Impact of glycogen synthase kinase-3β inhibition on rats’ temporomandibular joint collagen-induced rheumatoid arthritis with correlation to miRNA-155/miRNA-24 expression

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Abstract. – OBJECTIVE: The current study considered assessing the role of miRNA-155 and miRNA-24 in collagen-induced rheumatoid arthritis (RA) in rats’ temporomandibular joint (TMJ). Their role in histological aggressiveness of the disease and therapy response to glycogen synthase kinase (GSK) inhibitor 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) will be studied.

MATERIALS AND METHODS: Rats were randomly distributed to four groups (8 rats/group): group I negative control, group II collagen-induced rheumatoid arthritis (CIA), group III Control+TDZD-8 treated group, and group IV CIA+TDZD-8 treated group. Then were euthanized 42 days after the start of the experiment. H&E staining, Masson trichrome staining, and immunohistochemical antibodies against S100 were performed. qRT-PCR of miRNA-155 and miRNA-24 were done for frozen synovial tissues.

RESULTS: Histological analysis showed that the most affected structure in induced rheumatoid arthritis of TMJ is the articular disc, condylar head, and subchondral bone. Combined treatment with TDZD-8 improved histological status in the joint. Masson’s trichrome (MTC) histochemical staining revealed disarrangement of collagen fibers and adherence between the articular disc and condylar cartilage. Meanwhile, the morphology and collagen composition of the disc and condyle in CIA+TDZD-8 were similar to those of healthy tissues. Immunohistochemical analysis for S100A4 revealed increased immunoreactivity staining in the CIA group. The immunoreactivity was significantly decreased in CIA+TDZD-8 treated group. TDZD-8 significantly decreased the levels of miRNA-155 and miRNA-24 in synovial tissue.

CONCLUSIONS: Our results reveal for the first-time correlation of miRNA-155 and miRNA-24 that might be implicated in the onset of TMJ RA. Consequently, the treatment of CIA with GSK inhibitor (TDZD-8) yields encouraging results. We predicted the TDZD-8 might protect against CIA by suppressing miRNA-155, miRNA-24, and S100A4 protein levels.

Key Words: Temporomandibular, Rheumatoid arthritis, GSK-3b inhibitors, TDZD-8, miRNA-155, miRNA-24.

Introduction

One of human joints’ long-lasting, degenerative inflammatory disorders is rheumatoid arthritis (RA). Its pathogenesis still needs to be fully clarified; evidence states that interaction between environmental, genetic, and immunologic factors contribute to the disease. Both innate and adaptive immunity are implicated, and macrophages and T-cells have an imperative role in its pathogenic mechanism. The temporomandibular joint (TMJ) is a compound synovial joint composed of the temporal bone’s glenoid fossa and the mandibular condyle with interposed articular disc. The most prevalent
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type of inflammation-related arthritis linked to TMJ malfunction is this one. The overall incidence of rheumatoid arthritis-related TMD is underestimated, owing in most cases to a shortage of a routine rheumatologist assessment for TMD in the rheumatology practice. Sadura-Sieklucka et al reported that 50% of RA patients have TMJ dysfunction according to evaluation by the visual analog pain scale and TMJ palpation. Studies indicate that between 19-85.7% of RA patients have TMJ abnormatilities and symptoms in the form of joint discomfort, swelling, restricted jaw mobility, and even ankylosis.

Proteoglycan degeneration and weariness in the fibrocartilage of the articular eminence and condylar head are two key abnormalities that may be identified. Eventually, Cortical and subcortical bone destruction result in condylar damage associated with subchondral bone and osteoclastic bone destruction. In later stages, joint stiffness with limited movement and muscle spasms were prominent.

Rheumatoid factor (RF) has been detected as the primary serological test for diagnosing RA. Recently, Anti-citrullinated peptide antibody (ACPA) tests were developed and made commercially available. Since ACPA exist prior to the start of RA manifestations and is indicative of the RA progression, they are a valued diagnostic tool early in the prognosis of the disease.

Micro-RNAs (miRNAs) which are small non-coding RNAs (18-23 nucleotides in length) have been found to control the pre- and post-transcriptional levels of gene expression in a variety of targets. It has been evident over the past few years that RA patients exhibit changes to their cellular (miRNAs). In RA synovial tissue and synovium macrophages, miRNA-155 was shown to be highly expressed. Its overexpression elicited the production of chemokines and cytokines intensely involved in RA synovitis, namely interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α). miRNA-24 was established to be interrelated to RA pathogenesis through the Human Regulatory T Cells (Treg) pathway, and it is raised in arthritic disorders, including RA. Researchers have revealed that miRNA-24 is increased in all RA synovial fibroblast samples and synovial fluid as well.

Glycogen synthase kinase 3-β (GSK-3β) is a serine/threonine kinase characterized by site-specific dependent phosphorylation. In the literature, it was demonstrated that GSK-3 is tangled in various biological functions, including tumorigenesis, cell cycle progression, apoptosis and viability, cytoskeletal organization, and cell metabolism. The inflammation detected in RA is intensely linked with different pro-inflammatory triggers and transcription agents, which had been linked with GSK-3β. The thiazolidinedione compound TDZD-8 was recognized as a non-adenosine triphosphate (non-ATP) competitive inhibitor of GSK-3β. TDZD-8 has been proven to be a protective mediator in a variety of murine models of disease as arthritis, colitis, spinal cord injury, and septic shock. TDZD-8 can lessen the production of CIA in rats. Also, it can diminish paw edema and bone destruction in RA rats, as presented by the histological analysis of the knee joint.

The association between miRNAs and GSK inhibitors has been proven in several pathological processes; GSK-3β is essential in controlling neurogenesis and neuronal survival in the brain. Furthermore, exposure to GSK-3β inhibitors changes the expression of selective miRNAs (upregulated: miR-144; downregulated: let-7b, let-7c, miR-122a, miR-24a, miR-30c, miR-34a, and miR-221) that target proteins used in neurite outgrowth and neurogenesis.

S100 proteins are calcium-binding proteins with a low molecular weight generated by invertebrates. The family comprises more than 20 identified members, detected in various tissues and cells with a pivotal role in several cellular functions. In RA, S100A4, S100A8, S100A9, S100A11, S100A12, and S100B have been associated with inflammatory responses. These S100 proteins might encourage the promotion of arthritis by enhancement of catabolic signaling via the receptor for advanced glycation end products (RAGE) in cartilage. S100A4 levels in RA patients are related to radiographic damage and its progression. These proteins might be part of a novel group of biomarkers that predict radiographic progression and poor therapy response in RA patients.

Numerous challenges are met during the trip of RA diagnosis and therapy; thus, the current study aims at determining the impact of glycogen synthase kinase-3β inhibition on rats’ temporomandibular joint collagen-induced rheumatoid arthritis and correlation to miRNA-155/miRNA-24 Expression. In addition to evaluating the role of miRNA-155 and miRNA-24 in TMJ arthritis in rats and the interplay between miRNA-155, miRNA-24, and S100 protein in the pathogenesis of RA. The present study’s findings might help to understand the unexplored role of miRNA in RA pathogenesis, which might add to the diagnostic tests for TMJ arthritis and identify TDZD-8 as a prospective therapeutic drug for managing TMJ arthritis.
Materials and Methods

Animals

Adult male rats, Wistar albino species (170 ± 10 g, 8 weeks of age), were supplied from the animal facility at King Saud University in Riyadh, Saudi Arabia. Rats were accommodated as 4 rats per cage under pathogen-free conditions with ventilation, controlled humidity (50-60%), temperature (22-23°C), and a 12 h light/dark cycle with free access to standard pelleted chow and water. All attempts, including handling, housing, treatment, and operative procedures, were made to ensure proper care of all animals and diminish animal suffering.

Animal Model of Collagen-Induced Arthritis (CIA)

The procedures established by Brand et al.31 were followed to induce the CIA to the assigned groups of rats. Complete Freund’s adjuvant (CFA) (Cat. No. F5881, Sigma Aldrich, St. Louis, MO, USA) and Bovine Type II Collagen (CII) (Cat. No. C7806, Sigma Aldrich, St. Louis, MO, USA) were utilized. Collagen II was thawed in diluted acetic acid (10 mM) to a 4 mg/ml concentration by stirring overnight at 4°C. At 4°C, Freund’s complete adjuvant was mixed with collagen type II (1:1) to prepare the emulsion. For CIA induction, each rat received an intradermal (i.d.) injection of 100 µl of emulsified collagen type II on day 0 in the skin of the tail, about 1.5 cm distal to the base of the tail. After 14 days, the booster dose was administrated, applying the same concentration and route post-primary immunization. Following this procedure, RA is established in most rats 5-7 days post the second immunization.

Experimental Design

A total of thirty-two adult male rats were arbitrarily distributed into 4 groups (8 rats/group): Group I (control group): rats administrated vehicle of 100 µl normal saline (i.d) on day 0 and 14 and then given 100 µl of 0.1% DMSO (i.p) daily from day 21 to day 42; Group II (CIA-induced group): rats with pre-established CIA and received 100 µl of 0.1% DMSO as vehicle from day 21 to day 42; Group III (Control+TDZD-8 treated group): rats were treated as in group I, then treated daily with an i.p dose of 100 µl of 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), non-ATP competitive inhibitors of glycogen synthase kinase 3β (GSK-3β), (1 mg/kg) (Cat. No. Ab142372, Abcam, Cambridge, UK) starting from day 21 to day 42. Furthermore, Group IV (CIA+TDZD-8-combined treated group): rats with pre-established CIA received an i.p daily dose of 100 µl of TDZD-8 (1 mg/kg) from day 21 to day 42. TDZD-8 was dissolved in diluted DMSO (0.1%). TDZD-8 dose was approved from the previous studies23. DMSO of 0.1% has no impact on any of the tested parameters from our preliminary data (data not shown).

Arthritis Scoring

Following the second immunization of CII/CFA, each rat was tested for the prevalence and severity of RA by inspecting the scope of edema and erythema within the four paws starting from day 21 after the first intradermal injection every 2-days.

Inspections were done by two independent researchers who were uninformed of the experiment following the scoring system recognized by Cuzzocrea et al.32. The average score for the severity of arthritis in each of the hind paws of CIA rats was assessed by two veterinary investigators. The scoring scale was graded from 0-4 with a maximum score of 16.

Biochemical Serum Measurements

By the end of the experiment, all rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), the rats’ chest was open, and the blood sample was taken via cardiac puncture, and then collected in plain tubes, centrifuged at 3,000 rpm to collect serum. Then, rats were killed by cervical dislocation. Rheumatoid Factor (RF) serum levels were determined by a rat’s ELISA Kit (Cat. No. CSB-E13666r, CUSABIO Technology LLC, TX, USA). Anti-citrullinated protein antibodies (ACPA) levels in serum were quantified using a rat’s ELISA Kit (Cat. No. EKC38678, Biomatik, Ontario, Canada). All measurements followed the manufacturer’s instruction.

Synovium Isolation

The heads were fixed in a side position, and the skin was cut apart longitudinally in front of the ear tragus to expose the left TMJ of each rat. Under a dissecting microscope, the soft tissue was removed. To completely expose the synovial tissue, the fibrous/synovial layers were removed. The synovium was harvested, snap-frozen in liquid nitrogen, and stored at -80°C for further use.

Histological and Histochemical Evaluation

The right TMJ tissue was excised and fixated in 4% paraformaldehyde. For demineralization, the samples were placed in gauze bags and
suspended in 10% neutral buffered formalin. This was followed by 10% ethylene diamine tetra acetic acid (EDTA) for demineralization (pH 7). After demineralization was completed, which was assured by a sharp needle piercing the specimen, indicating easy penetration. Each rat’s right and left TMJs were dissected and embedded into paraffin blocks. For histological evaluation, deparaffinized 5-μm slices were rehydrated using a graduated ethanol series (100%, 90%, and 70%) and stained with hematoxylin and eosin (H&E) and Masson’s trichrome (MTC) staining. Images of histological sections were assessed using a light microscope equipped with a video camera and digital analysis system.

**Immunohistochemical Analyses**

Deparaffinized and rehydrated 5-μm sections were washed with polybutylene succinate (PBS) and blocked for 30 min in 0.1% H2O2 to quench endogenous peroxidase activity. Following PBS washing, the sections were incubated for 60 min at room temperature (RT) in a blocking solution (10% normal goat serum). The sections were then incubated with the primary antibody of S100 calcium-binding protein A4 (Anti-S100A4) (Code IS504, Dako A/S, Glostrup, Denmark) at RT for 1 h. Once washed in Tris Buffered Saline (TBS), sections were incubated with a biotinylated secondary antibody for 10 min at RT and rinsed. Next, sections were washed with PBS, and the enzyme conjugate streptavidin-horse-radish peroxidase solution was added to the sections for 10 min. Next, sections were washed with PBS, slides stained with two drops (100 μl) of hematoxylin, and then cleaned with distilled water. The slides were finally dehydrated in an ascending graded ethanol series, cleared in xylenes, mounted using Histomount, and covered with a coverslip.

**Computer-Assisted Morphometric Analysis of Digital Images**

Slides were photographed utilizing a digital camera Toupcam (XCAM1080PHA; Toup Tek, Hangzhou, China) fixed on a CX22 Olympus microscope (Olympus, Tokyo, Japan), using 100 and 400 objectives. The output images were assessed via Morphology Video Test software (Video Test, St. Petersburg, Russia) with a specified built-in routine for calibrating distance dimension and stain quantification. Five fields from each slide were randomly evaluated (2 slides/rat). Measurements of articular disc thickness were calibrated counter to a micrometer slide. This optical system measures the distance in micrometers (μm) instead of pixels. The measurements were averaged for each image. Images were captured from the camera using a MuTech frame grabber; next, the images’ color tones were enhanced depending on the target area’s hue. Images were thresholded at the level of the desired hue range to form a binary mask [region of interest (ROI)] that represents target areas. An independent, experienced investigator blindly assessed this. Calculation of percentage area (%A) was applied to obtain the % area of ROI in relation to the total field area. The results were exported in an Excel sheet. The values recorded for 8 animals per group were compared and statistically analyzed.

**Quantitative Real-time PCR (qRT-PCR)**

Forward primers used to amplify miR-155 (Cat. No. MPR00417) and miR-24 (Cat. No. MP-r00003) and Reverse primers (Cat. No. MPH00000) were purchased from ABM (Richmond, Canada). Primers sequence of internal control 18S (Accession No. M11188), F: 5'-CGGCTACCACATCCAGGAA-3, R: 5'-GCTGGAATTACCGCGGGCT-3 were purchased from Qiagen (Germantown, MD, USA). Total RNA was isolated from frozen synovium using an RNeasy Mini Kit (Cat. No. 74104, Qiagen, Victoria, Australia). RNA Purity and concentration were evaluated by calculating the absorbance at 260 and 280 using a NanoDrop ND-1000 spectrophotometer. cDNA was produced by a miRNA All-In-One cDNA Synthesis Kit (Cat. No. G270, ABM, Richmond, Canada). qPCR amplification was done with a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA) using BrightGreen miRNA qPCR Master-Mix (Cat. No. MasterMix-mM, Quimigen S.L., Madrid, Spain). Reactions of qPCR were made in a total volume of 2 μl/each as follows: 2 μl cDNA and 6 μl Nuclease-free water, 1 μl PCR Forward Primer (final concentration 300 Nm), 1 μl PCR universal Reverse Primer (final concentration 300 Nm), 10 μl (1x) Bright Green miRNA qPCR Master Mix, PCR reaction cycles were done for initial 10 min at 95°C, then denaturation 95°C (10 sec), annealing 63°C (15 sec), and extension 72°C (30 sec) for a total of 40 cycles, with a final extension step at 72°C for 5 min. The gene expression levels were calculated by 2^-ΔΔCq Livak method. The mRNA expression level was normalized by 18S as an internal control.
**Statistical Analysis**

Quantitative data were analyzed for normality by performing normality tests (Kolmogorov-Smirnov and Shapiro-Wilk tests). Data were displayed as mean ± (SD) values. A one-way ANOVA test followed by Bonferroni’s post-hoc test was applied for parametric data to compare the groups. Kruskal-Wallis test, followed by Dunn’s test, was performed for non-parametric data. The significance level was set at $p \leq 0.05$. Statistical analysis was performed with GraphPad Prism version 9 (La Jolla, CA, USA).

**Results**

**Arthritis Index**

Figure 1 reveals that the arthritis score of the control and control+TDZD-8 treated groups were zero. The CIA-induced group showed an increase in arthritis scores. The arthritis index of the CIA+TDZD-8 treated group was significantly less than the CIA group throughout the observation period.

**H&E Histological Examination**

Histological examination of untreated control revealed the typical histological architecture of the TMJ with its articular fossa, articular disc, and condylar head. Histological examination of the untreated control showed typical histological architecture of the TMJ with its articular fossa, articular disc, and condylar head.

The CIA group revealed thickening of the articular disc with fragmentation and splitting between collagen bundles. Thickening of the fibrous layer covering the condyle head with a gradual decline in the condylar cartilage thickness and a number of chondrocytes.

It also revealed destruction and loss of physiological layers of the subchondral bone, widening of bone marrow spaces with evident marrow degeneration, and irregular, disordered trabecular bone. The control+TDZD-8 treated group showed normal anatomical features of the TMJ, similar to those of the untreated group, with no evidence of histological changes. In contrast, CIA+TDZD-8 treated group showed a normal thickness of the articular disc and condylar cartilage and a regular arrangement of bone trabeculae. The thickness of the fibrous layer of the condylar head, temporal bone, and articular disc appeared normal in thickness. The bone marrow spaces of the condylar head showed regeneration with normal histological features (Figure 2A-D).

There was a statistically significant variation between the mean thickness of the articular disc in all groups ($p$-value $= 0.008$, Effect size $\eta^2 = 0.556$). Pair-wise comparisons using Dunn’s test revealed that the CIA group exhibited the statistically significant highest value. There was no statistically significant variation between control, control+TDZD-8, and CIA+TDZD-8 groups; all showed the statistically significantly lowest mean values (Figure 2E).

![Figure 1](image-url)
Figure 2. Temporomandibular joint micro-section morphology, H&E staining. A-A1, shows normal characteristics of the TMJ, articular fossa (AF), articular disc (AD), and Condylar head (CH) as shown by three distinct zones, an articular zone of fibrous connective tissue (FZ), a proliferative zone containing undifferentiated mesenchymal cells (PZ), a cartilage zone with its chondrocytes (CZ), and the deepest layer including mineralized cartilage and the subchondral bone with its normal regularly architecture. B-B1, The CIA-treated group shows thickening of the articular disc (AD) with fragmentation and splitting in between collagen bundles (arrow). The fibrous layer covering the condyle head thickening (FL), the separation between the cartilage and underlying bone (arrowhead) with a gradual decrease in the condylar cartilage thickness (double arrowhead) and chondrocytes number, which is lost in some areas (asterisk). Destruction and Loss of physiological layers of subchondral bone (SB), widening of bone marrow spaces with clear marrow degeneration (BM), and irregular, disordered trabecular bone (BT). C-C1, Control+TDZD-8 treated group shows normal anatomical features of the TMJ close similar to (A-A1) with no evidence of histological changes. D-D1, The CIA +TDZD-8 treated group shows apparently normal articular disc thickness (AD), condylar cartilage, and apparently normal bone trabeculae (BT) arrangement. The thickness of the fibrous layer of the condyle head, temporal bone, and articular disc featured normal. The bone marrow spaces in the condylar head expressed regeneration and normal morphology (BM). Note the separation between the cartilage and underlying bone (arrowhead). The photomicrographs (A), (B), (C), and (D) were 100 X magnified, while (A1), (B1), (C1), and (D1) were 400 X magnified. E, a graph showing the thickness of the articular disc in all groups. Data are shown as the mean (±SD). **p < 0.01 vs. control, and ###p < 0.01 vs. CIA.
**MTC Histochemical Staining Expression**

The control group showed normal histological features of collagen fibers distribution, condylar cartilage, and subchondral bone with mineralized regular trabecular bone structures. In the CIA-induced group, a marked increase in positive staining and collagen fibers disarrangement in the articular disc was observed, suggesting the presence of immature collagen fibers. Alterations were more aggravated in the subchondral bone, revealing newly formed unmineralized irregular disordered trabecular bone structures with large marrow cavities close to the cartilage remarkably increased. Changes were more provoked, with side adhesion detected between the articular disc and the condylar cartilage. The control+TDZD-8 treated group showed similar collagen distribution to the untreated group. CIA+TDZD-8 treated group revealed regenerated collagen fibers in some areas of regenerated cartilage; a near-normal arrangement of collagen fibers was observed around cell lacunae (Figure 3A-D).

There was a statistically significant variation between the area percentage of fibrosis in all groups (p-value < 0.001, Effect size η² = 0.897). Pair-wise comparisons using Bonferroni's post-hoc test revealed that the CIA group showed the statistically significantly highest mean area %. Control+TDZD-8 showed statistically significantly lower mean area %. There was no statistically significant difference between the control, control+TDZD-8 groups; both showed the statistically significantly lowest mean areas % (Figure 3E).

**Immunohistochemical Analysis**

Immunohistochemical analysis of the CIA group for S100A4 showed an increased expression, characterized by brown discoloration of chondrocytes, predominantly in the deep and, to some extent, in the superficial zone of the non-calcified articular cartilage. Cells in all joints and joint space bone marrow were also strongly positive for S100A4. In contrast, CIA+TDTZ-8 treated group revealed loss of chondrocyte S100A4 immunoreactivity in the non-calcified cartilage and superficial zone compared with group CIA group. However, mild S100 immuno-reactivity was still apparent in the bone marrow of a few osteocytes. Meanwhile, chondrocytes showed little positive S100A4 immunostaining in marginal regions in either control or control+TDZD-8 treated group, although bone marrow and some osteocytes were positive (Figure 4A-D).

There was a statistically significant difference in anti-S100A4 immunohistochemistry area percentage in the four groups (p-value < 0.001, Effect size η² = 0.994). Pair-wise comparisons using Bonferroni's post-hoc test revealed that the CIA group showed the statistically significantly highest mean area %. CIA+TDZD-8 showed statistically significantly lower mean area %. There was no statistically significant difference between control, control+TDZD-8 groups; both showed the statistically significantly lowest mean areas % (Figure 4E).

**Rheumatoid Factor (RF) And Anti-Citrullinated Protein Antibodies (ACPA) Serum Levels**

There was a statistically significant difference between mean RF levels in the four groups (p-value < 0.001, Effect size η² = 0.923). Pair-wise comparisons using Bonferroni’s post-hoc test revealed that the CIA group showed the statistically significantly highest mean RF level. CIA+TDZD-8 group showed a statistically significantly lower mean value. There was no statistically significant difference between the control and control+TDZD-8 groups; both showed the statistically significantly lowest mean RF level (Figure 5A).

There was a statistically significant difference between mean ACPA levels in the four groups (p-value < 0.001, Effect size η² = 0.959). Pair-wise comparisons using Bonferroni’s post-hoc test revealed that the CIA group showed the highest mean ACPA level, which was statistically significant. ACPA level declined significantly in the CIA+TDZD-8 group. There was no statistically significant difference between the control and control+TZD-8 groups; both showed the statistically significantly lowest mean ACPA level (Figure 5B).

**Quantitative Real-Time PCR**

There was a statistically significant difference between mean miRNA-155 levels in the four groups (p-value < 0.001, Effect size η² = 0.926). Pair-wise comparisons using Bonferroni’s post-hoc test revealed that the CIA group showed the statistically significantly highest mean miRNA-155 level. There was no statistically significant difference between the control and CIA+TDZD-8 groups; both showed significantly lower mean levels. Control+TDZD-8 group showed the lowest mean miRNA-155 level with non-statistically significantly different from CIA+TDZD-8 group (Figure 6A).
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Figure 3. Temporomandibular joint micro-section, Masson's trichrome (MTC) staining. **A-A1**, showing the control group with normal collagen fibers of condylar cartilage arranged in a normal mode. Normal subchondral bone with mineralized regular trabecular bone structures. **B-B1**, In the CIA group, showing a higher intensity of blue-stained disorganized collagen fibers in the articular disc, alterations were more aggravated in the subchondral bone, revealing irregular and disordered trabecular bone structures (stained blue) with large marrow cavities. **C-C1**, shows Control+TDZD-8 treated group, revealed similar collagen distribution of the untreated group. **D-D1**, shows the CIA+TDZD-8 treated group, regenerated collagen fibers in some areas of regenerated cartilage, near a normal arrangement of collagen fibers, was observed around cell lacunae. The images (A), (B), (C), and (D) 100 X magnification, while (A1), (B1), (C1), and (D1) were 400 X magnification. **E**, a graph showing the collagen fibers (%) area in all groups. Data are shown as the mean (±SD). ***p < 0.001 vs. control, and ###p < 0.001 vs. CIA.
Figure 4. Temporomandibular joint micro-section, anti-S100A4 immunohistochemistry (IHC). A-A1, The control group shows a negative immune reaction. B-B1, CIA group: shows a moderate immune reaction. C-C1, Control+TDZD-8 treated group shows no immune staining. D-D1, CIA + TDZD-8 treated group shows a mild immune reaction. The photomicrographs (A), (B), (C), and (D) were captured at 100 X magnification, while (A1), (B1), (C1), and (D1) were captured at 400 X magnification. E, a graph showing the area (%) of anti-S100A4 immunohistochemistry. Data are shown as the mean (±SD). ***p < 0.001 vs. control group, and ###p < 0.001 vs. CIA group.
There was a statistically significant difference between mean miRNA-24 levels in the four groups ($p$-value < 0.001, Effect size $\eta^2 = 0.919$). Pair-wise comparisons using Bonferroni’s post-hoc test revealed that the CIA group showed the statistically significantly highest mean miRNA-24 level. CIA+TDZD-8 group showed a statistically significantly decreased mean value. There was no statistically significant difference between the control and control+TDZD-8 groups; both showed the statistically significantly lowest mean miRNA-24 level (Figure 6B).

**Discussion**

The current work examined the temporomandibular joint in a rat model of collagen-induced rheumatoid arthritis (CIA). Given the similarities in pathology and arthritic manifestations between CIA model animals and individuals with rheumatoid arthritis (RA), the CIA model is the most frequently applied animal model for RA research. We applied this model to investigate the role of S100A4, miRNA-155, and miRNA-24 in the pathophysiology of RA with or without the application of the GSK-3β inhibitor, TDZD-8.
On day 21, the rats in the CIA model group began to exhibit joint abnormalities, swelling, and erythema in their paws. In our study, histochemical analysis by Masson trichrome revealed an increased amount of blue-stained collagen fibers in the articular disc of the CIA group. de Sousa et al.8 found an upsurge in type III collagen in the rats’ TMJ RA, indicating an attempt to regenerate the damage to the articular cartilage. We analyzed the blood and temporomandibular joint using ELISA, PCR, and immunohistochemistry. We believe our work is the first to demonstrate the role of S100A4 protein miRNA-155, and miRNA-24 in TMJ RA.

Glycogen synthase kinase 3β (GSK-3β) was primarily recognized as a fundamental regulator of insulin-dependent glycogen synthesis36. GSK-3β is also implicated in the regulation of fibrotic and inflammatory processes37. Wadhwa et al.18 demonstrated that GSK-3β activity is essential for the full enhancement of proinflammatory mediators’ elaboration, such as IL-6, IL-1β, and TNF-α. Additionally, GSK-3β specific inhibitors such as thiazolidinone-8 (TDZD-8) significantly attenuate proinflammatory cytokine elaboration and promote the production of anti-inflammatory cytokine39,40. It was observed that TDZD-8 in this experimental model attenuates the clinical and morphological manifestations of the disease23.

RA is a long-lasting inflammatory autoimmune disorder that causes synovial membrane degeneration, cartilage destruction, and bone loss. It is marked by the release of many inflammatory mediators that trigger significant swelling, discomfort, and stiffness in the joints41,42. In addition, RF and ACPA are frequent blood indicators linked to RA, and the levels of these markers are correlated with the degree of severity of the condition43,44. Our findings ascertained these reports, and the apparent improvement in the arthritis score and mitigation of joint damage brought on by CIA may be explained by a considerable decrease in the levels of these biomarkers after treatment with TDZD-823,45. In the current study, the CIA+TDZD-8 group showed a marked decrease in the mean area of fibrosis. Guo et al.46 reported that TDZD-8 suppressed Aldosterone-induced perivascular cardiac fibrosis. They attributed that to the role of TDZD-8 in the activation of autophagy. The proposed mechanisms by which TDZD-8 stimulates autophagy need further study.

S100 calcium-binding A4 (S100A4), also known as fibroblast-specific protein 1 (Fsp1), is one member of the S100 protein family that controls proliferative and inflammatory activities in RA47. Patients with RA showed high levels of S100 protein. Additionally, some S100 proteins can be used as biomarkers for RA activity monitoring48,49. RA patients had considerably greater levels of the S100A4 protein expression in the knee’s synovial membrane50,51, signifying that S100A4 overexpression positively correlates with RA development52. It considerably increases the expression of inflammatory cytokines50. S100A4 can also activate synovial fibroblasts and regulate apoptosis53. Decreased S100A4 protein might have a role in reducing fibrosis in RA. S100A4 is dysregulated in fibrotic diseases of lung, liver, kidney, and heart54. Therefore, our findings that emphasize the induction of S100A4 agree with the above reports.

The substantial role of miR-155 in the pathophysiology of RA has been well recognized over the past few decades55,56. miRNA-155 is a substantial inflammatory molecule identified in peripheral blood mononuclear cells, circulatory macrophages, Fibroblast-like synovial cells, and the joints of RA patients57. Additionally, mice lacking miRNA-155 did not establish CIA, displayed no apparent inflammatory cells in their synovial membranes, and exhibited normal synovial cell proliferation58. Therefore, our findings that highlight the induction of miRNA-155 by CIA are in accordance with the above reports.

However, the crosstalk between GSK-3β, S100 proteins, and miRNAs expression is underemphasized. GSK-3β was proven to regulate miRNAs expression in diverse healthy and cancerous cells. This relation has been documented with multiple miRNAs in different cancers, including (miR-1229) in breast cancer, (miR-96, miR-182, and miR-183) in gastric cancer, (miR-26a) in lung cancer, (miR-224) in colorectal cancer, (miR-940) in pancreatic cancer, (miR-769) in melanoma, (miR-433) in bladder cancer, (miR-15a) in nasopharyngeal carcinoma, and (miR-129) in endometrial cancer60. Also, prophylactic or therapeutic administration of TDZD-8 protects against ischemia/reperfusion (I/R) injury in the rat hippocampus with a reduction of infarct size and levels of S100B protein, a marker of cerebral injury. This was linked to a considerable decrease in oxidative stress, apoptosis, and the inflammatory reaction brought on by cerebral I/R injury. Furthermore, microRNA appears to control the expression of some S100 family members. Choe et al.62 showed that S100A4 is regulated by miRNA-124, as ascertained in our study. Moreover, the suppression of S100A4 in...
duces inhibition of proliferation and invasion of hepatocellular carcinoma (HCC) cells. Also, co-culturing with liver cancer-associated mesenchymal stem cells and ectopic overexpression of S100A4 significantly increased oncomic miRNA-155 expression in HCC. miRNA-155 inhibitor significantly mitigated the invasion-promoting effects of S100A4. This previous report proposes that S100A4 influences HCC cells by controlling miRNA-155 expression.

Conclusions

Our data showed that GSK-3β inhibition caused a significant decrease in miRNAs gene expression and S100A4 protein. Our results demonstrate for the first-time correlation of miRNA-155 and miRNA-24 that might be implicated in the onset of TMJ RA. Consequently, the treatment of CIA with GSK inhibitor (TDZD-8) yields encouraging results, which warrant additional research in humans. We predicted that the TDZD-8 might protect against CIA by suppressing miRNA155, miRNA-24, and S100 protein levels.

Authors’ Contributions

Conceptualization, methodology, writing-review and editing, supervision, project administration, funding acquisition, R.H. Al-Serwi, G. Othaman, and M. El-Sherbiny. Analysis, investigation, writing-original preparation, A. F. Dawood. Software, Validation, analysis, and Interpretation of Data A.K. Alhumaidan, H. S. Alharbi, M.E. Almadani, and D.M.A. Elsherbini. All authors read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare that they have no conflict of interest to declare.

Informed Consent

Not applicable.

Ethics Approval

The research design applied in our study meets Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Animal experiments involved in this research were approved by the Ethical Committee of the Faculty of Pharmacy, Ain Shams University, Cairo, Egypt (6-2020) and conducted following the guiding principle of laboratory research animal species and utilize, issued by the US National Institutes of Health (NIH publication No. 85-23, reviewed 1996).

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Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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