colonies (80–90% confluence), the cultures were washed twice with phosphate buffer saline (PBS) followed by trypsinization of the cells with 0.25% trypsin for 5 min at 37 °C in 1ml EDTA (GIB- CO/BRL). Following centrifugation, cells resuspension was performed using a serum-supplemented medium for the incubation in a 50 cm^2 culture flask (Falcon). The resultant cultures were described as first-passage cultures. Fusiform shape adherent BM-MSCs were attained and utilized in the experiment[24].

Intravenous transplantation of BM-MSCs

This was done out by the injection of stem cells via the

lateral tail vein. The rats were laid under a heat lamp to induce the peripheral vasodilation, followed by a mechanical restrain utilizing a home-made Plexiglas chamber. The tail was maintained in a horizontal straight posture to ease the injection. The injections of the stem cells were applied using syringes with 25 gauge needles. For each rat, the resuspension of the BM-MSCs in 0.1 mol/ l phosphate buffered saline was accomplished at a concentration of $3x10^6$ cells/ml for each rat. The animals were placed in their cages after handling for recovery[25,26].

At the end of the experimental period (12 weeks), all animals were sacrificed at morning by intracardiac administration of anesthetic overdose (sodium thiopental 80 mg/kg BW)[27]. The animals' heads were separated and fixed for 24 hours in 10% buffered formalin. Getting rid of the sacrificed rats' bodies was implemented regarding the ethical committee rules in the incinerator of Ain Shams University Hospital. The temporomandibular joints (TMJs) were dissected and rinsed in a water beaker and then placed in capsules carrying distinct codes for each rat.

Histopathological examination

The dissected TMJs were immersed in the fixative for 24 hours at 4°C. Decalcification of the specimens was done with a 10% ethylene-diamine-tetraacetic acid disodium solution for 6 weeks at 4°C then the specimens were embedded in paraffin sections. Routine hematoxylin and eosin histological and immunohistochemical examinations of the sagittal serial 5μm sections of TMJ specimens[28] were executed by a light microscope (Olympus® BX 60, Tokyo, Japan) containing ApogeeKX85 digital camera (Apogee Biotechnology Corporation, USA) and the images were acquired by Image-Pro Plus software (Media Cybernetics, Inc., USA).

Immunohistochemical examination

Standard immunohistochemical labeling for the vascular endothelial growth factor (VEGF) using anti-VEGF antibody (rabbit polyclonal antibody to VEGF-A) was carried out regarding the manufacturer's protocol. Quenching of endogenous peroxide by 3% hydrogen peroxide incubation for 20 minutes at room temperature was followed by the sections reaction with the primary antibodies overnight at 4 °C. The primary antibody was substituted by PBS as a negative control. The slides were consequently washed twice with PBS, incubated for 30 minutes with biotinylated secondary antibody at room temperature, washed and then incubated for 15 minutes with streptavidin-peroxidase complex at room temperature. Afterwards, diaminobenzidine/ peroxidase staining (DAB, Sigma Chemical Co., St. Louis, MO, USA) and counterstaining of the nucleus for 15 seconds with hematoxylin were achieved. The cover slipping of the slides was done after drying. The localization of immunopositive cells to VEGF was microscopically examined by a light microscope (Olympus® BX 60, Tokyo, Japan)[28].

Detection of AChE protein by Western blot analysis

(V3 Western Workflow™ Complete System, Bio-Rad® Hercules, CA, USA) was used. Extraction of proteins was performed from tissue homogenates utilizing ice-cold radio-immuno-precipitation-assay (RIPA) buffer with supplementation of phosphatase and protease inhibitors (0.5m Mphenylmethylsulphonyl fluoride, 50 mmol/L sodium vanadate, 0.5mg/mL leupeptin and 2mg/mL aprotinin). Afterwards, centrifugation for 20 minutes at 12,000 rpm was attained. By Bradford assay, the protein concentration in favor of each sample was established. Separation of equal protein amounts (20-30 μg of total protein) was completed with SDS/ polyacrylamide gel electrophoresis (10% acrylamide gel) utilizing a Bio-Rad Mini-Protein II system. The transfer of the protein to polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA) was acted by a Bio-Rad Trans-Blot system. Subsequently, membranes wash with PBS and block at room temperature for 1 h with skimmed milk powder 5% (w/v) in PBS was carried out. The manufacturer's regulations were followed for the reactions of the primary antibody. After blocking, the blots were developed using antibodies for beta-actin and acetylcholinesterase (Thermo scientific, Rockford, Illinois, USA) incubated overnight at 4°C in pH 7.6 with gentle shaking. Following washing, the addition of peroxidase-labeled secondary antibodies was done and the membranes were incubated for 1h at 37°C. Using ChemiDoc TM imaging system through Image Lab TM software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA), band intensity was analyzed. After normalization for β-actin protein expression, the results were expressed as arbitrary units[23].

Real-time PCR for quantitative analysis of collagen I and II gene expression

The extraction of the total RNA from tissue homogenate (TMJ specimens) was made using SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's regulations. Both RNA purity and concentrations were measured using the ultraviolet spectrophotometer. For complementary DNA (cDNA) synthesis, utilizing SuperScript III First-Strand Synthesis System, cDNA was synthesized as illustrated in the protocol of the manufacturer (#K1621, Fermentas, Waltham, MA, USA) from 1 μg RNA and as previously exemplified by other studies[29,30]. For real-time quantitative PCR, real-time PCR amplification along with analysis was executed using the Applied Biosystem with software version 3.1 (StepOne™, USA). The reaction included SYBR Green Master Mix (Applied Biosystems), gene-specific primer pairs which were displayed in (Table 1) designated with Gene Runner Software (Hasting Software, Inc., Hasting, NY) for RNA sequences from a gene bank. Data calculation from real-time assays using the v1•7 sequence detection software from PE Biosystems (Foster City, CA) was accomplished. Calculation of the relative expression of studied gene mRNA using the comparative Ct method was implemented. Normalization

of all values to beta-actin which regarded as the control housekeeping gene was achieved and described as fold change over background levels perceived in the studied groups as described by other authors[29,30].

Table 1: The primer sequence of the studied gene

	Primer sequence
Collagen I	Forward primer: 5'- CCCTGTGCACTAAAGTGCCCC-3 Reverse primer: 5'- GTCAGATGGACACATGGTG-3
Collagen II	Forward primer: 5'- CTACGAGTGGGATGCTGGAG-3 Reverse primer: 5'- TTCTTCACGATGGTGAGCG-3
Beta-actin	Forward primer: 5'- GGTCGGTGTGAACGGATTTGG-3 Reverse primer: 5'- ATGTAGGCCATGAGGTCCACC-3

Histomorphometric and statistical analysis

The integrated optical density of the VEGF stained TMJ bony components and synovium was statistically measured and analyzed. This analysis was estimated by a digital camera as well as the software (Leica Qwin 500) of Leica microscope. The pixels produced with the image analysis program by the image analyzing system transformed into real micrometere units[28]. Data obtained from histomorphometry, western blotting and PCR were coded and analyzed by statistical package SPSS (Statistical Package for the Social Sciences) version 25. Data was summarized utilizing mean and standard deviation. One way Analysis of Variance (ANOVA) was used and after its illustration of the statistically significant result, the significant difference with the multiple comparison post hoc test (Tukey's) was also utilized for comparing the studied parameters between the three studied groups. *P values* less than 0.05 were regarded as statistically significant[1,31].

RESULTS

Histopathological results

Group I (control group)

The sagittal view of the temporomandibular joint (TMJ) of adult control rats exhibited the glenoid fossa of the temporal bone, mandibular condyle, an articular disc with the retrodiscal bilaminar zone posteriorly and a synovial membrane that lined the non articulating disc surfaces. Both of the temporal fossa and mandibular condyle presented normal architecture and stainability with smooth articular surfaces (Figure 1a). The mushroom-like and wide mandibular condyle revealed smooth fibrous coverage continuous with the periosteum of the condylar neck and normal cortical bones (Figures 1a, 1b). Both central and posterior regions of the condyle demonstrated a clear cartilaginous structure that gradually thinned at the anterior region (Figure 1c). Articulating temporal bone and mandibular condyle almost displayed a similar structure. From the condylar surface, the fibrous articular zone was composed of several layers of flattened cells and dense parallel collagen fibers. Subjacently, the thin layered, densely packed, flattened and proliferative precondroblastic cells and undifferentiated mesenchymal cells were surrounded by a basophilic ground substance. Some specimens showed an underneath mature cell layer with well-differentiated chondrocytes and intercellular matrix. Then, the thin calcified cartilage contained small mature ovoid chondrocytes with condensed nuclei. Moreover, the dark basophilic wavy tidemark was observed at the mineralizing front of the calcified cartilage parallel and near to the articular surface. A clear osteochondral junction (interface between subchondral bone and calcified cartilage) was recognized along with the osteon-like osteogenesis around blood vessels at this border. The ossified subchondral bone displayed lamellar bone with dense bone matrix, normal osteoid seam, osteocytes, osteoblasts, bone marrow cavities with stromal tissue and endosteum. The articular disc with its normal biconcave configuration, thick at the peripheries and thin at its centre, was formed of dense collagenous fibrous tissue and some chondrocytes (Figures 1a,1b,1c,1d). Areolar, fibrous and mixed types of the synovial membrane were recognized in different TMJ portions. Loosely arranged synovial intimal cells were distinguished in a compact not continuous one to three layers deep (Figures 1e,1f,1g). The synovial subintima was composed of loose fibrous connective tissue with high vascularity in the areolar type (Figure 1e), dense collagenous tissue with less vascularity in the fibrous type (Figure 1f) and numerous adipocytes found in the mixed type (Figure 1g). Numerous folds protruded into the articular cavity facing the joint space, particularly in the posterior-superior region of the articular cavity with rich vascular subintima enclosing some adipocytes (Figure 1h).

Group II (ALZ group)

Careful examination of the H&E sections in the Alzheimer's group demonstrated degenerative changes of TMJ. Articulating bones surfaces appeared irregular. Pannus-like tissue (hyperplastic synovium with inflammatory cell infiltrates in synovial stroma) and synovial villi were evident (Figure 2a). The condylar head was irregularly outlined with almost distorted surface and apparently thinner outer cortical bone. The condylar deformation arose in two regions: at the chondro-osseous border as well as in the anterior region that showed such thin articular cartilage (Figure 2b). Yet, endochondral ossification appeared to be impaired in this group so that the condylar cartilage appeared thicker compared to the age-matched control group, particularly in the central region. The TMJ disc of this group lost its distinctive biconcave shape and thinned along with inflammatory infiltrates besides the loss of chondrocytes (Figure 2c). The articulating bones were lined by an apparently irregular fibrous layer in thickness and cellularity. Disorganization, decreased cellularity and discontinuity of the four-layered cartilaginous coverage were observed with no clear borders and indistinct distorted tidemark. Obvious degenerative signs in both chondroblastic and hypertrophic cell layers were represented by disordered cartilage matrix, irregularly arranged reduced chondrocytes in number and size, noticeable cells clustering, areas of acellularity, extensive areas of chondrocytes degeneration and apoptosis where cell debris as well as shrunken cells with fragmented pyknotic nuclei and hypereosinophilic cytoplasm were seen (Figures 2c, 2d). Moreover, multifocal large erosion cavities

were evident in the cartilage layer and subchondral bone with apparently increased osteoclastic activity along with congested vascular channels at the osteochondral junction surrounded by disordered or even apoptotic chondrocytes. The subchondral bone illustrated observable microarchitectural deterioration, apparently impaired density, discontinuous rod-like rather than plate-like bone trabeculae with osteopenia and apparently wide osteoid seam lined by single or several rows of clustered large osteoblasts. Furthermore, the expanded bone marrow cavities appeared with increased adipose vacuoles, chronic inflammatory cell infiltrates and thin-walled congested vascular channels with extravasations (Figures 2b, 2c, 2d). The severity of these destructive changes seemed slightly more in the condyle (Figures 2b, 2c, 2d) than in temporal fossa (Figure 2e). Marked signs of synovitis included hypertrophy and hyperplasia of the synovium and its folds forming pannus-like tissue. Thickened multi-layer synovial intima as well as inflammatory cell infiltrates including macrophages was noted. Fibrin deposits, congested dilated blood vessels with extravasation and an apparent increase of the adipocytes were displayed in different regions of the synovial subintima. Moreover, erosive fronts of cortical bone, as well as the apparently increased osteoclastic activity, were revealed between bone surfaces and pannus-like tissue (Figures 2f, 2g, 2h).

Group III (BM-MSCs group)

The histological examination of TMJ specimens of this group showed marked amelioration of the pathologic changes detected in group II. The architecture of articular bones involving glenoid fossa of the temporal bone and mandibular condyle, together with the articular disc, were renovated being almost similar to those of the control group. In the articulating bones, the articular cartilage illustrated improved organization of the superficial articular fibrous layer, proliferative layer and the underlying thin calcified cartilage containing mature ovoid-flattened chondrocytes. A clear cement line was demonstrated at the chondro-osseous junction implying the frequent bone deposition and thus contributed to the increased condylar thickness, particularly in the central region. An apparently enhanced bone deposition, ossification thickness and quality of subchondral and cortical bone were elucidated with an apparent decrease of the osteoid seam. The apparent increase of bone cells (osteocytes and osteoblasts), an apparent decrease of osteoclasts, and attenuated resorption, together with the restoration of bone marrow cavities to the normal structure, were also distinguished (Figures 3a, 3b). The treatment with BM-MSCs in this group alleviated the identified synovitis in group II through the substantial reduction of inflammatory cells, restoration of intimal thickness simulating that of the control group. Fewer blood vessels and decreased cellularity including adipocytes were found in the subintimal layer. The detected synovial folds in this group appeared smaller in size and number compared to those of both control group and group II (Figure 3c).

Immunohistochemical and statistical results for VEGF immunoreactivity

Group I (control group)

The TMJ specimens of the control group displayed a mild expression to VEGF. Faint cytoplasmic immunopositivity could be observed in the superficial fibrous and proliferative cell layers of the condyle. Minimal VEGF expression was distinguished in scarce chondrocytes of the calcified cartilage as well as in vascular endothelium, endosteum cells and osteoblasts of the dense subchondral bone. Mild reactivity to VEGF in synoviocytes and subintimal fibroblasts besides vascular endothelium were also evident (Figure 4a).

Group II (ALZ group)

A significant upregulation of the VEGF expression (*P-value* <0.001) was elucidated in the articular bony components of TMJ in comparison to the control group (Figure 5a). The increased VEGF expression in the articular cartilages mainly in the non-apoptotic clustered cells of the chondroblastic layer and some non-apoptotic hypertrophic chondrocytes were found (Figure 4b,5a). Intense cytoplasmic and nuclear positivity to VEGF was detected in both subchondral and cortical bones, in the apparently increased osteoclasts on eroded surfaces in addition to the positive large osteoblasts. The high expression of VEGF was also noticed in bone marrow in the recruited inflammatory cells and stromal cells. Furthermore, the endothelium of the congested vascular channels was obviously reacted (Figures 4c, 4d, 5a). VEGF was significantly expressed (*P-value* <0.001) in the synovium compared to the control group. The synovial pannus-like tissue and folds exhibited increased cytoplasmic and/or nuclear VEGF immunoreactivity in the thickened synovial intima. The cytoplasmic positivity was also displayed in the apparently increased adipocytes, some inflammatory cells besides the endothelium of the apparently increased congested and dilated subintimal blood vessels (Figures 4e, 4f, 5b)

Group III (BM-MSCs group)

Comparing to group II, a significant decrease in the VEGF immunoreactivity (*P-value* <0.001) was detected in the articular bony components of the herein TMJ specimens but with a significant increase (*P-value* <0.001) when compared to the control group. Mild cytoplasmic expression of VEGF was perceived in the fibrous articular layer, some cells in the proliferative layer and calcified cartilage along with the borderline chondrocytes of the condyle. Some positively reacted bone cells (osteoblasts and osteocytes) were illustrated in the dense subchondral bone and bone marrow stroma as well as the positive vascular endothelium (Figures 4g, 5a). For synovium, the expression of VEGF displayed a significant decrease (*P-value* <0.001) in comparison to group II but in relation to the control group, a less significant increase (*P-value* = 0.002) was evident. This was represented by the mild VEGF reaction in some synoviocytes, subintimal fibroblasts and vascular endothelium (Figures 4h, 5b).

AChE protein Western blotting and statistical results

Regarding cholinesterase, there was a statistically significant increase in the expression levels of AChE protein in group II compared to the control group (*P-value* <0.001). On the other hand, a significant decrease in AChE expression levels was elucidated in the BMSCs treated group in relation to group II (*P-value* <0.001) but with an insignificant increase as compared with the control group (P-value =0.4) (Figures 6a, 6b).

Quantitative real-time PCR of collagen I & II gene expression and statistical results

Concerning collagen type I, a statistically significant decrease in the collagen gene expression was distinguished in group II compared to the control group (*P-value* <0.001). While the gene expression was significantly upregulated in the BMSCs treated group when related to group II (*P-value* <0.001) and to the control group (*P-value* = 0.02). For collagen type II, there was a statistically significant downregulation in the collagen gene expression in group II as compared with the control group (*P-value* <0.001). This expression was significantly increased in the BMSCs treated group in comparison to group II (*P-value* <0.001) but with an insignificant increase in relation to the control group (*P-value* = 0.08) (Figure 7).

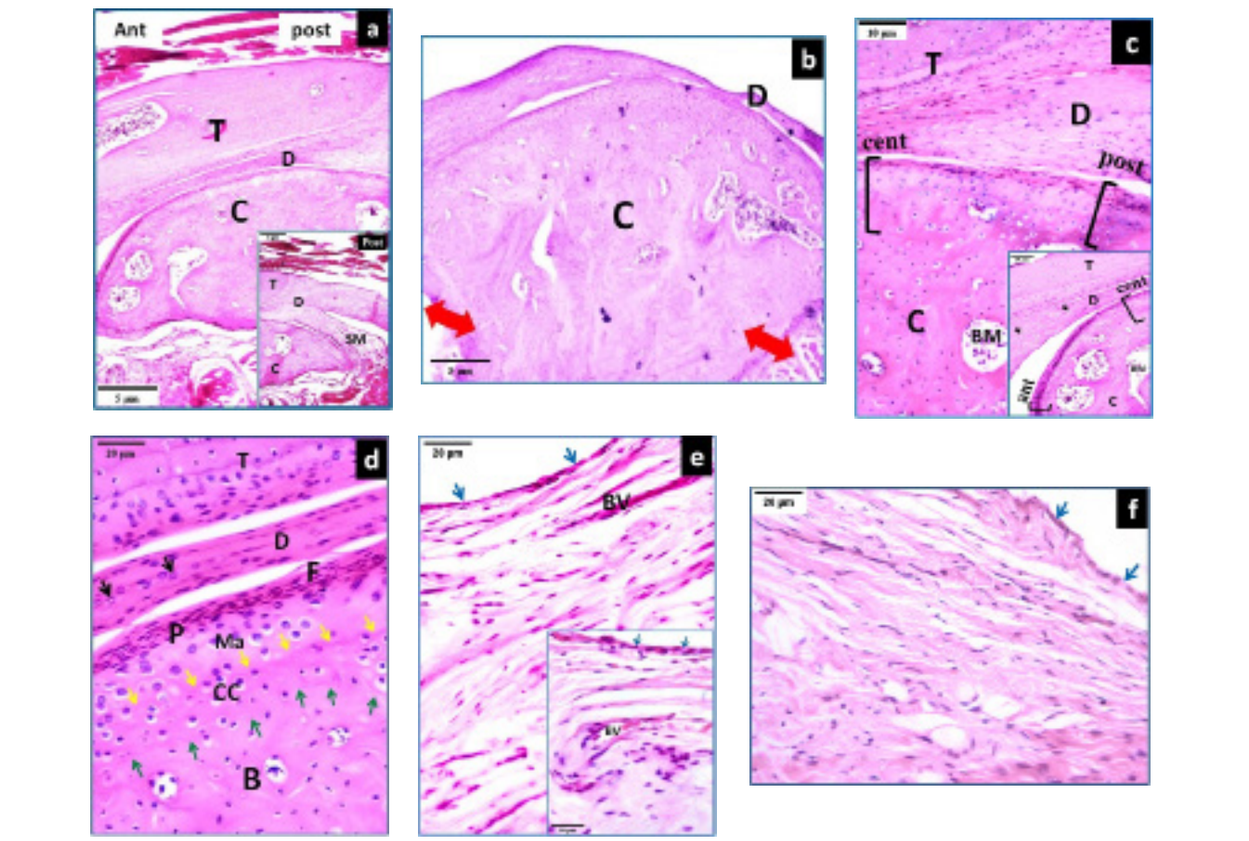

Fig. 1: Photomicrographs of sagittal H&E sections of TMJ in adult control rats; **(a)** showing regular architecture temporal bone, condyle, TMJ disc with retrodiscal bilaminar zone posteriorly and, Inset: synovial membrane (H&E, x40), **(b)** showing mushroom wide condyle with normal cortical bone (H&E, x40), **(c)** showing central and posterior condylar cartilaginous regions, Inset: condylar cartilage gradually thinned anteriorly with some chondrocytes in articular disc (black arrows) (H&E, x100), **(d)** regular structure of fibrous layer, proliferative layer, thin calcified cartilage and osteons of subchondral bone, distinct tidemark (yellow arrows), obvious osteochondral junction (green arrows) and some chondrocytes in articular disc (black arrows) (H&E, x200), **(e)** showing areolar synovial membrane (H&E, x200 ; Inset: x400), **(f)** showing fibrous synovial membrane (H&E, x200), **(g)** showing mixed type synovial membrane and some subintimal adipocytes (H&E, x200 ;Inset: x400), **(h)** showing synovial folds in posterior-superior region with subintimal adipocytes and vascularity (H&E, x40; Inset: x200).Temporal fossa (T), mandibular condyle (C), articular disc (D), synovial membrane (SM), fibrous articular layer (F), proliferative layer (P), mature chondrocytic layer (Ma), calcified cartilage layer (CC), subchondral bone (B), bone marrow (BM), blood vessels (BV), synovial intima (blue arrows), synovial folds (red arrows), adipocytes (black asterisks)

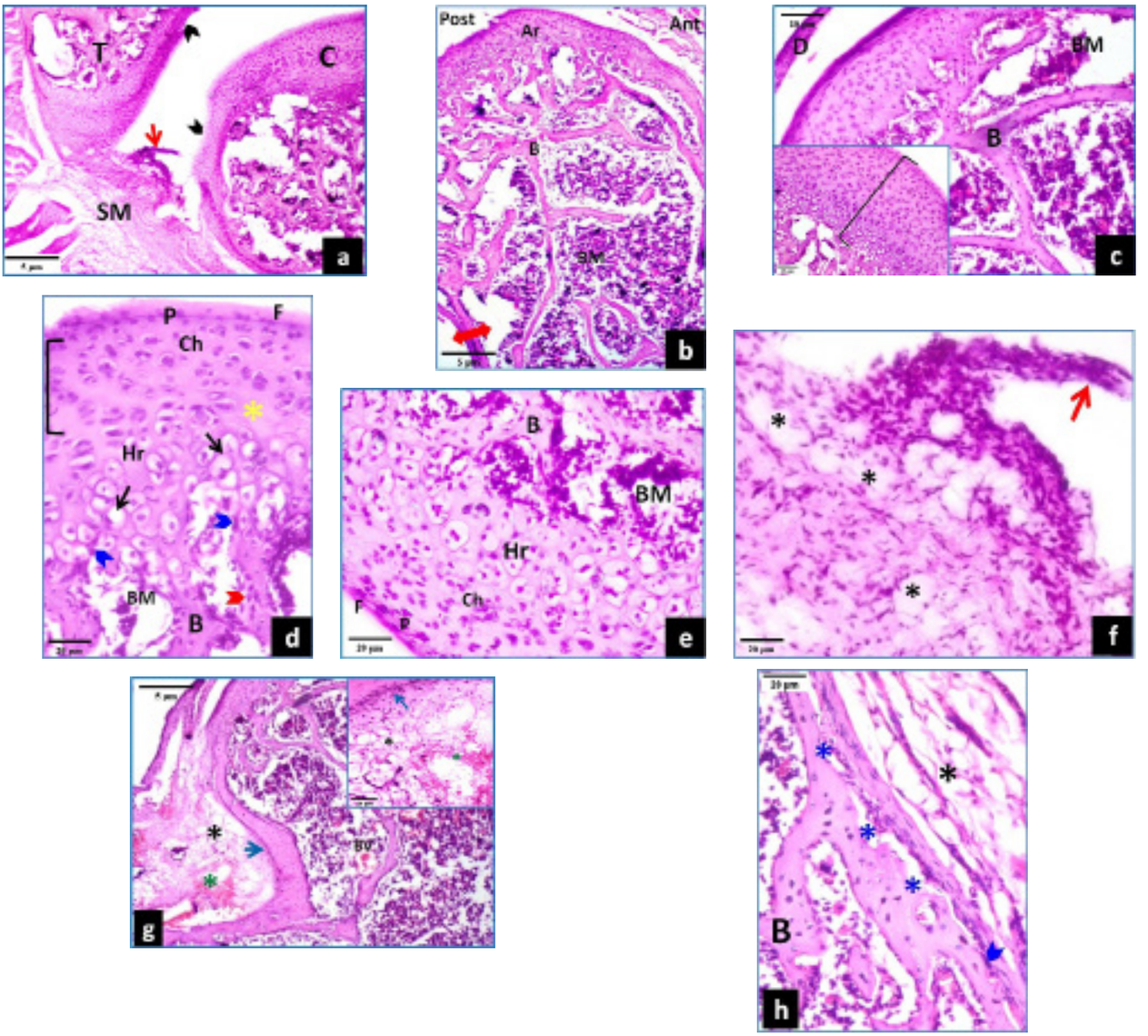

Fig. 2: Photomicrographs of sagittal H&E sections of TMJ in Alzheimer group; (a) showing irregularity of articular bony surfaces (black arrowheads), pannus like tissue and synovial villi (H&E, x40), (b) showing irregularly outlined condyle,thin articular cartilage anteriorly, deteriorated bone structure, rod like trabeculae, expanded marrow cavities with increased fat vacuoles, apparently thin cortical bone (red double arrow) (H&E, x40), (c) showing thin deformed disc, congested vascular channels at osteochondral junction, eroded bone surfaces, (H&E, x100), Inset: cartilaginous hyperplasia in condylar central region (H&E, X200), (d) showing apparently irregular fibrous layer, proliferative layer with decreased cellularity, clustering (bracket), irregularly arranged and apoptotic chondrocytes (black arrows), areas of acellularity (yellow asterisk), wide osteoid seam with large osteoblasts (red arrowhead), increased osteoclastic activity (blue arrowheads) and chronic inflammatory infiltrates in marrow cavities (H&E, X200). (e) showing apparently lesser destructive changes in temporal fossa (H&E, X200), (f,g) showing thickened synovial intima with inflammatory infiltrates and increased subintimal adipocytes (H&E, X200), (g) showing subintimal congested dilated blood vessels with extravasation (green asterisk) (H&E, x40 ; Inset: x200), (h) showing osteoclasts (blue arrowheads) at erosive fronts (blue asterisks) between bone surfaces and pannus like tissue (H&E, X200). Glenoid fossa of temporal bone (T), articular disc (D), mandibular condyle (C), synovial membrane (SM), articular cartilage (Ar), fibrous articular layer (F), proliferative layer (P), chondroblastic layer (Ch), hypertrophy chondrocytic layer (Hr), subchondral bone (B), bone marrow (BM), blood vessels (BV), synovial intima (blue arrows), synovial villi (red arrows), adipocytes (black asterisks).

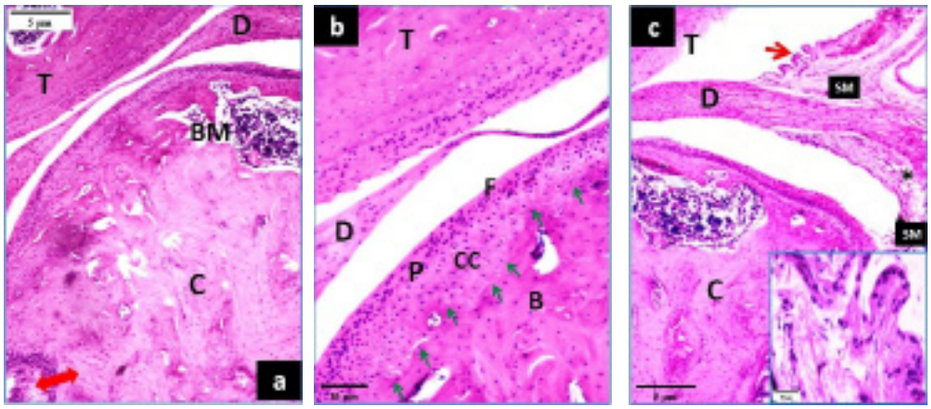

Fig. 3: Photomicrographs of sagittal H&E sections ofTMJ in BM-MSCs treated group; (a) showing almost normal architecture of TMJ components including TMJ disc, thickness and quality of articular bones; trabecular and cortical bones; (red double arrow) (H&E, X40), (b) showing properly organized articular fibrous layer, proliferative layer and underlying calcified cartilage of articulating bones, enhanced osteogenesis of subchondral bone, normal bone marrow, apparent increase of bone cells, clear cement line at chondro-osseous junction (green arrows) (H&E, X100), (c) showing restored thickness of synovial intima, reduced subintimal cellularity, vascularity and adipocytes, less synovial folds in number and size (H&E, X40), Inset: synovial folds (H&E, X400). Glenoid fossa (T), articular disc (D), mandibular condyle (C), synovial membrane (SM), fibrous articular layer (F), proliferative layer (P), calcified cartilage layer (CC), subchondral bone (B), bone marrow (BM), blood vessels (BV), synovial intima (blue arrows), synovial folds (red arrows), adipocytes (black asterisks).

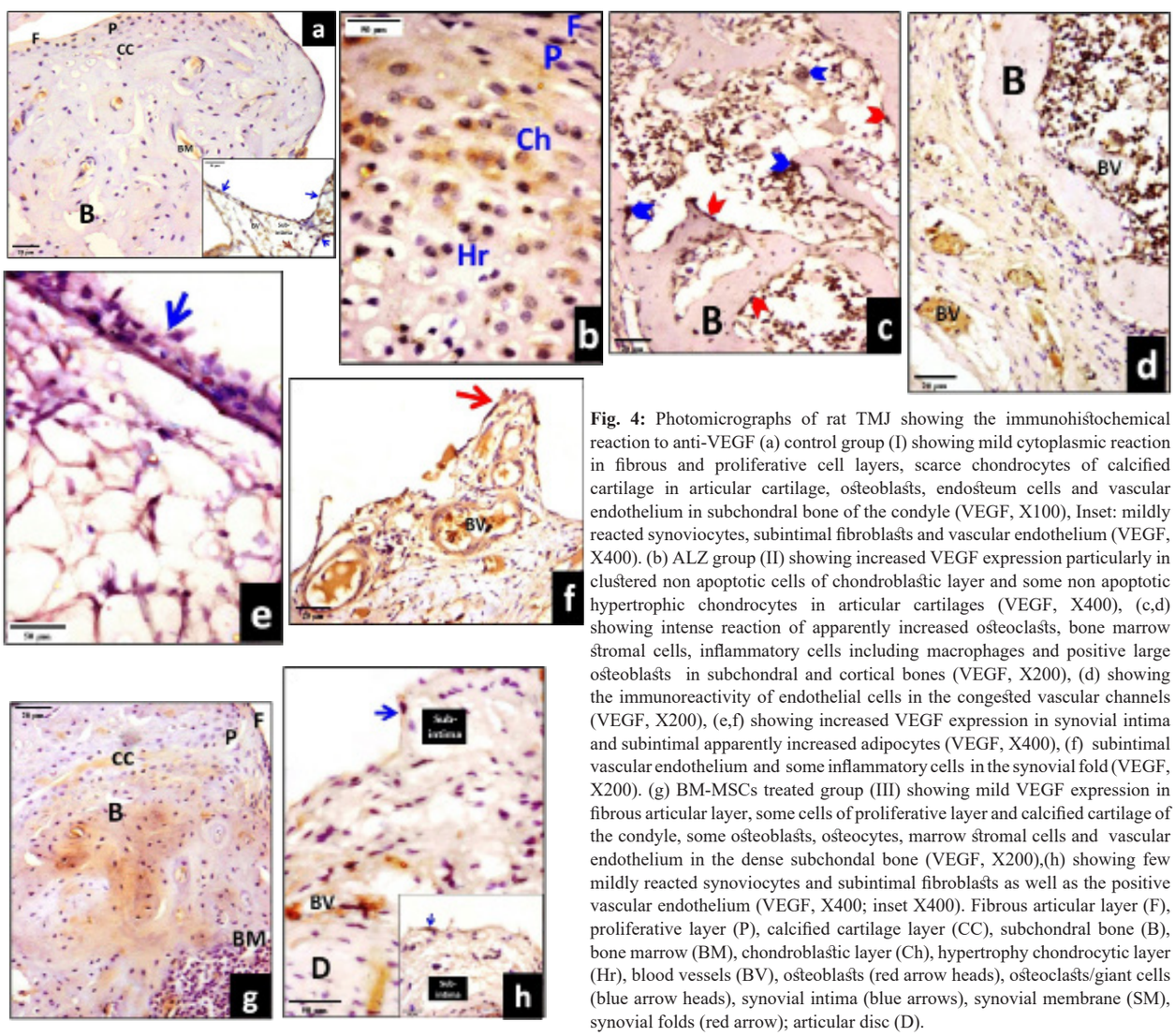

Fig. 4: Photomicrographs of rat TMJ showing the immunohistochemical reaction to anti-VEGF (a) control group (I) showing mild cytoplasmic reaction in fibrous and proliferative cell layers, scarce chondrocytes of calcified cartilage in articular cartilage, osteoblasts, endosteum cells and vascular endothelium in subchondral bone of the condyle (VEGF, X100), Inset: mildly reacted synoviocytes, subintimal fibroblasts and vascular endothelium (VEGF, X400). (b) ALZ group (II) showing increased VEGF expression particularly in clustered non apoptotic cells of chondroblastic layer and some non apoptotic hypertrophic chondrocytes in articular cartilages (VEGF, X400), (c,d) showing intense reaction of apparently increased osteoclasts, bone marrow stromal cells, inflammatory cells including macrophages and positive large osteoblasts in subchondral and cortical bones (VEGF, X200), (d) showing the immunoreactivity of endothelial cells in the congested vascular channels (VEGF, X200), (e,f) showing increased VEGF expression in synovial intima and subintimal apparently increased adipocytes (VEGF, X400), (f) subintimal vascular endothelium and some inflammatory cells in the synovial fold (VEGF, X200). (g) BM-MSCs treated group (III) showing mild VEGF expression in fibrous articular layer, some cells of proliferative layer and calcified cartilage of the condyle, some osteoblasts, osteocytes, marrow stromal cells and vascular endothelium in the dense subchondal bone (VEGF, X200),(h) showing few mildly reacted synoviocytes and subintimal fibroblasts as well as the positive vascular endothelium (VEGF, X400; inset X400). Fibrous articular layer (F), proliferative layer (P), calcified cartilage layer (CC), subchondral bone (B), bone marrow (BM), chondroblastic layer (Ch), hypertrophy chondrocytic layer (Hr), blood vessels (BV), osteoblasts (red arrow heads), osteoclasts/giant cells (blue arrow heads), synovial intima (blue arrows), synovial membrane (SM), synovial folds (red arrow); articular disc (D).

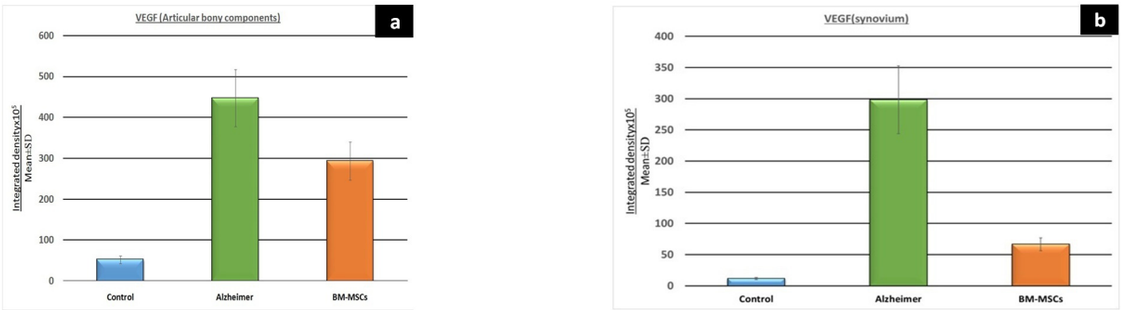

Fig. 5: Bar charts showing the mean ±SD of VEGF Immunoreaction (Integrated density x10^5) among all studied groups in (a) TMJ bony components and in (b) TMJ synovium.

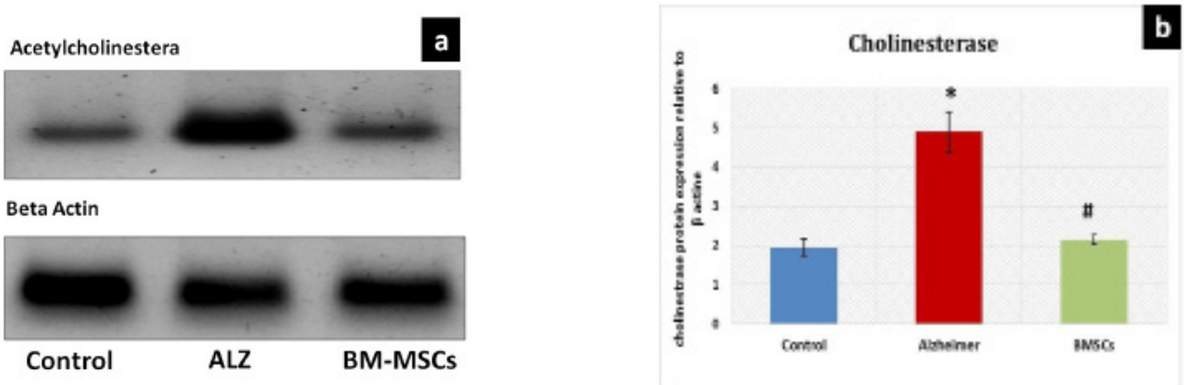

Fig. 6: (a) Representative immunoblotting of the expression levels of AChE proteins detected by anti-AChE antibody in control, ALZ and BM-MSCs groups. (b) Bar chart showing the mean ±SD of the densitometric quantification of AChE-immunoreactive bands applying β-actin as a control and expressed in arbitrary units in theTMJ specimens among all studied groups.P value <0.05 is considered significant, (*): denotes significant difference versus control group, (#): denotes significant difference versus ALZ group.

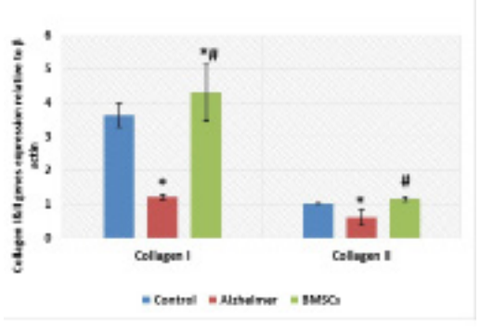

Fig. 7: Bar charts showing the mean ±SD of the changes in the gene expression of collagen type I and collagen type II evaluated by real-time PCR among all studied groups. P value <0.05 is considered significant, (*): denotes significant difference versus control group, (#): denotes significant difference versus ALZ group.

DISCUSSION

Temporomandibular joint (TMJ) is one of the major habitually working joints because of mastication. Hence, TMJ could serve as an appropriate indicator to profile the age-related changes and disorders such as Alzheimer's disease (AD), osteoarthritis (OA) and osteoporosis[32]. Rat TMJ provided some anatomical and functional similarities to that of humans[33]. Two assumptions have been made about the association of AD with joint and bone disorders, whether bone diseases are the risk factor for the development of AD or vice versa[4,9]. Since the latter proposition has been adopted in this study, we induced AD in adult male rats' model by the neurotoxic and cholinotoxic AlCl3[1] to evaluate the possible effects of AD as well as the subsequent bone marrow mesenchymal stem cell therapy (BM-MSCs) on rat TMJ. The routine histological examination of the control TMJ sagittal specimens presented the normal architecture of TMJ components characteristic to the middle-aged adult rats (9-10 months old) at the end of the experimental period with the morphofunctional changes related to the occasion of endochondral ossification. This occurred with the disappearance of the hypertrophic layer of articular cartilages akin to other experimental data[32,34]. The authors added that the ossification of the articular cartilage in adult and older rats varied from the endochondral ossification process in the younger rats due to the functional alteration from growth to articular function[32,34]. In parallel to our results, Inoue *et al*.[35] attributed the synovial intimal discontinuity to the existence of laminin around synovial fibroblasts and subintimal blood vessels for anchorage and adhesion but not around the synovial macrophages because of their phagocytic migratory function. The fibroblastic synoviocytes which secrete collagen types I and II besides other proteins could act as MSCs due to their potentiality to differentiate into osteocytic, chondrocyte and adipocytic lineages while the synovial macrophages predominated with age in humans and rodents[29,35].

Because of the evident TMJ degenerative changes in the specimens of group II (induced Alzheimer group) in this work and since the relationships of AD with osteoporosis[9] and with OA[36] were assessed, we believed that they all form a triad in the AD-related joint disorders. Particularly that bone loss along with reduced bone mineral density could be early indicators of brain degeneration in AD. This is due to the high correlation of the detrimental bone alterations with cholinergic degradation in the hypothalamus and with areas of the brainstem that generates most of the brain's serotonin and also regulates skeletal tissues[6,37]. Likewise, the complex degenerative cartilaginous disease OA, which was described as M. Alzheimer of chondrocytes and articular cartilage[4], might intensify the occurrence of neuroinflammation and Aβ pathology by means of circulating cytokines in the context of AD[38,39]. In the current study, the TMJ articular cartilages of group II specimens histologically showed four layers including hypertrophic chondrocyte layer observed in the TMJ of growing rats younger than 8-9 months[34,40] but with degenerative signs simulating those of osteoarthritic joints[4,11]. These findings confirmed our thoughts regarding the retardation of endochondral ossification in this group compared to the age-matched control rats. In agreement with our results, other studies assigned the apparent thickening of TMJ articular cartilages to the osteoarthritic changes. This thickening would reduce over a longer time in TMJ-OA with the condylar anterior region considered a critical weak area because it normally encompasses a thin cartilage[28,41]. The very low proliferative capacity of osteoarthritic chondrocytes could lead to the typical clustering feature of OA cartilage with no significant adding to the matrix anabolism [4]. In synchronization, Wang *et al*.[30] found lesser erosive changes in the temporal fossa when compared to the condyle in the early stages of TMJ-OA whereas, in the late stages, sclerosis was evident. Given that both articular cartilage and subchondral bone act as a functional unit of joints[42], subchondral bone has been lately believed to play a substantial role in OA pathogenesis that was initially considered a cartilage driven process[39].The illustrated microarchitectural deterioration together with the reduced sinusoids in the subchondral bone of group II specimens reflected the porosity of bone, reduced bone quality and insufficient blood flow. These were all described in different osteoporotic bone studies[43,44,45]. Moreover, our osteoporotic findings of the more compromised cancellous bone than cortical bone, marrow adiposity, reduced myeloid content and osteopenia that were attributed to the diminished differentiation of the stromal cell precursors into osteoblasts, were also affirmed by other authors[46,47]. The adipose synovial membrane most likely occurs with age or in TMJ OA where the subintimal vascular dilatation and extravasation were also detected[33,35]. Normally the proliferation rate of synoviocytes has been extremely low[33]. But, the drastic increase of the synovial macrophages, together with the inflammatory infiltrates in OA possibly contributed to synovitis, the increase in size and number of synovial folds beside the formation of the pannus-like tissue[48,49] which was atypical to that in rheumatoid arthritis[30]. Some investigators believed that the synovial tissue has been the primary trigger for OA[39].

Oxidative stress, which was likely accompanied by significant DNA injury and loss of cells, has been believed to play a significant role in the development of AD, osteoporosis and OA[3,9,11]. High levels of inflammatory mediators and cytokines were released by bone, cartilage and synovium cells such as inducible nitric oxide synthase and nitric oxide in response to the synovial fibroblastic release of nuclear factor-κB[35,48] causing neoangiogenesis in OA for repair[39,49]. In response to these cytokines, the production of proteolytic enzymes like cysteine proteases and MMPs[39], degradation of articular cartilage and resorption of subchondral bone matrix were induced in OA. This is due to the imbalanced activity between these enzymes and their inhibitors, together with the inhibition of new matrix formation[24,30]. Furthermore, Aβ accumulation in both AD brain and osteoporotic bone tissue has interfered with RANKL signaling and bone remodeling, suppressed osteoblastic differentiation, enhanced adipogenic differentiation and osteocytic apoptosis[45,50]. Concurrently, two positively correlated and independent pathways have

been involved in the enhanced osteoclastic activity. First, the abnormal provoked RANKL pathway which is associated with osteoprotegerin downregulation, while the other is the inflammatory cell infiltrates and osteoblasts pathway. Both could result in the release of inflammatory cytokines thus promoting the associated bone diseases including osteoporosis and arthritis which exhibit some similarities as detected before[10,51,52].

Since there is no effective prevention or therapeutic approach to cure or even to slow the progression of AD[15], mesenchymal stromal stem cells (MSCs) are now broadly used in clinical trials as a promising regenerative treatment for AD. Functions of MSCs like BM-MSCs are monitored by different signaling systems[53,54]. Because of the deficient renovative capacity of articular cartilage, stem cells depletion in OA as well as local osteoporosis, it was ideal the repair of the diseased articular cartilage and subchondral bone by stem cells therapy[24,55]. The H&E sections of the BM-MSCs treated group presented an obvious restoration of the AD-associated pathologic changes. This appeared in the apparently improved architecture and cellularity of the articular calcified cartilage and subchondral bone together with the enhanced ossification when compared to group II and control group. Additionally, the synovial folds appeared smaller in size and number in this group. The articular calcified cartilages in the TMJ specimens showed properly organized layers akin to those found in the age-matched normal rats used in the Ohashi *et al*.[34] study. In harmony with these results, it has been proposed that the therapeutic effect of MSCs is by migrating to distant sites such as draining lymph nodes near inflamed tissues where the activated inflammatory cells can give an appropriate proinflammatory environment for induction of MSCs to inhibit the local and systemic inflammatory responses[17,56]. Likewise, certain factors such as chemokines secreted by hippocampal cells might serve as homing signals for the coming MSCs from peripheral blood and thus explicate the protracted survival of BM-MSCs in the hippocampus and their beneficial effects as perceived in mice studies. Consequently, Aβ plaques and their associated pathologies other than stimulating neuron formation were targeted and removed[54,57]. BM-MSCs functionality has been achieved in a paracrine manner rather than direct trans-differentiation since the systemically transplanted BM-MSCs could poorly survive at the damage sites for around 4 weeks[58]. This has been attained by cell-cell interaction, engrafting and replacing for apoptotic plus injured cells in addition to the release of several extracellular vesicles like microvesicles and exosomes that enclose bioactive paracrine factors including cytokines, chemokines as well as the growth factors which were either directly released or induced by MSCs[54,56,59]. These bioactive factors were described as anti-inflammatory (suppress inflammatory cells), anti-apoptotic, proliferation, differentiation (into osteogenic and chondrogenic lineages) and angiogenic promoting factors in BM-MSCs[17,53,58]. Also, BM-MSCs and their progenies are involved in bone repair and remodeling[12] that exhibit an association between the osteogenic and angiogenic pathways[60]. In the resolution phase of bone resorption, the phagocytosis of apoptotic neutrophils by inflammatory macrophages promoted the switch of the latter cells to reparative macrophages which together with the pre-osteoclasts and bone matrix could release osteogenic factors like TGF-β1 to suppress inflammation, induce neovascularization and thus initiate the coordinated bone repair[61].

Vascular endothelial growth factor (VEGF), the angiogenic and mitogenic cytokine, plays a significant role in different physiological and pathological conditions. It promotes angiogenesis, vasculogenesis and capillary permeability by provoking endothelial proliferation and migration through its paracrine mechanism. It also contributes to cell survival by its intracrine/autocrine mechanisms[61]. In this work, the mild expression of VEGF in the control specimens could be attributed to the balance between the anti- and pro-angiogenic factors synthesized by the chondrocytes of the normal proliferative layer and resting articular cartilage and thus resisted the vascular invasion[28,62]. Since the paracrine function of the osteoblastic VEGF can influence the osteoblastic and osteoclastic activities along with angiogenesis, therefore the normal levels of VEGF are essential for organizing the physiologic bone remodeling[61,63].

TMJ specimens of group II exhibited a significant upregulation of VEGF in the articular cartilage and subchondral bone compared to the control group thus ascertained our proposition concerning the retarded endochondral ossification. Analogous to our findings, the normal levels of VEGF provided by vascularization together with the autocrine VEGF signaling in mature and hypertrophic chondrocytes were presumed to regulate chondrocyte differentiation, cartilage remodeling, angiogenesis as well as endochondral ossification in the condyle in rat studies[31]. On this basis, the VEGF overexpression most likely potentiated the noted thickening of the calcified cartilage in this work and also accelerated the catabolic activity of chondrocytes by enhancing MMPs[28,64]. This upregulation may be induced with the cytokines released by the surrounding inflammatory infiltrates that liberate angiogenic factors to indirectly stimulate revascularization along with that the direct target of VEGF are endothelial cells[61]. On the other side, the marked tidemark distortion in this study might result from the thin-walled vascular invasion to the non-calcified cartilage that could damage the subchondral bone / articular cartilage barrier allowing the massive diffusion of VEGF to the osteochondral junction from cartilage and bone as reported by Wang *et al*.[28]. The subsequent contact of articular chondrocytes with the migrated endothelial cells was indicative of pathological neovascularization in arthritis[62]. What is more, the expressed VEGF by osteoporotic and osteoarthritic osteoblasts generated a potent angiogenic response more than normal cells in the condyle in mice studies[60,64]. However, the intracellular intracrine VEGF mechanisms, consistent with the osteoporotic features, were progressively lost in osteoblasts and MSCs favoring the adipogenic over osteoblastic differentiation

under the regulation of PPARγ and Runx2 transcription factors respectively[63]. Therefore, the typical OA changes as well as osteoporosis were supposed to be angiogenesis-dependent and provoked by the imbalance between anti- and pro-angiogenic factors such as VEGF[28,44]. In OA and osteoporosis reports, uncontrolled osteoblast derived VEG was associated with increased osteoclastic differentiation by the paracrine mechanism through VEGFR2 signaling on osteoclastic precursors[55,65]. In concurrence to the herein significant increase of synovial VEG in group II compared to the control group, the upregulated VEGF in synovitis and OA may be involved in the prevention of synovial fibroblastic apoptosis and supports the endothelial chemokines production, which in turn can induce both synoviocytes lineages to secrete more VEGF. Through the paracrine and autocrine mechanisms, this might denote angiogenesis, direct osteoclastogenesis from monocytes or indirectly by synovial fibroblastic RANKL which stimulates the osteoclast precursors to differentiate[66]. Consequently, the paracrine and/or autocrine VEGF signaling has been correlated to the pathogenesis of both osteoporosis and osteoarthritis[67].

Comparing to group II, the articular bony components and synovium of BM-MSCs treated specimens presented a significant decrease in the VEGF reactivity but with a significant increase when compared to the control group. In explanation of these results, the expression of VEGF and its receptors in the transplanted BM-MSCs proved its intracellular intracrine potency to elicit proliferation and migration of undifferentiated cells, BM-MSCs/osteoblastic differentiation and inhibition of adiposity. Whereas the VEGF paracrine potency in the generation of new and proper vascularity[21,57,63] needed for stem cell migration and survival has also been reported[68]. Moreover, VEGF is essential for effective angiogenicosteogenic coupling required for bone repair and regeneration; directly through the VEGF chemotactic effect on osteoblasts, along with the vascular supply of bone cell precursors, cytokines, osteogenic factors such as TGF-β and minerals[44,61]. The indirect effect arises from the increased osteoblastic activity by inducing the endothelial cells with VEGF to enhance the BMPs expression and thus ensuing osteoblastic proliferation and differentiation[44]. Likewise, the expressed VEGF by maturing osteoblasts can act in an intracrine, autocrine as well as paracrine mechanisms to support cellular maturation, survival, transcriptional pathway and mineralization by promoting alkaline phosphatase and osteocalcin levels[44]. Also, the binding of VEGF to VEGFR2 and VEGFR1 expressed on osteoclasts and their precursors, respectively contributes to the regulation of bone remodeling and repair[61]. In parallel to the herein results of BM-MSCs treated synovium, the restored balance between pro- and anti-angiogenic factors donated alleviation of VEGF upregulation in osteoarthritic synovium, inflammation, pannus formation and joint erosion[49].

Western blotting is a rapid and sensitive assay for the detection of proteins concentration. Hence, it was used to determine the alterations in the expression levels of AChE protein in the studied groups using specific anti-AChE antibody similar to other reports[69]. Corresponding to AChE expression in the control group, AChE expression in bone and cartilage tissue plus cells was illustrated besides its effect on tissue remodeling in both osteogenesis and hematopoiesis processes in rat studies. It is regulated by hormones, growth and osteogenic factors such as Cbfa1 which have binding sites on the extended promoter regions of the AChE gene. AChE (ectoprotein) acts as a bone matrix protein secreted in osteoid, along cement line and interacts with other AChE substrates besides the other matrix proteins as collagen type I. It is involved in cell-cell, cell-matrix minerals and cell-matrix adhesive interactions in bone[70]. The expression levels of AChE protein were significantly higher in group II specimens compared to the control group similar to Campanari *et al*. study[69]. In synchronization, despite the decreased enzymatic levels of the extracellular tetrameric true cholinergic AChE in AD brain, a large pool of intracellular monomeric AChE species having diverse physiological roles other than cholinergic role increased and amplified the severity of the disease. This increase in the AChE levels and activity was associated with the direct action of Aβ accumulation[69,71] in neuronal and non-neuronal tissue and cells (like chondrocytes and osteoblasts)[3]. Aβ deposits could reduce the AChE degradation by its binding to the nicotinic receptors and inhibit ACh synthesis by Aβ-provoked leakage of choline across the cell membranes due to the Aβ induced lipid peroxidation along with the hydrolytic activity of AChE. Likewise, AChE itself may potentiate Aβ toxicity in AD[72,73]. Up-regulated AChE could inhibit cell proliferation and promote apoptosis in AD in AChE positive cells such as osteoblasts and chondrocytes, which are considered more sensitive to apoptotic stimuli than negative cells. Particularly that excess pro-inflammatory cytokines at damaged sites in bone destructive disorders (like OA and osteoporosis) could incite this apoptotic sensitivity[74]. AChE contributes to Ca2+ mobilization, interaction between cytochrome c and apoptotic protease activating factor-1 in the mitochondrial apoptotic pathways, caspase-3 activation, morphological impairments and cell death[75,76]. Furthermore, Sato *et al*.[13] affirmed that AChE overexpression in osteoclasts and their precursors was involved in the RANKL-provoked osteoclastic differentiation of BM-MSCs with the upregulation of RANK mRNA expression on the osteoclastic precursors. For that reason, the occasion of AD-associated osteoporosis and/ or OA was suggested to occur subsequent to the influence of AChE on bone[43].

Significant decrease of AChE protein levels in BM-MSCs treated group compared to group II was found but with an insignificant increase compared to the control group. Accordingly, the aforesaid target and removal of Aβ deposits with BM-MSCs therapy[57] could in turn suppress the Aβ associated monomeric AChE levels[71]. Weist *et al*.[18] confirmed that suppression of upregulated AChE in its producing cells accelerated the endochondral ossification in mice and inhibited apoptosis of osteoblasts[77]. The improbable proliferation of MSCs in the homeostatic conditions was

elucidated to be enhanced by ACh accumulation via mAChR following AChE suppression and in coordination with Wnts, BMP, IFN-γ signaling molecules affecting the intracellular messenger Ca2+ concentration and cAMP of the BM-MSCs to proliferate, migrate and differentiate into osteogenic and chondrogenic lineages[12,53]. Although, AChE mRNA expression was correlated directly with cell differentiation and inversely with cell proliferation so its suppression could increase cell density in a sequence as well as dose-dependent manner[78]. Sato *et al*.[13] found that the regular AChE expression was stimulated by RANKL in BM-MSCs and bone cells while its secretion and activity increased in differentiated osteoblasts[14] in parallel with the alkaline phosphatase levels and activity[70]. Especially that, AChE subserves ACh - independent and/or dependent functions regarding mineralization[79]. Moreover, the decrease of endothelial AChE occurred with ACh accretion in response to VEGF via the proteasome degradation of AChE, could mediate the endothelial VEGF secretion and angiogenesis[77]. The distinct cholinergic as well as growth factor-mediated angiogenic pathways were strongly correlated since the ACH/nAChRs signaling might contribute to the growth factor-mediated angiogenesis in pathological disorders[80]. More than that, MSCs can restrain inflammatory cells by means of the AChE suppression/ACh accrual /nAChR signaling[17] which has also been involved in osteoclastic apoptosis and blockage of RANK-RANKL signaling hence improved the bone mass and quality. At last, AChE suppression could be a favored approach for the treatment of AD-associated osteoporosis and /or OA[13,43].

The requisite for TMJ homeostasis in mice studies is the well coordinated remodeling of the major extracellular components such as collagen types I, II[81]. Collagen I, as an osteogenic protein, plays significant roles in osteoblastic differentiation besides bone mineralization and could be used as a biomarker for osteoblastic differentiation at different stages[14]. Likewise, collagen II is essential for cartilage growth and maintenance and is used as a specific marker for chondrogenic differentiation as well as osteoarthritis[42,82]. That is why we quantified the genes expression by real-time PCR for both collagen types I and II from TMJ specimens. The significantly increased expression of the collagen I anabolic genes than those of collagen II in the control group was assigned to their different distribution in the TMJs of the adult rats implying their diverse function. For collagen II, it is mainly synthesized by chondrocytes in both mature transitional and hypertrophic layers of the TMJ articular cartilage forming 80-90% of its collagenous matrix but the hypertrophic layer was reported to be absent in the TMJ articular cartilages of 9-10 month-old adult rats or older akin to the control animals of this study. Likewise, positive collagen I areas in the 9 month-old rats extended to the whole cartilaginous region suggesting that condylar cartilage becomes fibrocartilaginous tissue with articular function by means of maturity-associated ossification. In addition, collagen I was also detected in the articular disc and subchondral bone[34,83].

As confirmation of all the aforementioned results, a significant diminishing in the anabolic genes of both collagens was illustrated in group II specimens compared to the age-matched control group but with higher significance for collagen I than collagen II. We attributed the decreased gene expression of both collagens to AD-associated TMJ pathologic changes involving disturbed metabolism of subchondral bone and cartilaginous tissue in TMJ-OA as proved by Wang *et al*.[30]. Diverse cellular reactions that were described in OA included deactivation or activation of specific catabolic or anabolic pathways, cell death and severely altered gene expression. However, osteoarthritic chondrocytes were believed to stay in para- or pre-apoptotic phenotype rather than apoptosis with a dis-coordinated micro-heterogeneous pattern of gene expression[4]. These cells might produce collagen type I[34] otherthan type II besides the inhibition of new matrix formation including collagen II[11,42], which hardly turnover in the adult articular cartilage[4]. So, the variable amount of both collagen types was related to age changes, injuries and repair[84]. Comparable to group II and control group, the BM-MSCs group exhibited a significant upregulation in the gene expression of both collagen types but with a particular increase for collagen I than collagen II due to the previously illustrated enhanced ossification subsequent to the properly restored angiogenesis and regular levels of VEGF. Likewise, the suppression of the upregulated AChE permitted the induction of ACh/mAChRs signaling in BM-MSCs resulting in increased proliferation, proline incorporation and enhanced collagen synthesis[85]. Collagen I (the majority of bone organic contents) has a significant role in bone remodeling, maintenance and mineralization[70]. MSCs including BM-MSCs were revealed to promote the osteogenesis-related gene expression such as collagen I by the binding of Runx2 to its gene promoter[14,86]. The gene expression of collagen II has been enhanced in the repair of damaged articular cartilage by MSCs under the control of its gene promoter SOX9 which enhance endochondral ossification in adult rats[42,87]. The herein fluctuated levels of both collagens genes may refer to the different mechanisms by which each collagen type renovates and remodels[84].

CONCLUSIONS AND RECOMMENDATIONS

The detected histopathologic, immunohistochemical and molecular changes of the TMJ components in AD induced rat model highlighted the possible associated alterations with disease progression and subsequent stem cell therapy. The alleviated findings might exceed the normal in some respects particularly for enhanced ossification. Hence, MSCs most probably can treat neuronal and aging-related diseases besides the ensuing peripheral changes. Yet, we have concerns regarding the safety profile of the future therapeutic approach. Regardless of the valuable therapeutic outcomes in our study, several disadvantages of stem cells therapy were reported relating to a reliable source for cells with constant phenotype and tumor formation in some studies. Despite the currently recommended use of MSC-derived exosomes as an alternative therapy since they could recapitulate the biological potentiality of MSCs. Yet, further characterization for numbers of injections and doses should

be précised carefully since increased doses in the MSC field could attenuate the therapy because of the host immune responses[56]. Therefore, we recommend conducting further studies for a therapeutic approach that has the potential to mediate both self-renewal and differentiation of host stem cells to overcome all adverse effects of exogenous grafting.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

ABBREVIATIONS

(TMJ) Temporomandibular joint; **(ALZ)** Alzheimer; **(AD)** Alzheimer's disease; **(BM-MSCs)** bone marrow mesenchymal stem cells; **(OA)** Osteoarthritis; **(VEGF)** Vascular endothelial growth factor; **(AChE)** Acetylcholinesterase; **(ACh)** Acetylcholine; **(nAChR/mAChR)** nicotinic/ muscarinic acetylcholine receptor.

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الملخص العربى

هل تعمل الخلايا الجذعية على التغلب على التغيرات المتوقعة فى هستولوجيا مفصل الفك السفلي وتعبير أستيل كولين استريز في مرض الزهايمر المستحث تجريبياً؟

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نبذة مختصرة: مرض الزهايمر هو اضطراب تنكسي عصبي يتميز باضطراب الدماغ التنكسي التدريجي
الهدف من التجربة: يهدف هذا العمل إلى إلقاء الضوء على الآثار المحتملة لمرض الزهايمر والعلاج اللاحق للخلايا الجذعية الوسيطة لنخاع العظم على المفصل المؤقت للفئران
التجربة: تم تقسيم ٣٠ من الفئران البيضاء الذكور البالغين إلىثلاثة مجموعات متساوية: (عشرة فئران لكل منها) تلقوا حقنة يومية داخل الصفاق ؛ المجموعة الأولى : ٠,٩٪ محلول ملحي ، المجموعتان الثانية والثالثة: ٧٠ مجم / كجم من وزن الجسم الالومنيوم كلوريد المذاب في ماء مقطر. ، تلقت المجموعة الثالثة جرعة واحدة من الخلايا الجذعية بعد ٦ أسابيع من التلقي اليومي من الالومنيوم كلوريد. بعد ٦ أسابيع أخرى ، تم تحضير عينات المفصل الفكي الصدغي للفحص الهستوباثولوجي و الفحص المناعي الكيميائي باستخدام الأجسام المضادة لعامل النمو البطاني الوعائي وأيضا لعمل لطخة ويسترن للأستيل كولين استريز والكشف عن التعبير الجيني الكولاجين (I & II) بواسطة تفاعل البوليمراز المتسلسل بالزمن الحقيقي.
النتائج: بالمقارنة مع المجموعة الأولى ، اظهر الفحص الهستولوجي انه بالمقارنة مع المجموعة الأولى ، اظهرت عينات المجموعة الثانية تعظمًا غضروفيًا معوقًا وفُصال عظمي وهشاشة لعظام لمكونات المفصل الفكي الصدغي و تم التحقق من خلال زيادة التعبير لعامل النمو البطاني الوعائي الملحوظة. كل هذه النتائج تناقصت جميعًا بشكل كبير في المجموعة الثالثة مع استمرار وجود زيادة كبيرة لإيجابية لعامل النمو البطاني الوعائي مقارنة بالمجموعة الأولى. الزيادة الكبيرة في تعبير الأستيل كولين استريز والتناقص الملاحظ في التعبير الجيني لكولاجين I & II في المجموعة الثانية مقارنة بالمجموعة الاولي وجد تحسنهم في المجموعة الثالثة مع استمرار وجود زيادة مقارنة بالمجموعة الاولي وقد كانت هذه الزيادة ملحوظة بالنسبة لجين الكولاجين I وغير ملحوظة لكل من التعبير الجيني الأستيل كولين استريز ولكولاجين II.
الاستنتاج: النتائج التي تحسنت بعد العلاج بالخلايا الجذعية قد تتجاوز المعدل الطبيعي في بعض النواحي كالتعظم المحسن. على الأرجح يمكن للخلايا الجذعية ان تعالج الأمراض العصبية و الامراض المرتبطة بالشيخوخة المرتبطة بالاضافة إلي التغيرات الطرفية التي تلت ذلك. ومع ذلك ، نوصي بإجراء المزيد من الدراسات للنهج العلاجي الذي يمتلك القدرة على التوسط للتجديد الذاتي ولتمايز الخلايا الجذعية الموجودة بالفعل للتغلب على جميع الآثار السلبية المحتملة للتطعيم الخارجي.