Mesenchymal stem cells-derived microvesicles versus platelet-rich plasma in the treatment of monoiodoacetate-induced temporomandibular joint osteoarthritis in Albino rats

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ABSTRACT
Temporomandibular joint osteoarthritis (TMJ-OA) is a serious disease, designated by severe joint pain and dysfunction. Limitations of current therapeutics have led to an increased interest in regenerative strategies. Recently, the non-surgical treatment of OA has seen increased use of biologic injectable therapies like mesenchymal stem cells (MSCs) and platelet-rich plasma (PRP). Although these biotherapies represent an admirable effort, more studies are necessary to determine their efficacy. Thus, the aim of this study was to assess the curative potential of a single intra-articular injection of bone marrow MSCs-derived microvesicles (BM-MSCs-MVs) versus a single intra-articular injection of PRP in monoiodoacetate (MIA)-induced TMJ-OA model in Albino rats. Forty-eight male rats were used. A single intra-articular unilateral MIA injection was utilized to induce TMJ-OA. One week post induction, rats were sorted into 3 groups (16 rats each): group (I): received no treatment, groups (II) & (III): received BM-MSCs-MVs and PRP respectively. Scarification was done at 2 and 4 weeks from onset of treatment. Histological changes of the condylar TMJ were examined with H&E staining. Expression of IL-1β, TNF-α, NF-κB, MMP-13, MMP-3, and collagen II markers was detected using real-time PCR. Histologically, the osteoarthritic group exhibited degenerated condylar tissues which were aggravated at 4 weeks. Oppositely, a marked improvement in the condylar TMJ histology was noticed in both the BM-MSCs-MVs-and PRP-treated groups at both time intervals. Additionally, the treated groups showed a decrease in IL-1β, TNF-α, NF-κB, MMP-13 and MMP-3 and an increase in collagen II genes expression in contrast to the untreated group. Moreover, this difference was significant in the BM-MSCs-MVs group as compared to the PRP-treated group. Our results concluded that BM-MSCs-MVs as well as PRP treatments were able to target the key pathological features in OA, mainly inflammation and matrix degradation, and helped in restoring condylar structure in TMJ-OA rat model. However, BM-MSCs-MVs treatment exhibited more efficient therapeutic potential as compared to PRP treatment.

1. Introduction
Temporomandibular joint (TMJ) disorders are a collection of illnesses that result in discomfort and dysfunction in the jaw joint and the muscles controlling jaw movements. Such disorders have numerous and complex etiologies, including occlusal abnormalities, psychological, social, cognitive, and systemic factors [1, 2]. It was reported that patients with systemic sclerosis had trouble opening their mouth, besides difficulties in mandibular movements excursion, among other indicators of TMJ dysfunction [3]. Moreover, patients with juvenile idiopathic arthritis can suffer from unstable occlusion, changes in masticatory function, asymmetric loading of the joints and muscles, and TMJ pain [4].

TMJ osteoarthritis (OA) is a common form of TMJ disorders. It is a debilitating illness that is marked by progressive cartilage deterioration, synovitis, subchondral bone remodelling, and chronic pain [5]. The majority of TMJ-OA cases have a multifactorial or unclear etiology. One of the main causes of this disease is overloading of the TMJ, which involves significant malocclusion, muscle overuse, and skeletal jaw asymmetry [6]. The patients of TMJ-OA suffer from TMJ arthralgia, limited

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mandibular range of motion, impairment in chewing function, clicking sound, and stiffness [7].

Most of the traditional non-surgical clinical treatments for TMJ-OA including, occlusal splints, physical therapies, arthrocentesis, and nonsteroidal anti-inflammatory drugs, are directed towards alleviating pain and slowing the progression of the disease. However, these treatments can’t fully heal deteriorated cartilage or subchondral bone lesions, as well as disc degeneration [8, 9].

Mesenchymal stem cells (MSCs) served as a precious cell reservoir for repair and regeneration of the TMJ different animal models [10, 11]. The majority of their therapeutic effects are attributed to the secretion of a wide spectrum of mediators with potent regenerative, anti-inflammatory, and immunomodulatory properties [12, 13]. These mediators are transported within extracellular vesicles (EVs) released by the MSCs [14].

MSCs-EVs’ regenerative activity has been reported in several animal models, including those for myocardial infarction, brain, lung, liver and acute renal injury [15], rheumatic diseases [16], fracture healing [17], and neurodegeneration [18]. When compared to direct cell transplantation, EVs are considerably simpler, safer, more practical, and easier to manipulate [19]. There are various forms of EVs, however the most typically reported vesicles are microvesicles, exosomes, and apoptotic bodies [20].

Microvesicles (MVs) are a diverse population of spherical structures with diameters ranging from 50–1000 nm [21]. Their biosynthesis necessitates vertical transportation of molecular content to the cell membrane, membrane lipid reallocation, and the utilization of contractile machinery at the surface to allow for vesicle pinching [22]. Researchers have realized the important role of MVs in modifying the extracellular environment and intercellular signaling via their capability to convey biologically active compounds [23].

Platelet-rich plasma (PRP) is blood plasma composed of concentrated platelets. It represents a promising regenerative therapy for injuries in the orthopaedic field, having anti-inflammatory, analgesic, and antibacterial properties [24, 25]. It has been used in intra-articular injections to treat temporomandibular disorders in a number of clinical trials with encouraging results where the included patients had to have imaging verified TMJ osteoarthritis or TMJ dysfunction. However, those with systemic illnesses were excluded [26, 27, 28]. PRP has been reported to restore intra-articular hyaluronic acid, enhance chondrocyte glycosaminoglycan synthesis, balance joint angiogenesis, and act as a scaffold for the migration of stem cells [26].

Due to the limitations of the current TMJ disorder therapies, there has been a surge in interest in regenerative approaches, which needs more supportive studies to draw a definite conclusion about their efficacy. So far, preclinical in-vivo studies comparing the effectiveness of different kinds of biotherapies in relation to each other in TMJ osteoarthritic models are very limited. Also, the ability of a single injection to induce positive therapeutic effect needs to be clarified. Thus, the aim of the present research was to assess and compare the curative potential of a single intra-articular injection of bone marrow (BM)-MSCs-derived MVs and PRP on TMJ-OA in Albino rats, through histopathological and quantitative real-time PCR (qRT-PCR) examination techniques.

2. Materials and methods

2.1. Preparation, isolation and identification of BM-MSCs

Extirpated bone marrow cells from 5 male Albino rats’ (6 weeks old) tibia were washed in phosphate buffer saline (PBS) three times. 15 ml of extirpated bone marrow cells were layered over 15 ml. Ficoll-Paque” (Gibco/Invitrogen, USA) then centrifuged (400g, 35 min). After aspiration of the upper cell layer, the undisturbed mononuclear cell layer was aspirated, washed twice in PBS, and centrifuged (200g, 10 min). The isolated cells were then grown in RPMI-1640 media supplemented with 0.5% penicillin and streptomycin, 10% PBS (Gibco/Invitrogen, USA) and kept at 37 °C in a cell culture incubator (5% CO2) until reaching 80–90% confluence. BM-MSCs were then detached by trypsin/EDTA for 5–10 min at 37 °C and sub-cultured in new plates. BM-MSCs at third passage were used. MSCs were recognized by their shape. They were suspended (1×10^6 cells/ml) for further identification by fluorescence activated cell sorting (FACS) through assessment of CD90^+ , CD105^+ positivity, and CD14^- negativity [29, 30].

2.2. Preparation, isolation and characterization of BM-MSCs-MVs

MVs were acquired from the supernatant of BM-MSCs (5×10^6 cells/ml), cultured in RPMI without FBS and the cells were starved for 24 h, using a filtration/centrifugation-based protocol. Centrifugation at 2000g for 20 min at 4 °C was used to remove cellular debris. The supernatant was ultracentrifuged at 100000g for 1 h at 4 °C to pellet MVs, after which it was washed in serum-free media and subjected to another round of ultracentrifugation. The resulting pellets containing MVs were washed once with sterile PBS [31]. Protein concentration was quantified by Bradford protein assay kit (Thermo Scientific, USA). MVs were identified using transmission electron microscope (TEM) and by detecting CD63 and CD81 via enzyme-linked immunosorbent assay (ELISA).

2.3. Characterization of MVs using TEM

MVs were washed, then fixed for 2 h with 2.5% glutaraldehyde. 1% osmium tetroxide in 0.1 M PB was employed for 1 h for post-fixation. After rinsing with sodium maleate buffer, the fixed MVs were subjected to dehydration in graded ethanol followed by infiltration and embedding in beam capsules. Sections were mounted onto a carbon-formvar grid, negatively stained for 1 min with 1% uranyl acetate, and observed by TEM (JEOL, JEM -2100, Japan) [32].

2.4. Characterization of MVs using ELISA

Binding buffer (200 µl) (System Biosciences Inc.) was applied to the pellets. Pellets were then incubated for 10 min at room temperature. 50 µl of each marker’s preparation was then applied to microtiter plate’s wells. The plate was incubated overnight at 37 °C. Following washing with a working buffer (System Biosciences Inc.), 50 µl of anti-CD63 and anti-CD81 (ELISA kits) (System Biosciences Inc.) were added to the wells, and then incubated at 37 °C for 60 min. After another wash, the plate was incubated for an hour at room temperature with 50 µl of horseradish peroxidase (HRP) enzyme-linked secondary antibody (goat anti-rabbit). To stop the reaction, a colorimetric substrate and stop solution from Sunnyvale, CA. Protein amount was compared to the standard curve generated by the kit. For each concentration (pg/ml) of the two investigated proteins, the results were expressed as mean ± SD [32].

2.5. Preparation of PRP

A total of 3 ml of blood was taken from the retro-orbital vein of previously anesthetized animals. The tubes containing the blood samples were centrifuged at 500g for 10 min resulting in three layers; inferior red cells, intermediate white cells, and superficial plasma. The upper portion of the supernatant containing platelets and plasma was collected into fresh tubes and centrifuged for 10 min at 2200g. The resulting upper poor-platelet plasma was separated and stored in different tubes, while the residual material bearing the platelet pellet was gently aspirated and placed in a sterile tube originating the platelet-rich plasma portion [33].

2.6. Experimental study design

2.6.1. Animals

All animal protocols were carried out according to the Institutional Animal Care and Use Committee (IACUC) of Cairo University (approval number; CU/III/F/31/19). In this investigation, forty-eight adult male
Albino rats (3–4 months), weighing 150–170 gm, were employed. The animals were housed in a climate-controlled setting with a 12-hour light/ dark cycle, as well as easy access to water and food.

2.6.2. Induction of TMJ-OA model
An intraperitoneal injection of a ketamine and xylazine mixture was used to anaesthetize all of the experimental rats. OA was induced with a single intra-articular injection of 2 mg/joint of monosodium iodoacetate (MIA) (Sigma-Aldrich Chemical Co., St. Louis, USA) in 50 μL sterile 0.9% saline solution into each rat's right TMJ [34].

2.6.3. Animal grouping
One week after OA induction, the animals were randomly separated into 3 groups, each with n = 16. Group I (OA) received 50 μL PBS. Group II (OA + MVs) received 100 μg MVs/50 μL PBS [35]. Group III (OA + PRP) received 50 μL PRP [36]. The injections were administered as a single intra-articular injection in the right TMJ.

2.6.4. Animals' scarification and tissue preparation
At 2 and 4 weeks post-treatment, animals from each experimental group (n = 8) were euthanized, using anaesthetic overdose. The heads were then sagittally dissected. The rats' TMJ tissue specimens were then collected.

2.6.4.1. Histopathological examination.
The obtained samples were fixed in 4% paraformaldehyde, and demineralized in 15% ethylene diamine tetra-acetic acid (EDTA). The samples were then dehydrated in alcohol blocks. Haematoxylin and Eosin (H&E) stained sections (4–5 μm thick) were put on standard glass slides for histological assessment.

2.6.4.2. Quantitative real-time PCR.
The mRNA gene expression of interleukin-1-beta (IL-1β), tumour necrosis factor-alpha (TNF-α), nuclear factor kappa B (NF-κB), matrix metalloproteinase-13 (MMP-13), matrix metalloproteinase-3 (MMP-3), and collagen II was investigated. GAPDH was used as the internal control. The condylar tissue samples were homogenized and the total RNA was extracted in accordance with the manufacturer's instructions utilizing a QiaGen extraction kit (Qiagen, USA). Total RNA was converted to complementary DNA (cDNA) using cDNA reverse transcription kit (Fermentas, USA).

Subsequent real-time qPCR amplification and analysis were performed. SYBR Green Master Mix (Applied Biosystems), and gene-specific primer pairs (Table 1) were used in the reaction. Amplification protocol involved: enzyme activation (95 °C, 15 min) then followed by 40 cycles of denaturation (95 °C, 30 s) and annealing/extension (60 °C, 60 s). The comparative threshold cycle approach was used to calculate the relative expression of the studied genes using Applied Bio System software [37].

2.7. Statistical analysis
Values were analyzed using SPSS (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) computer software version 22. Data were expressed as mean ± SD. Data normality was explored using the Kolmogorov-Smirnov test. Results revealed normally distributed data. Thus, analysis of variance (ANOVA) test followed by Post-hoc Tukey test were employed to compare between groups. Comparisons between two durations within the same group were analyzed using a paired t-test. Correlation between various studied variables was performed using Pearson's correlation. P value < 0.05 was considered as statistically significant.

3. Results

3.1. Characterization of BM-MSCs
BM-MSCs were recognized by their spindle-fusiform appearance (Figure 1a). Furthermore, FACS analysis indicated the purity of BM-MSCs where they showed positivity for CD90+ (99.3%), CD105+ (97.5%), and negativity for CD14− (3.6%) (Figure 1b).

3.2. Characterization of BM-MSCs-MVs
BM-MSCs-MVs were characterized by their size (average diameter 79 nm) and their spherical shape as identified under TEM (Figure 2a). In addition, MVs were positive for CD63 (179.4 ± 5.662 Pg/ml), and CD81 (241.6 ± 8.817 Pg/ml) markers as shown by ELISA (Figure 2b).

3.3. Histopathological results

3.3.1. At 2 weeks

The articular surface of the rat condylar TMJ of group I (OA) revealed the condyle with abnormal configuration. The fibrocartilaginous layer demonstrated disrupted cellular and fibrillar arrangement. The cartilaginous layer displayed focal regional loss of chondrocytes as well as areas of vacuolization and atrophy. Cartilage chondrocytes showed abnormal arrangement, clustering, as well as signs of degeneration with empty lacunae. The subchondral bone presented distorted bone architecture with absence of regular bone trabecular alignment. Most of the osteocytes were degenerated and showed disorganized alignment. Widened bone marrow cavities with apparent loss of osteoblastic lining as well as abnormal marrow fibrosis was noticed (Figure 3a).

Regarding group (II) (OA + MVs), well organized fibrocartilaginous layer overlying relatively thin but properly aligned hyaline cartilage layer was observed. The chondrocytes displayed well stained basophilic nuclei. Some mitotic figures were also observed in the cartilaginous layer. Subchondral bone demonstrated closely packed lamella enclosing organized osteocytes in their lacunae. Multiple resting lines were also detected. Osteoblasts were seen partially lining the intervening marrow cavities. Most of the marrow cavities enclosed obvious cellular infiltrate (Figure 3b).

In group III (OA + PRP), the fibrocartilaginous tissue demonstrated dense acellular fibrous layer that seemed to be hyalinized. The cartilaginous layer displayed appropriate chondrocyte arrangement. Empty chondrocytic lacunae were observed in some areas. The underlying subchondral bone assumed relatively regular appearance enclosing few osteocytes. Widened bone marrow cavities enclosing high cellular infiltrate, extravasated red blood cells, and vascular congestion were also detected (Figure 3c).

3.3.2. At 4 weeks

Marked histological distortion in the microstructure of the condylar TMJ of group I (OA) was observed. The fibrocartilaginous tissue showed abnormal fibers’ orientation and disrupted cellular arrangement. Complete loss of the underlying hyaline cartilaginous layer was
demonstrated. The structure of the subchondral bone was obviously deteriorated. Fibrous tissue infiltration was seen advancing into some areas of subchondral bone replacing distorted bony trabeculae. Other bone areas enclosed irregular oriented few osteocytes where some lacunae were empty. Spaces resembling marrow cavities were also observed where most of them were obscured by fibrous tissue as well as extravasated red blood cells (Figure 3d).

In group II (OA + MVs), a fibrocartilaginous layer with evenly aligned fibers and cells was seen overlying a well-developed cartilage layer. The chondrocytes were intact and properly oriented. The subchondral bone displayed uniform configuration with regular trabeculation enclosing orderly arranged osteocytes. Most of the bone marrow cavities were outlined by osteoblastic cells and filled with fibrocellular tissue (Figure 3e).

A thick fibrocartilaginous layer with focal matrix discontinuity was observed in group III (OA + PRP). Its cellular and fiber orientation were relatively disoriented in many areas. The underlying cartilaginous layer was well identified with its characteristic chondrocytes. In some areas, the arrangement of the chondrocytes was disordered. The subchondral bone enclosed osteocytes in their lacunae. The bone trabecular boundaries enclosed multiple widened marrow cavities lined by osteoblasts and partially filled with fibrocellular tissue (Figure 3f).

3.4 Quantitative real-time PCR analysis

A difference was assessed in the expression of genes associated with pro-inflammatory (IL-1β, TNF-α and NF-κB), matrix degradation (MMP-13 and MMP-3) and matrix synthesis (collagen II) at 2 and 4 weeks, between all groups using ANOVA (p ≤ 0.001). Multiple pairwise group comparisons were then performed within each time interval (Figure 4).

Within group I (OA), there was an increase in IL-1β (p = 0.001), TNF-α (p = 0.017), NF-κB (p = 0.015), MMP-13 and MMP-3 (p = 0.017) expression at 4 weeks compared to 2 weeks. On the other hand, the expression was decreased with time within group II (OA + MVs) and III (OA + PRP); IL-1β (p = 0.017, p = 0.002), TNF-α (p < 0.001, p = 0.001), NF-κB (p < 0.001, p = 0.008), MMP-13 (p = 0.038, p = 0.004) and MMP-3 (p = 0.004, p < 0.001) respectively (Figure 4a-e).

Relative to OA group, mRNA gene expression of IL-1β, TNF-α, NF-κB, MMP-13 and MMP-3 in the OA + MVs group was decreased at 2 and 4 weeks (p < 0.001). Similarly, OA + PRP group showed a decrease in IL-1β (p = 0.002 at 2 weeks and p < 0.001 at 4 weeks), TNF-α, NF-κB, MMP-13 and MMP-3 (p < 0.001 at 2 and 4 weeks) as compared to OA group (Figure 4a-e).

For IL-1β gene expression in MVs treated group, it was lower compared to that of PRP treated group, however this decrease was not statistically significant at 2 weeks (p = 0.079). By 4 weeks, this decrease was statistically significant (p = 0.021). Regarding other markers, a decrease in their expression was recorded in MVs group as compared to PRP group during 2 and 4 weeks time intervals (TNF-α, p = 0.025, p = 0.007; NF-κB, p < 0.001, p = 0.038; MMP-13, p < 0.001, p = 0.03 and MMP-3, p = 0.01, p = 0.002) respectively (Figure 4a-e).

For collagen II gene expression, within group I, there was a decrease in its expression at 4 weeks compared to 2 weeks (p = 0.01) while, within group II and III, the expression was increased with time (p = 0.001 and p < 0.001 respectively). Moreover, an increase in collagen II expression in OA + MVs group (p < 0.001 at 2 and 4 weeks) compared to OA group was evident. In parallel, the OA + PRP group demonstrated an increase in the gene expression (p = 0.003 at 2 weeks and p = 0.001 at 4 weeks) relative to OA group. The increase in collagen II expression in MVs group as compared to PRP group was non-significant at 2 weeks (p = 0.1), however, it was significant at 4 weeks (p = 0.02) (Figure 4f).

At 2 and 4 weeks time interval, Pearson’s correlation among the studied genes showed a positive correlation present among IL-1β, TNF-α, NF-κB, MMP-13 and MMP-3. While collagen II showed a negative correlation with IL-1β, TNF-α, NF-κB, MMP-13 and MMP-3 (Tables 2 and 3).
Figure 3. Photomicrographs of sagittal sections in the articular surface of rat condylar TMJ (a) Disrupted fibrocartilaginous layer (bracket), matrix vacuolization (V), misaligned cartilaginous layer (C), regional loss of chondrocytes (asterisk), chondrocytes clustering (dotted circle), degenerated chondrocytes (dotted arrow), distorted subchondral bone (Sb), widened bone marrow cavities (Bm), loss of osteoblastic lining (arrows), abnormal marrow fibrosis (star). (b) organized fibrocartilaginous layer (bracket), properly aligned cartilaginous layer (C), mitotic figure (dotted arrow), subchondral bone (Sb), resting lines (notched arrows), marrow cavities (Bm), partial osteoblastic lining (arrows), marrow cellular infiltrate (asterisks). (c) fibrocartilaginous layer with cellular atrophy (bracket), relatively aligned cartilaginous layer (C), empty chondrocytic lacunae (dotted arrow), subchondral bone (Sb), widened marrow cavities (Bm), marked marrow cellular infiltrate (asterisks), extravasated red blood cells (arrow), congested blood vessels (Bv). (d) disrupted fibrocartilaginous layer (bracket); Note: complete loss of underlying cartilaginous layer, distorted bone trabeculae (stars), fibrillar infiltration (asterisks), empty osteocytic lacunae (dotted arrows), resting lines (arrows), extravasated red blood cells (notched arrow). (e) well-arranged fibrocartilaginous layer (bracket), well developed cartilaginous layer (C), subchondral bone (Sb) with osteocytes in their lacunae (dotted arrows), marrow cavities (Bm), osteoblastic lining (arrows), fibrocellular tissue (asterisks). (f) fibrocartilaginous layer (bracket) with focal matrix discontinuity (arrows), relatively disordered cartilaginous layer (C), subchondral bone (Sb), osteocytes (dotted arrow), marrow cavities lined by osteoblasts (notched arrows), fibrocellular tissue (asterisks) (a-f; H&E, Orig. Mag. × 400).

4. Discussion

TMJ disorders are a broad category of clinical conditions that affect the TMJ, and other structures connected to the masticatory system. They have a range of etiologies, including congenital, inflammatory, and traumatic [38]. Due to the shortcomings of existing treatments for TMJ problems, interest in regenerative approaches; combining cells, implantable scaffolds, and carefully targeted bioactive chemicals has increased [39]. TMJ-OA is a serious form of TMJ dysfunctions [40]. The use of biologic injectable treatments such as MSCs and PRP in the non-surgical therapy of OA have recently become popular [41]. Although these biotherapies are a commendable effort, further research is required to determine their effectiveness.

In this research, the therapeutic potential of a single intra-articular injection of BM-MSCs-MVs in MIA-induced TMJ-OA model was assessed and compared with a single intra-articular PRP injection. We demonstrated that injection of BM-MSCs-MVs could effectively restore the damaged condylar tissue better than PRP.

Male rats were employed in this study to prevent hormonal fluctuations from affecting the findings since the expression of estrogen receptors α and β in TMJ suggests that the TMJ is an estrogen target. Additionally, it was established that estrogen accelerated the progression of TMJ-OA as it caused cartilage deterioration, subchondral bone sclerosis, and erosion in a rat model of induced TMJ-OA [42].

In this investigation, the TMJ-OA model was set up by injection of MIA. The intra-articular injection paradigm of MIA is frequently utilized for pain research and therapeutic intervention efficacy evaluation, since this chemical model closely resembles the alterations seen in patients [43]. However, one of the MIA-induced TMJ-OA model disadvantages is the lack of serious histopathological alterations associated with TMJ-OA, such as cartilage vertical splitting and subchondral bone exposure [44].

Regarding the histological results in the current study, the condylar tissue including the fibrocartilage, cartilage as well as subchondral bone of the untreated OA group (I), showed time dependent degenerative changes in response to intra-articular MIA injection. Marked chondrocyte degeneration was observed at 2 weeks. These changes were aggravated after 4 weeks with complete loss of the cartilaginous layer. Our results were in line with previous studies which demonstrated successful OA-like lesions induced by MIA [35, 44]. It has been previously reported that MIA blocks glyceraldehyde-3-phosphate dehydrogenase, which causes chondrocyte death [45]. Moreover, MIA was found to induce an imbalance between anabolic and catabolic genes, concerned with cartilage and subchondral bone metabolism, which is considered a crucial aspect that contributes to condylar disintegration [46, 47]. Obvious distortion of osteochondral junction was also observed in the OA group of the present research. This might suggest a disturbance in the biochemical and biomechanical crosstalk between cartilage and bone that may contribute to joint deterioration [48].

In this study, rats with induced TMJ-OA were treated with either BM-MSCs-MVs (group II) or PRP (group III). An intra-articular injection was the administration method used for both types of treatment. This was based on previous reported protocols which proved the effectiveness of such a method of administration [49, 50].

The present results showed a marked histological improvement with almost complete structural restoration in condylar cartilage as well as subchondral bone after 4 weeks in the BM-MSCs-MVs-treated group (II). Chondrocyte proliferation was denoted by the presence of mitotic figures. Consistent with our findings, MSCs-EVs were reported to enhance migration and proliferation of chondrocytes as well as stimulate the
expression of chondrocyte markers in-vitro [14]. Moreover, improvements in cartilage histological parameters with subsequent cartilage regeneration and prevention of OA progression in osteoarthritic models treated with MSC-EVs were demonstrated in-vivo [14, 51, 52].

In the current study, some signs of condylar tissue degeneration as well as bone marrow lesions were still evident in the PRP-treated group. This suggests incomplete restoration of condylar tissue histology. However, as compared to the OA untreated group, PRP treatment had

Figure 4. A graph showing the gene expression of (a) IL-1β, (b) TNF-α, (c) NF-κB, (d) MMP-13, (e) MMP-3, (f) Collagen II, in all study groups at 2 and 4 weeks. Mean values with different capital letters indicate statistically significant difference between groups utilizing Post-hoc Tukey test at 2 weeks. Mean values with different small letters indicate statistically significant difference between groups utilizing Post-hoc Tukey test at 4 weeks. * denotes significant difference within each group across various time intervals (2 and 4 weeks) using paired t-test. Significance level was adjusted at P < 0.05.

Table 2. Correlation between all 6 examined genes at 2 weeks.

|       | IL-1β | TNF-α | NF-κB | MMP-13 | MMP-3 | Collagen II |
|-------|-------|-------|-------|--------|-------|-------------|
| **r** | 1     | .936**| .867**| .864** | .881**| -.767**     |
| p value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.001       |
| **r** | .936**| 1     | .897**| .927** | .896**| -.760**     |
| p value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.001       |
| **r** | .867**| .897**| 1     | .912** | .942**| -.899**     |
| p value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001      |
| **r** | .936**| .927**| .912**| 1     | .899**| -.885**     |
| p value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001      |
| **r** | .881**| .896**| .942**| .899**| 1     | -.902**     |
| p value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001      |
| **r** | .767**| .760**| -.899**| -.885**| -.902**| 1           |
| p value | 0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001      |

** Correlation is significant at the 0.01 level (2-tailed).
noticeably attenuated condylar tissue degeneration. This is consistent with previous studies, where they reported decreased cartilage degenerative changes and increased cartilage thickness in OA models treated with PRP [53, 54]. Oppositely, a former study reported no statistical difference in cartilage and subchondral bone healing between the OA and the PRP-treated group in the TMJ-OA model in rabbits [55]. This inconsistency among different reported research may be attributed to the different methods utilized to prepare PRP solutions in the different experimental models [56, 57], leading to a lack of standardized procedure and thus the evaluation of outcomes may have been influenced. Furthermore, the PRP content, such as the concentration of certain growth factors or cytokines, could affect the PRP properties and thus affect its effectiveness [58].

IL-1β and TNF-α are two important proinflammatory cytokines associated with TMJ-OA [59]. These mediators play an important role in the degeneration of cartilage and bone joint by releasing proteinases and other inflammatory molecules [60]. Both inflammatory cytokines were found to activate NF-κB signaling pathway [61]. NF-κB proteins are found inactively attached to inhibitory protein IκB in the cytoplasm. Cell stimulation through IL-1β or TNF-α induces nuclear translocation of NF-κB to trigger the expression of a variety of regulatory genes involved in inflammation, apoptosis, and other immune responses [62]. NF-κB is an important transcription factor in the pathology of OA [63]. Several studies have shown that NF-κB signaling is highly activated in OA [59, 64, 65]. Thus, mRNA gene expression of IL-1β, TNF-α and NF-κB was examined by qRT-PCR in the current work.

We demonstrated that treatment with either MVs or PRP was associated with a decrease in the expression of IL-1β, TNF-α and NF-κB genes in contrast to the OA control group. These findings indicate that both types of treatment were able to downmodulate NF-κB pathway mainly through down regulation of the inflammatory cytokines (IL-1β and TNF-α), thereby triggering the resolution of inflammation in TMJ-OA.

In line with our results, a previous study showed that weekly intra-articular administration of human embryonic MSCs derived vesicles in immunocompetent rat model of TMJ-OA reduced the expression of genes associated with pro-inflammation such as IL-1β and TNF-α compared to OA untreated rats [35]. Another study reported that binding of NF-κB to DNA in the nucleus of OA chondrocytes was reduced by MVs-derived from human adipose tissue MSCs [66]. Moreover, it was demonstrated that the phosphorylation status of NF-κB signaling molecules was blocked by human BM-MSCs−EVs [67].

As for PRP, it was found that PRP inhibited the inflammatory process in OA chondrocytes through the reduction of IL-1β-induced NF-κB activation [68]. Additionally, PRP in a dose-dependent manner suppressed the NF-κB pathway, with a marked reduction in IκB phosphorylation and NF-κB nuclear translocation [69]. On the contrary, another study demonstrated that pure platelet-rich plasma showed no effect on the translocation of NF-κB in human articular osteoarthritic chondrocytes [70].

In the pathophysiology of OA, matrix metalloproteinases (MMPs) are a class of enzymes that induce cartilage extracellular matrix turnover and disintegration [71]. MMP-13 is linked to the aggressive breakdown of type II collagen in OA, which leads to articular cartilage deterioration [72]. Other matrix molecules targeted by MMP-13 include types I, III, IV, IX, X collagen, perlecan, osteonectin, and proteoglycan [73]. A former study documented that increased MMP-13 mirrored the cartilage disintegration in MIA-induced OA [74]. MMP-3 is another MMP that is primarily expressed in OA cartilage. It is capable of cleaving denatured collagens, aggrecan, link proteins, laminin, and fibronectin [75]. MMP-3 levels have been found to be higher in arthritic conditions [76, 77], pointing to the significance of this MMP in cartilage proteoglycan release [78].

In our work, MMP-13 and MMP-3 mRNA expression was assessed in the condylar tissue. We observed that MVs treatment reduced the gene expression of both MMPs as contrasted to the OA control group at time intervals of 2 and 4 weeks. Our findings went in accordance with former investigations that demonstrated the ability of MSCs-EVs to reduce MMP-13 and MMP-3 expression in an OA model [35, 66].

In line with group II, treatment of osteoarthritic rats with PRP (group III) in this work, decreased the expression of MMP-13 and MMP-3 in contrast to the OA untreated group. Our results supported a previous publication which found that PRP treatment reduced catabolic markers including MMP-3, MMP-13, and IL-6 in osteoarthritic chondrocytes in-vitro [79]. The statistical reduction in MMP-13 and MMP-3 expression in the current study, observed in both groups II and III, suggests attenuation in tissue matrix degradation, which could provide a way to restore matrix homeostasis.

The extracellular matrix (ECM) of articular cartilage is composed mainly of a network of type II collagen, other minor collagen including VI, IX, X, XI, proteoglycans as well as non-collagenous proteins [80]. The integrity of cartilage is maintained by the presence of type II collagen and proteoglycans which are degraded by the expression of MMPs [81]. Thus, in the present study, the restoration of cartilage ECM was analyzed through investigating gene expression of collagen type II. Our results demonstrated that the gene expression of collagen II was increased in both groups II and III in comparison to group I. This finding indicated that MVs as well as PRP treatment succeeded in stimulating the anabolic response of the OA damaged cartilage.

A series of previous studies agreed with our results. BM-MSC-EVs were shown to improve the content of proteoglycans and upregulates COL2A1 gene expression in OA chondrocyte cultures [82]. Additionally, Zhang et al. [51] demonstrated that MSC-EVs promoted cartilage regeneration and enhanced matrix synthesis of type II collagen in a model.
of osteochondral defect in immunocompetent rats. Similarly, the beneficial effect of PRP in stimulating the deposition of collagen type II, and regeneration of articular cartilage in arthritis model was previously reported [83, 84, 85].

Statistical correlation between all the studied genes in the current investigation was also examined. A positive correlation was observed among IL-1β, TNF-α, NF-κB, MMP-13 and MMP-3. While there was a negative correlation present with collagen II and (IL-1), TNF-α, NF-κB, MMP-13, MMP-3. Our findings demonstrated that there is a collaborative activity between the inflammatory mediators and the induction of matrix degradation as well as inhibiting collagen synthesis. It was reported that in OA, the chondrocytes shift to a degenerative phenotype and produce various NF-κB mediated catabolic chemokines and cytokines, which enhance the production of numerous degenerating enzymes, including MMP-13, reduce proteoglycan and collagen formation, causing articular cartilage damage and act in a positive feedback loop to enhance NF-κB stimulation [86, 87].

Going through analyzing our qRT-PCR data at 4 weeks time interval, there was a decrease in the expression of inflammatory and matrix degrading genes as well as an increase in matrix construction gene in the MVs-treated group in comparison to the PRP-treated group. These results supported our histological results in that BM-MSCs-MVs had better therapeutic potential as compared to PRP treatment.

MVs are one of many other trophic secretions including exosomes, growth factors, chemokines, cytokines as well as ECM released by MSCs. All these secretions are contained in the conditioned medium of MSCs [88]. The potentiality of BM-MSCs-MVs to exert a protective and pro-regenerative effect in this study, might be explained by its ability to communicate with recipient cells in a variety of ways. They can activate intracellular signaling pathways by interacting with cell surface receptors via their transmembrane proteins. Furthermore, they can delivercargo to target cells either directly by fusing into the cell membrane or through endocytosis [21]. The combined functional complexity of MSCs-EVs cargo provides them with the ability to exert broad therapeutic efficacies. MSCs- MVs were reported to possess complex regulatory components including proteins, and different nucleic acids including messenger RNAs, non-coding RNAs, and microRNAs [89, 90]. Cargo proteins play a significant role in cellular communication, structure and dynamics, immunological regulation, and tissue regeneration [91]. Notably, microRNAs are considered the key component of EVs. Through their modulation of the inflammatory response, chondrocyte survival, and extracellular matrix deposition, microRNAs play crucial roles in treatment of OA [92].

The presence of various important biological active molecules in PRP is considered the biological rationale behind the positive therapeutic effect demonstrated by PRP-treated group in the current study. PRP was reported to contain a great amount of growth factors released from the highly concentrated platelets, such as platelet-derived, transforming, vascular endothelial, insulin-like, fibroblast, and epidermal growth factors [93]. It has been suggested that these growth factors work synergistically, resulting in stimulating the proliferation and differentiation of fibroblasts, osteoblasts, chondrocytes, and MSCs [94]. PRP alpha granules are also considered a source of chemokines, cytokines, and other proteins that aid in chemotaxis, cell proliferation and maturation, inflammatory molecule modulation, and leukocyte attraction [95]. Moreover, histamine, adenosine, dopamine, serotonin, and calcium ions stored in platelets’ dense granules play a complex function in tissue regulation and regeneration [96].

Finally, according to our discussion, although several previous preclinical studies provided evidence on the therapeutic effect of both MVs and PRP, comparing the effectiveness of a single injection dose of each kind of therapy in relation to each other was not previously reported as far as we know. Our results provided evidence that BM-MSCs-MVs treatment was more effective than PRP treatment regarding delaying the progression of OA and that a single injection of both kinds of treatment succeeded in providing positive therapeutic activity.

However, this study still has several limitations. First, we did not evaluate the effect of the used treatments in-vitro as our primary aim was to evaluate the structural changes involved in cartilage reconstruction in vivo. Second, we did not analyze post-treatment OA progression using OA pathology grading system and we didn’t detect cytokines protein expression using Western Blot, which are recommended to be performed as additional tools in studying OA models.

5. Conclusion

In conclusion, we showed in a rat model of TMJ-OA, that intraarticular injection of BM-MSCs-MVs or PRP helped in restoring damaged condylar structure via attenuating inflammation and matrix degradation genes and promoting matrix synthesis gene collagen II. As discussed, microRNAs contained in MSCs-MVs, or growth factors contained in PRP could be strong candidates involved in the regenerative activity of these biotherapies. Our findings suggested a superior therapeutical potential for BM-MSCs-MVs over PRP.

Recommendations

Additional research about the superiority of biological approaches, especially stem cell derived-EVs, with respect to traditional approaches in the treatment of osteoarthritic lesions, need to be fully proven in order to draw clear clinical application. Also, the signaling pathways involved in cartilage reconstruction are worth exploring in future research. Moreover, further studies are required to set up a standardized preparation method, and to determine the optimal dosage and best protocol for administration of various types of biological treatments.

Declarations

Author contribution statement

Nermeen AbuBakr, Amira E. Fares, Aber Mostafa and Dina B.E. Farag: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Additional information

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