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A Rat Model of Rheumatoid Arthritis: TDZD-8 is Associated with the Protection Against the Induction of the Synovium Knee Joint IL-17A/GSK3β/ROS/α-SMA Axis of Fibrosis

Un Modelo de Rata de Artritis Reumatoide: TDZD-8 está Asociado con la Protección Contra la Inducción del Eje de Fibrosis de la Articulación Sinovial de la Rodilla IL-17A/GSK3β/ROS/α-SMA

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SUMMARY: Rheumatoid arthritis (RA) that affects the synovial knee joint causes swelling of the synovial membrane and tissue damage. Interleukin-17A (IL-17A) and the enzyme glycogen synthase kinase-3β (GSK3β) are involved in the pathogenesis of RA. The link between IL-17A, GSK3β, the oxidative stress, and the profibrogenic marker alpha-smooth muscle actin (α-SMA) with and without TDZD-8, GSK3β inhibitor has not been studied before. Consequently, active immunization of rats was performed to induce RA after three weeks using collagen type II (COII) injections. The treated group received daily injection of 1 mg/kg TDZD-8 for 21 days following the immunization protocol (COII+TDZD-8). Blood and synovium tissue samples were harvested at the end of the experiment. RA development was confirmed as corroborated by a substantial increase in blood levels of the highly specific autoantibody for RA, anti-citrullinated protein antibody as well as augmentation of reactive oxidative species (ROS) levels measured as lipid peroxidation. RA induction also increased synovium tissue levels of IL-17A and the profibrogenic marker, α-SMA. All these parameters seemed to be significantly ($p<0.0001$) ameliorated by TDZD-8. Additionally, a significant correlation between IL-17A, ROS, and α-SMA and biomarkers of RA was observed. Thus, knee joint synovium RA induction augmented IL-17A/GSK3β/ROS/α-SMA axis mediated arthritis in a rat model of RA, which was inhibited by TDZD-8.

KEY WORDS: Rheumatoid arthritis; IL-17A; GSK3β; ROS; α-SMA; Fibrosis; TDZD-8.

INTRODUCTION

Chronic inflammation of the joints is a hallmark of the autoimmune disease, rheumatoid arthritis (RA) that is more common in women, and can lead to degraded cartilage and bone erosion (Ahlmén *et al*., 2010; Smolen *et al*., 2018). RA is the model of a chronic disease without indication for spontaneous resolution and affects about 1% of the people worldwide (Chen *et al*., 2016). The systemic inflammation observed in RA shows extensive damage beyond the joints to include for example the cardiovascular, skin, lungs, and eyes (Scott *et al*., 2010; Bordy *et al*., 2018). The pro-inflammatory cytokine IL-17 that is produced by specialized CD+ T helper (Th17) cells and oxidative stress such as ROS, are associated with the pathogenesis of RA in both, human and animal models (Gaffen, 2009; García-González *et al*., 2015). IL-17A increased autoimmunity which is a characteristic feature of RA (Binger *et al*., 2017),

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ROS production (Dhillion *et al*., 2012), and fibrosis (Zhang *et al*., 2019). ROS is believed to cause augmentation of the inflammatory cytokines and increased dysregulation of fibroblast-like synoviocytes (Lou *et al*., 2021), which can lead to inflammation of the synovial membrane, synovial angiogenesis, and cartilage degradation and bone erosion which involves the induction of osteoblast activity (Nygaard & Firestein, 2020). This can eventually lead to disability (Scott *et al*., 2010; Nygaard & Firestein, 2020).

The glycogen synthase kinase-3β (GSK3β) enzyme is involved in the pathophysiology of rheumatoid arthritis and osteoarthritis as well as many metabolic disorders (Zhou *et al*., 2016; Zhang *et al*., 2018; Shu *et al*., 2020). Indeed, in a rat model of RA, GSK-3β, and tissue and blood levels of inflammatory mediators such as histamine, prostaglandin E2, and proinflammatory cytokines are linked to the affected joints (Zhou *et al*., 2016). Whereas, GSK-3β inhibited the anti-inflammatory cytokine IL-10 in human peripheral blood mononuclear cells (Chan *et al*., 2009). Furthermore, (i) GSK-3β was reported to be involved in the ROS-induced necrosis in malignant cells (Ciotti *et al*., 2020); (ii) GSK3β/ROS axis stimulated the growth and metastasis of murine breast cancer (Jin *et al*., 2019); and (iii) GSK-3β inhibitor was reported to increase bone thickness in mouse model of osteoporosis (Zahoor *et al*., 2014). Therefore, this report investigated the IL-17A/GSK3β/ROS/α-SMA fibrosis axis in a rat model of RA with and without the specific inhibitor of GSK3β, TDZD-8.

MATERIAL AND METHOD

Animals. The work was performed on Wistar rats (160 ± 10g) that were provided by the animal facility located at King Saud University, Riyadh, Saudi Arabia. Free access to water and food were provided for these animals that were housed in a clean facility under a constant room temperature and a cycle of 12 h light/dark. All animal procedures were approved by the Princess Nourah University (Ethical Committee, IBR No. 17-0201).

Experimental design. After few days of acclimatization , a total of 24 rats were divided equally into three groups: Firstly, the experimental group (RA) of rats that was immunized (via active immunization method) with bovine collagen type II (COII, Sigma-Aldrich, MO, USA) as previously reported (Alzamil *et al*., 2020), which was confirmed after three weeks; Secondly, the treated group (COII+TDZD-8): between day 21-42, rats with RA had received a daily dose of TDZD-8 (1 mg/kg) (Zhou *et al*., 2016); Thirdly. The control group of rats which received vehicles; normal saline on days 0 and 14 received a daily dose of 0.1 % DMSO between days 21-42 via i.p. route. Blood was collected and rats were then culled following anaesthesia. The synovium was removed under a dissecting microscope, snap-frozen in liquid nitrogen and stored at -80 ºC until being used.

IL-17A, α-SMA, and p53 Immunohistochemistry and assessment of disease phenotype. As previously described (Dawood *et al*., 2022), 5 μm thick sections of deparaffinized synovium tissue were dehydrated and antigen retrieval was performed. In a humidity chamber, these tissue sections were incubated at room temperature for 1 hour with the primary antibodies, anti-IL-17A, anti-α-SMA, and p53 obtained from Abcam, Cambridge, UK. Tissue sections were then washed and incubated at room temperature for 30 min with the secondary antibody. Finally, sections were counterstained with Meyer hematoxylin. The areas % of IL-17A and α-SMA immunohistochemistry staining was assessed using "Leica Qwin 500 C" image analyzer (Cambridge, UK). The ANOVA followed by post-Hoc analysis (Tukey test) were used for comparing the quantitative data, which is presented as means ± standard deviations (SD). P-values < 0.05 was deemed statistically significant.

Anti-citrullinated protein antibody (ACPA), malondialdehyde (MDA), and superoxide dismutase (SOD) blood determination. Six weeks post the active immunization procedure, ACPA levels were assessed in the blood of all rats' groups using rat ELISA assay Kits purchased from Biomatik (Kitchener, Ontario, Canada) as recommended by the manufacturer. ELISA kits (Cayman Chemical, MI, USA) for the determination of liver malondialdehyde (MDA) and superoxide dismutase (SOD) were done as recommended by the manufacturer.

Western Blotting Analysis of IL-6 and Bcl-2: As previously reported (Dawood *et al*., 2022), 40 μg extracted protein (synovial tissues) per sample were immunoblotted with anti-IL-6 and anti-Bcl-2 (Santa Cruz Biotechnology). To visualize the protein bands, ECL detection kit obtained from Thermo Fisher, Waltham, MA, USA was used. Image analysis software (C-Di Git blot scanner; LI-COR, Lincoln, NE, USA) was used to measure the intensity of bands.

Statistical analysis: GraphPad Prism statistical software package (version 6) to perform the statistical analysis was used. One-way ANOVA was done followed by Tukey's test to assess the differences among the four groups involved in the study. Data were expressed as mean ± SD, and results were considered significant when P≤0. 05.

RESULTS

Rheumatoid arthritis (RA) induction in rats. To investigate the aim of this study, we first induced the disease in rats 42 days after active immunization with COII. A sharp increase in the blood levels of the anti-citrullinated protein autoantibodies (ACPA) (Fig. 1A) together with the upregulation of the protein expression of the survival biomarker, B-cell lymphoma-2 (Bcl-2) (Fig. 1B) in synovial tissue of the experimental group, as well as the observed changes in the macroscopic features of paws in rats (data not shown) confirmed the disease induction. In addition, the negative effect of apoptosis measured as synovial p53 (biomarker of apoptosis) expression (Figs. 1C and 1D) further confirmed the development of RA.

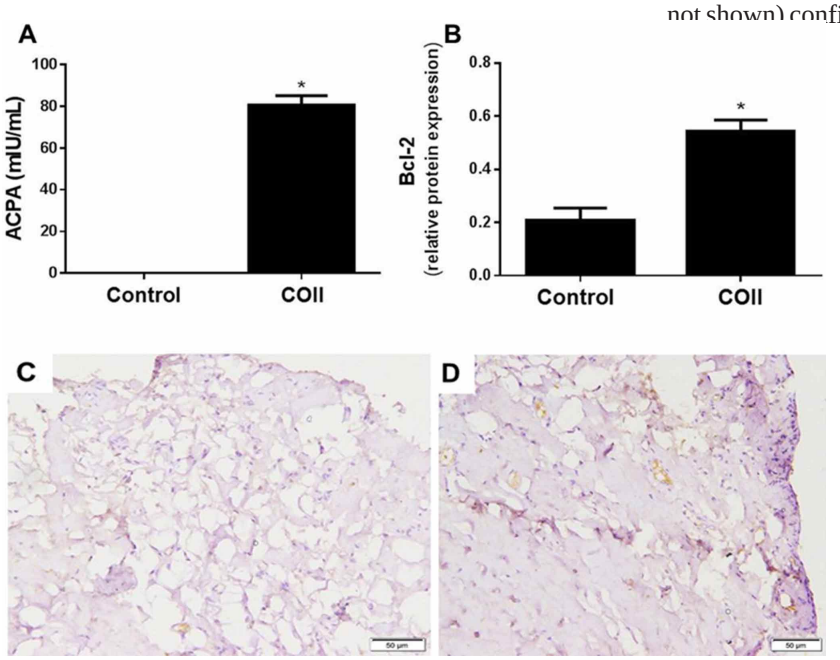

Fig. 1. Induction of rheumatoid arthritis in rats by COII immunization. ACPA blood levels (A) and synovium tissue levels of Bcl-2 (B) and p53 (C and D) were assessed at the end of the experiments in the control and experimental groups of rats. Presented p values are significant. *p<0.0001 versus control. COII: collagen type II; TDZD-8: thiadiazolidine derivative; ACPA: anti-citrullinated protein autoantibodies; Bcl-2: B-cell lymphoma-2; p53: tumour suppressor p53.

Collagen type II (COII) immunization augments synovium IL-17A, is inhibited by TDZD-8. IL-17A is a well-known inducer of autoimmunity including RA (Gaffen,2009). Therefore, synovium tissue levels of IL-17A were measured in all animal groups with and without TDZD-8 incorporation as well as the RA biomarker, ACPA. Compared to weak IL-17A+ve immunostained cells in the control group (Fig. 2A), COII immunization caused a substantial increase in IL-17A+ve immunostained cells in the stroma of the synovial tissue (arrows) of the experimental group (RA) (Fig. 2B), which was significantly (p<0.0001) inhibited by TDZD-8 in the treated group (COII+TDZD-8) (Figs. 2C and 2D). However, the level of IL-17A+ve immunostained cells in the treated group (COII+TDZD-8) was significantly higher compared with the control rats. This means incomplete inhibition by TDZD-8.

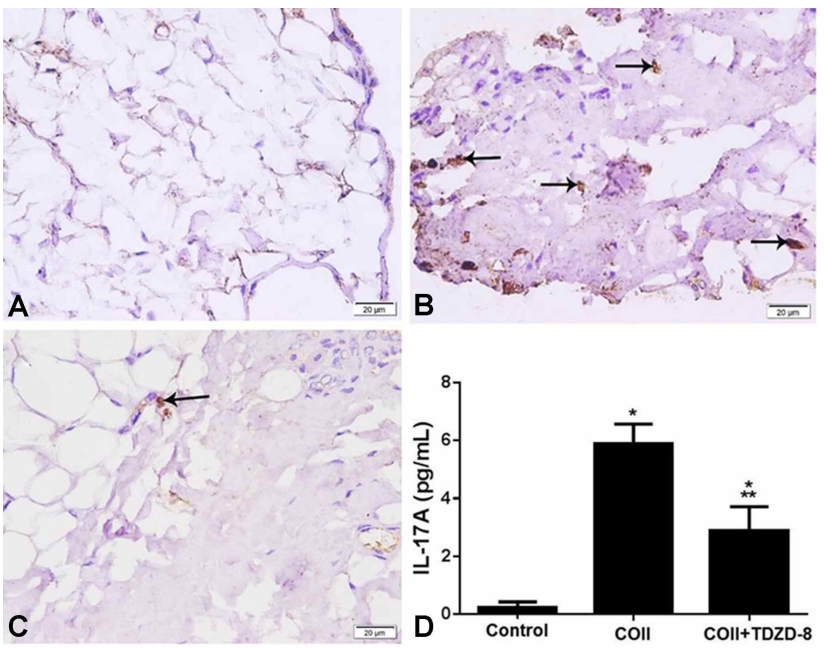

Fig. 2. COII immunization activates synovium IL-17A protein expression with inhibition being associated with TDZD-8. IL-17A immunohistochemistry representative images (x400) of synovium sections prepared at the end of the experiment, end of week 6 from the control rats (A), model rats (COII) (B), and treated rats (COII+TDZD-8) (C) are displayed. A quantitative analysis of IL-17A immunostaining deduced from these images is shown (D). Presented p values are significant. *p<0.0001 versus control, **p<0.0001 versus COII. IL-17A: interleukin-17A; COII: collagen type II; TDZD-8: thiadiazolidine derivative.

TDZD-8 inhibits ROS and inflammation biomarkers induced by RA. ROS is located downstream of IL-17A (Dhillion *et al*., 2012). Therefore, levels of biomarkers of oxidative stress and inflammation were evaluated in all rats groups in order to determine whether these biomarkers are also augmented in RA, and whether they are inhibited by TDZD-8. As shown in Fig. 3, active immunisation with COII caused a sharp increase in the blood levels of malondialdehyde (MDA) measured as lipid peroxidation (Fig. 3A) and a significant ($p<0.0001$) decline in the antioxidant levels of superoxide dismutase (SOD) (Fig. 3B). In addition, synovium tissue levels of the inflammatory marker IL-6 were substantially increased upon COII immunisation (Fig. 3C). All these parameters were significantly ($p<0.0001$) modulated by TDZD-8 (Fig. 3).

Collagen type II (COII) immunization augments a-SMA protein levels in injured synovium, is inhibited by TDZD-8. In cell signalling, α-SMA is located downstream of ROS (Yang *et al*., 2020). To assess the

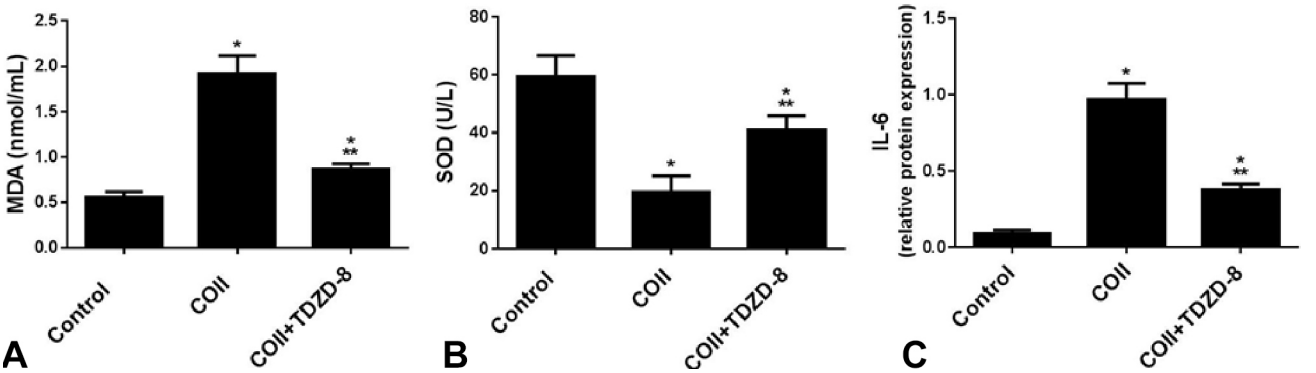

Fig. 3. COII immunization activates biomarkers of oxidative stress and inflammation with inhibition being associated with TDZD-8. Blood levels of MDA (A) and SOD (B) as well as synovium tissue levels of IL-6 (C) were measured end of week 6 in all rats' groups; Control rats, model rats (COII), and treated rats (COII+TDZD-8). Presented p values are significant. *$p<0.0001$ versus control, **$p<0.0001$ versus COII. MDA: malondialdehyde; SOD: superoxide dismutase; IL-6: interleukin-6; COII: collagen type II; TDZD-8: thiadiazolidine derivative.

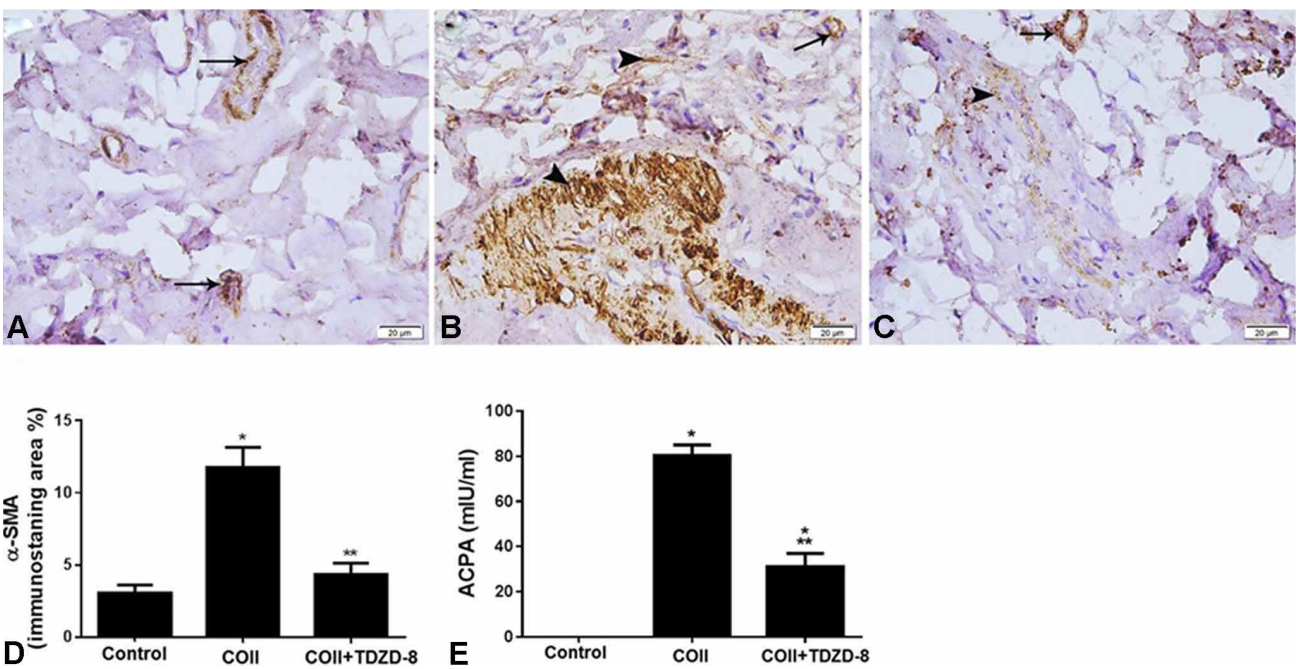

Fig. 4. COII immunization activates synovium a-SMA protein expression with inhibition being associated with TDZD-8. α-SMA immunohistochemistry representative images (x400) of synovium sections prepared end of week 6 from the control rats (A), model rats (COII) (B), and treated rats (COII+TDZD-8) (C) are displayed. A quantitative analysis of α-SMA immunostaining deduced from these images is shown (D). (E) Blood levels of ACPA were determined in all rats' group at the end of the experiment, end of week 6. Presented p values are significant. *$p<0.0001$ versus control, **$p<0.0001$ versus COII. a-SMA: alpha-smooth muscle actin; COII: collagen type II; TDZD-8: thiadiazolidine derivative; ACPA: anti-citrullinated protein autoantibodies.

association of IL-17A/GSK3β/ROS axis with knee joint synovium fibrosis, levels of the profibrogenic biomarker a-SMA were evaluated in the synovial tissue of knee joints in all rats groups with and without TDZD-8 incorporation. The image representing the control rats (Fig. 4A) depicted weak +ve immunostaining in the blood vessels' smooth muscles (arrow), compared to a strong positive immunostaining in the stroma of the synovial tissue (arrow head) besides the wall of the blood vessels (arrow) shown in the experimental group (COII) (Fig. 4B). Treatment of the immunized rats with TDZD-8 for three weeks (COII+TDZD-8) appeared to significantly ($p<0.0001$) inhibit α-SMA +ve cells in the synovial tissue (arrow head) and besides the wall of the blood vessels (arrow) (Figs. 4C and 4D). However, the level of α-SMA +ve immunostained cells in the treated group (COII+TDZD-8) was significantly higher compared with the control rats. This means incomplete inhibition by TDZD-8. TDZD-8 also significantly ($p<0.0001$) inhibited COII-induced the blood levels of the specific biomarker of RA, ACPA (Fig. 4E), but still higher compared with the control rats.

Correlation between marker of fibrosis and IL-17A as well as biomarkers of RA and oxidative stress. To claim a link between IL-17A/GSK3β/ROS axis and fibrosis in RA animal model, we assessed the correlation between these parameters as well as RA biomarker, ACPA. α-SMA score exhibited a significant ($p<0.0001$) positive correlation with ACPA ($r = 0.934$) (Fig.5A) IL-17A ($r = 0.897$) (Fig. 5B), and MDA ($r = 0.941$) (Fig. 5C). Whereas, α-SMA score exhibited a significant ($p<0.0001$) negative correlation with the antioxidant, SOD ($r = - 0.864$) (Fig. 5D).

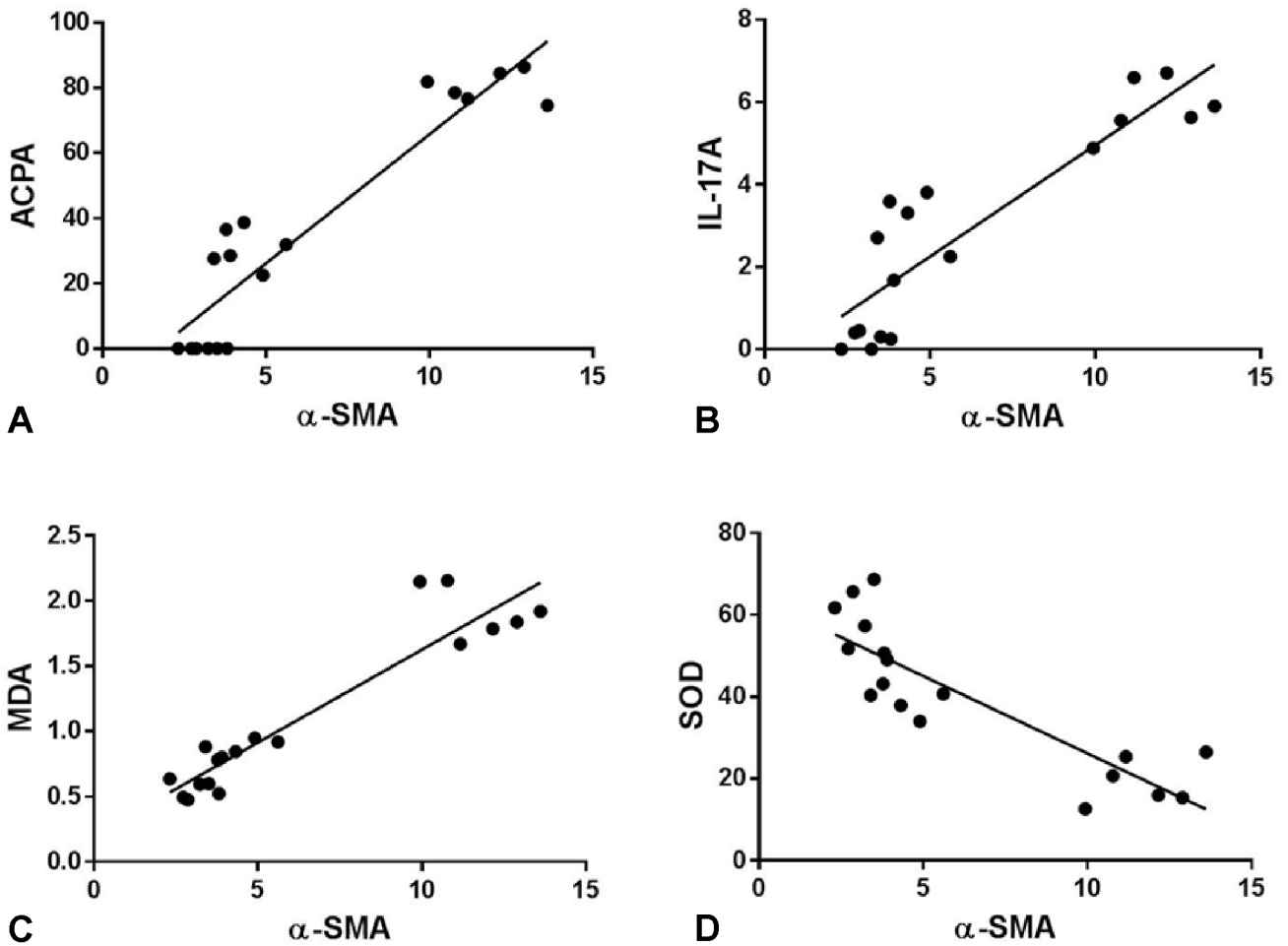

Fig. 5. Correlation between the scoring of α-SMA and IL-17A/GSK3β/ROS axis mediated fibrosis and arthritis. Degree of the profibrogenic marker α-SMA in synovium was evaluated in all rats' group end of week 6 to link between α-SMA and ACPA (A), IL-17A (B), MDA (C), and SOD (D). α-SMA: alpha-smooth muscle actin; ACPA: anti-citrullinated protein antibody; IL-17A: interleukin-17A; malondialdehyde; SOD: superoxide dismutase.

DISCUSSION

To assess the working hypothesis that rheumatoid arthritis (RA) can augment IL-17A/GSK3β/ROS axis-mediated fibrosis in knee joint synovium that can be inhibited with TDZD-8, the autoimmune and inflammatory disease RA was modelled in rats. This was demonstrated in Fig. 1 by (i) a sharp increase in the blood levels of the specific RA biomarker ACPA that commonly observed in RA patients (Kurowska *et al*., 2017); and (ii) upregulation of the synovium Bcl-2 (synovial hyperplasia), but not the apoptosis biomarker p53, which agreed with previous work (Audo *et al*., 2007). In addition, the correlation between the above mentioned parameters (IL-17A/GSK3β/ROS) as well as the biomarker of RA was investigated to further corroborate such a link. Here, the data showed that induction of RA in rats using an active immunization method with bovine type II collagen injections after 6 weeks caused a profound increase in knee synovial tissue IL-17A, IL-6, and α-SMA, as well as blood ROS and ACPA, which appeared to be inhibited by TDZD-8 (Fig. 6). Also, the correlation data (Fig. 5) that linked all these parameters is further supported the working hypothesis stated in this report.

Currently, there is no cure for this chronic autoimmune disease (RA) that causes damage to the joints. Therefore, exploring new pathway(s) or further understanding the known tools involved in the pathophysiology of RA would help to treat or minimize the damage incurred by RA. IL-17A increased both autoimmunity and ROS production which causes augmentation of the inflammatory cytokines that can lead to inflammation of the synovial membrane and bone erosion is a characteristic feature of RA (Dhillion *et al*., 2012; Binger *et al*., 2017). The other important parameter in this investigated axis that is known to be involved in the pathophysiology of RA and osteoporosis is the GSK-3β enzyme (Zahoor *et al*., 2014; Zhou *et al*., 2016). (i) GSK-3β is activated by IL-17A (Xu & Cao, 2010); and (ii) GSK-3β induces mitochondrial ROS production (Yang *et al*., 2017). These reports are in agreement with the data presented in this report that demonstrated the activation of IL-17A/GSK3β/ROS axis, which appeared to be inhibited by the inhibitor of GSK3β (Figs. 2 and 3). Furthermore, fibrosis mediated by IL-17A/GSK3β/ROS axis (Singh *et al*., 2015; Zhang *et al*., 2019; Lou *et al*., 2021) is also in agreement with this study showing the upregulation of the profibrogenic marker α-SMA that was inhibited by the GSK3β inhibitor (Fig. 4).

In summary, using a rat model of knee joint RA induced by active immunization with COII, this study demonstrated the stimulation of IL-17A/GSK3β/ROS axis mediated fibrosis and arthritis, which appeared after 42 days, to be inhibited by TDZD-8 treatment. Therefore, this report represents an important contribution to the study of the autoimmune and inflammatory disease, rheumatoid arthritis induced by actively immunizing rats with bovine type II collagen for 21 days followed by treatment for another 21 days with the inhibitor (TDZD-8) of the enzyme GSK3β that is proven to be involved in the pathophysiology of rheumatoid arthritis. Augmentation of synovium and blood levels of IL-17A/GSK3β/ROS/α-SMA axis mediated arthritis that was inhibited by TDZD-8 was demonstrated in this animal model, which can be a useful model to test specific pharmacological and molecular inhibitors to this investigated axis-mediated arthritis.

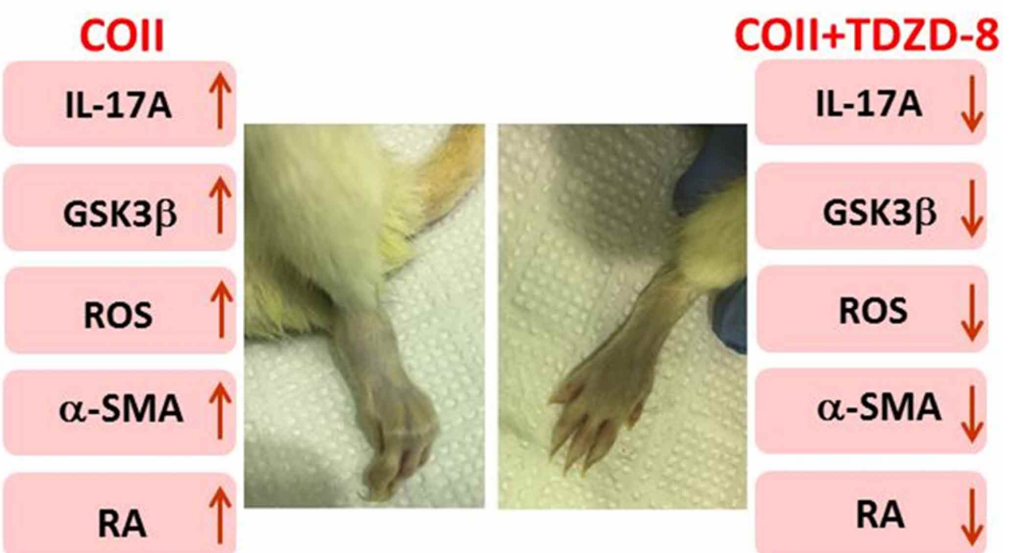

Fig. 6. Proposed model for rheumatoid arthritis which appears to be inhibited by TDZD-8. COII: collagen type II; TDZD-8: thiadiazolidine derivative; IL-17A: interleukin-17A; GSK3β: glycogen synthase kinase-3β; ROS: reactive oxygen species; α-SMA: alpha-smooth muscle actin; RA: rheumatoid arthritis.

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RESUMEN: La artritis reumatoide (AR) que afecta la articulación sinovial de la rodilla provoca inflamación de la membrana sinovial y daño tisular. La interleucina-17A (IL-17A) y la enzima glucógeno sintasa quinasa-3β (GSK3β) están involucradas en la patogenia de la AR. No se ha estudiadol vínculo entre IL-17A, GSK3β, el estrés oxidativo y el marcador profibrogénico actina de músculo liso alfa (α-SMA) con y sin inhibidor de TDZD-8, GSK3β. En consecuencia, se realizó una inmunización activa de ratas para inducir la AR después de tres semanas usando inyecciones de colágeno tipo II (COII). El grupo tratado recibió una inyección diaria de 1 µg/kg de TDZD-8 durante 21 días siguiendo el protocolo de inmunización (COII+TDZD-8). Se recogieron muestras de sangre y tejido sinovial al final del experimento. El desarrollo de AR se confirmó como lo corroboró el aumento sustancial en los niveles sanguíneos del autoanticuerpo altamente específico para AR, el anticuerpo antiproteína citrulinada, así como el aumento de los niveles de especies oxidativas reactivas (ROS) medidos como peroxidación lipídica. La inducción de AR también aumentó los niveles de tejido sinovial de IL-17A y el marcador profibrogénico, α-SMA. Todos estos parámetros parecían mejorar significativamente ($p<0,0001$) con TDZD-8. Además, se observó una correlación significativa entre IL-17A, ROS y α-SMA y biomarcadores de AR. Por lo tanto, la inducción de AR en la sinovial de la articulación de la rodilla aumentó la artritis mediada por el eje IL-17A/GSK3β/ROS/α-SMA en un modelo de rata de AR, que fue inhibida por TDZD-8.

PALABRAS CLAVE: Artritis reumatoide; IL-17A; GSK3β; ROS; α-SMA; Fibrosis; TDZD-8.

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