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Suppression of Nitrosative Stress and Inflammation of the Knee Joint Synovium in Collagen Type II-Induced Rheumatoid Arthritis by the Inhibition of Glycogen Synthase Kinase-3ß

Supresión del Estrés Nitrosativo e Inflamación de la Sinovial de la Articulación de la Rodilla en la Artritis Reumatoide Inducida por Colágeno Tipo II Mediante la Inhibición de la Glucógeno Sintasa Quinasa-3β

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SUMMARY: Rheumatoid arthritis (RA), an inflammatory autoimmune disease that causes cartilage degradation and tissue destruction, can affect synovial joints such as the knee joint. The link between the nitrosative stress enzyme inducible nitric oxide synthase (iNOS) and the cytokine interleukin-1 (IL-1β) in RA-induced knee joint synovial membrane damage with and without the incorporation of the GSK3β inhibitor TDZD-8 has never been studied. As a result, we used active immunization method with collagen type II (COII) for twenty one days to induce RA in rats. TDZD-8 (1 mg/kg; i.p.) was given daily into matched immunized rats for three weeks after day 21 (COII+TDZD-8). Blood and tissue samples were taken 42 days after immunization. A dramatic increase in rheumatoid factor (RF) blood levels, as well as considerable synovial tissue damage and inflammatory cell infiltration of the synovial membrane, were used to validate the onset of RA following COII immunization. COII immunization increased tissue levels of iNOS protein and IL-1β mRNA and protein expression, which TDZD-8 suppressed considerably ($p<0.0001$). Furthermore, there was a significantly ($p<0.001$) positive correlation between iNOS, inflammatory biomarkers, and RF. We concluded that TDZD-8 reduced RA-induced IL-1β -iNOS axis-mediated arthritis in the rat knee joint synovium.

KEY WORDS: Rheumatoid arthritis; IL-1β-iNOS axis; TDZD-8; Nitrosative stress; Rat model.

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory illness distinguished by persistent joint inflammation s that can lead to tissue loss and cartilage erosion (Ahlmén *et al*., 2010; Smolen *et al*., 2018). Infiltration of inflammatory cells into the synovium (also known as the synovial membrane) and proliferation of fibroblast-like synoviocytes (FLS) enhance synovial inflammation (synovitis), which draws in additional immune cells (Guo *et al*., 2018). FLS promotes osteoblast activity, bone erosion and cartilage breakdown, synovial angiogenesis, and immune cell infiltration in RA patients (Nygaard & Firestein, 2020). This accelerates joint destruction, resulting in joint swelling and discomfort, bone remodelling and distortion, and finally incapacity (Scott *et al*., 2010; Nygaard & Firestein).

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The involvement of glycogen synthase kinase-3 (GSK3b) in the pathogenesis of metabolic disorders and diseases like diabetes, multiple sclerosis, Alzheimer's disease, and RA is well-established (Miao *et al*., 2013; Maixner & Weng, 2013; Zhang *et al*., 2018). In the afflicted joints of patients suffering fromRA, there is a relationship between GSK-3, inflammation, and proliferation (Yoshino & Ishioka, 2015; Zhou *et al*., 2016). Indeed, GSK-3 regulates innate and adaptive immune responses like T-cell proliferation, differentiation, and survival (Beurel *et al*., 2010), as well as upregulating the pro-inflammatory cytokines IL-6 and monocyte chemoattractant protein 1 and downregulating anti-inflammatory cytokines like IL-10 (Steinbrecher *et al*., 2005; Chan *et al*., 2009).

Furthermore, the inflammatory cytokine IL-1 β is located upstream of iNOS in cell signaling, such as in primary chondrocyte tissue culture and in osteoarthritis animal models (Mäki-Petäjä *et al*., 2008), and iNOS has been observed to be raised in the blood of patients with RA (Mäki-Petäjä *et al*.; Zhou *et al*., 2020). As a result, the goal of the current study was to see if the IL-1 β -iNOS axis is involved in rheumatoid arthritis that affects the knee joint synovium with and without TDZD-8 incorporation.

MATERIAL AND METHOD

Animals. All investigations were performed on Wistar male rats (150 - 170g, 8 weeks old) supplied by the animal house at King Saud University (Riyadh, Saudi Arabia). Rats had unrestricted access to food and water and were kept a constant temperature (twenty two degree Celsius) and a twelve hour light/dark cycle. All experimental techniques were authorized by the Ethical Committee of Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia, and followed the criteria for laboratory animal care and use issued by the US National Institutes of Health (NIH publication No. 85-23, amended 1996).

Induction of rheumatoid arthritis (RA) by type II collagen (COII): As previously described, active immunization was used to produce RA in rats (Brand *et al*., 2007). In a nutshell, lyophilized bovine type II collagen (Sigma-Aldrich, MO, USA) emulsified in an equal volume of complete Freund's adjuvant (Sigma-Aldrich, MO, USA) was injected intradermal (i.d) in a volume of 100 L at a final concentration of 200 g COII on day 0 in a volume of 100 L. At day 14, a booster dose (two hundred g in one hundred letters) of incomplete Freund's adjuvant (Sigma-Aldrich, MO, USA) was given intradermal. At day 21, RA was established.

Experimental design. 24 rats were divided into four groups (each with six members): (1) Control group: rats were given 100 µl of 0.9% saline by intradermal route on days zero and fourteen, and then 100 µl of 0.1 percent DMSO (i.p.) daily from day twenty one to day fourty two; (2) TDZD-8 control group: rats were given the same treatment as before and then given TDZD-8 (100 µl of 1 mg/kg; i.p.) (Abcam, Cambridge, UK) every day from day twenty one to day fourty two; (3) the model group (RA): rats with RA were given 100 µl of 0.1 percent DMSO as a vehicle from day twenty one to day forty two; and (4) the treated group (COII+TDZD-8): rats with RA were given a daily i.p injection of of TDZD-8 (100 µl of 1 mg/kg; i.p.) (Zhou *et al*., 2016) from day twenty one to day forty two. TDZD-8 was dissolved in diluted 1% DMSO.

Collection of blood and isolation of the synovium. After being anesthetized with fifty mg/kg sodium pentobarbital, rats were culled by cervical dislocation. Blood samples were drawn, and sera were separated and stored at minus twenty degrees Celsius for biochemical analysis. Under a dissecting microscope, the soft tissue and ligaments above the patella of the exposed knee joints were removed, and the synovium was extracted, snap-frozen in liquid nitrogen, and kept at minus eighty degree Celsius until use.

Histological examination. After tissues were dried in successive grades of alcohols, collected specimens from synovium tissues were fixed for twenty four hours in 10% formal saline and paraffin blocks were made. For tissue histological investigation, tissue sections (5 µm thick) were stained with the basic histology stain, hematoxylin and eosin (H & E).

iNOS and CD45 immunohistochemistry. Antigen retrieval was done on deparaffinized synovium tissue sections (5 µm thick). The primary antibodies, anti-inducible nitric oxide synthase (iNOS) (Abcam, cat # ab15323) and anti-cluster of differentiation (CD) 45 (Abcam, cat # ab10558), were incubated in a humidity chamber for 1 hour at room temperature (RT), washed, and incubated with the secondary antibody for 12 hours at RT. Meyer hematoxylin was used to counterstain the sections.

Determination of rheumatoid factor (RF) blood levels. RF was measured using an ELISA kit (CUSABIO Technology LLC, TX, USA) that was utilized according to the manufacturer's instructions.

Western blotting analysis of interleukin -1beta (IL-1β), tumor necrosis factor-alpha TNF-α, and interleukin-6 (IL-6): Anti- interleukin -1beta (Santa Cruz Biotechnology), anti- tumor necrosis factor-alpha (Santa Cruz

Biotechnology), and anti- interleukin-6 antibodies were used to Western blot extracted protein (40 µg per sample) from the synovium, as previously stated (Al-Ani *et al*., 2010). (Santa Cruz Biotechnology). The protein bands were seen using an ECL detection kit (Thermo Fishe
r Scientific Inc, Rockford, IL, USA). To assess band intensities (C-Di Git blot scanner; LI-COR, NE, USA) image analysis software was used.

Statistical analysis. The statistical analysis was carried out using the GraphPad Prism statistical software tool (version 6). To analyze the differences between the four groups in the study, one-way ANOVA was used, followed by Tukey's test. The results were judged significant when $P < 0.05$ was used to express the data.

RESULTS

Induction of rheumatoid arthritis in rats by type II collagen immunization. We began by simulating RA in rats. Active immunization of rats with COII triggered RA, resulting in a significant rise in RA biomarkers and aberrant changes in synovial membrane architecture (Fig. 1). In comparison to the control group (Control), COII immunization of the model group of rats (COII) resulted in a significant ($p<0.0001$) rise in blood rheumatoid factor (RF) (Fig. 1A) and synovial TNF-α (Fig. 1B). In synovial tissue slices from the model group (COII), immunohistochemical labeling for CD45 (leukocyte common antigen) revealed robust CD45 stained cells (Fig. 1D), compared to negative to weak CD45 stained cells in the control group (Fig. 1C). In comparison with a normal tissue histology in the control group, basic histology stained synovium sections of the model group exhibited extensive hyperplasia of the synoviocyte and inflammatory cells infiltration of into the subsynovium (data not shown).

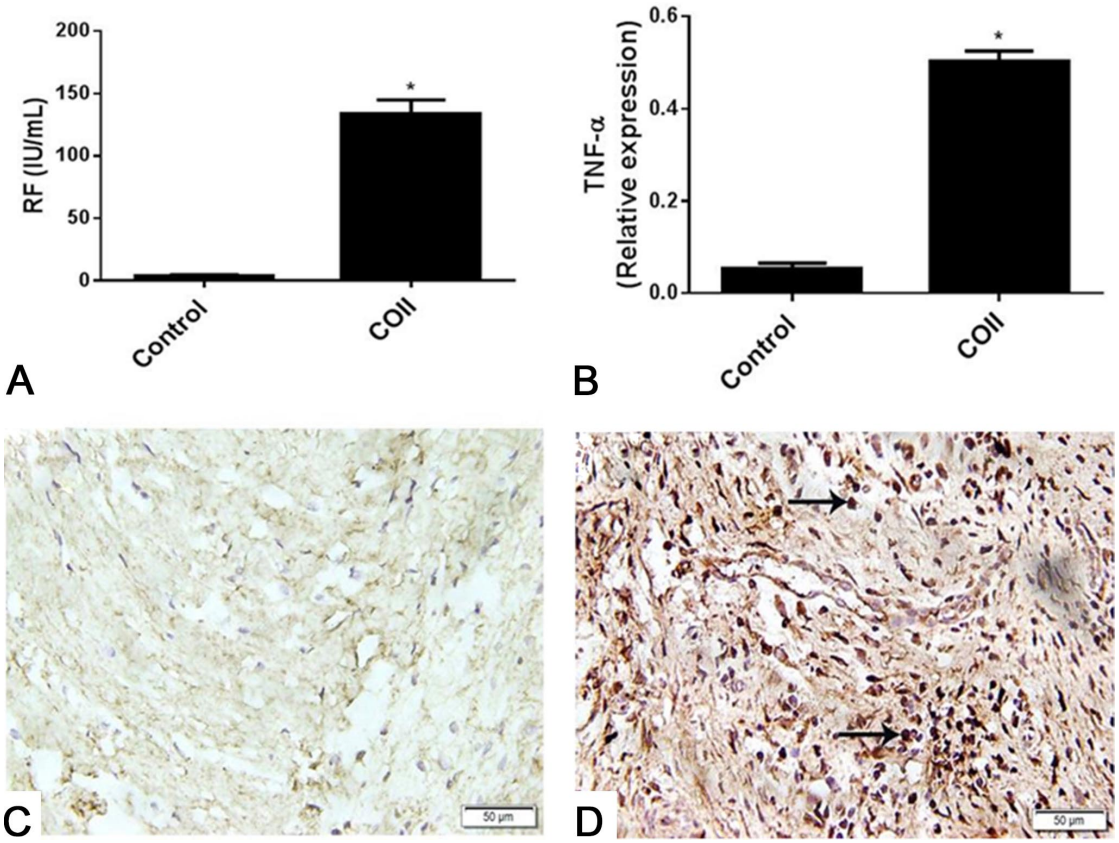

Fig. 1. In rats, collagen type II (COII) causes rheumatoid arthritis (RA). At the end of the experiment, at day 42, blood levels of RF (A) and synovium levels of the inflammatory biomarker TNF- a (B) were assessed in the model group of rats (COII) compared to the control group of rats (Control). For each group, the results are the mean (±SD); n=6. All of the *p* values shown are significant. * $p<0.0001$ when compared to the control group. (B, C, and D) The immunohistochemistry of CD45 in synovium sections from control (C) and COII (D) rats is shown. RF stands for rheumatoid factor, TNFα stands for tumor necrosis factor α, COII stands for collagen type II, and CD45 stands for cluster of differentiation (CD) 45.

TDZD-8 inhibits biomarker of nitrosative stress in injured synovium induced by rheumatoid arthritis. We measured the levels of iNOS in all animal groups to see if iNOS is increased in our RA animal model and if TDZD-8 can prevent the predicted iNOS induction (Fig. 2), In synovial tissue sections from the RA group (COII), immunohistochemical staining for iNOS revealed strong positive iNOS stained cells in the synovial tissue (arrows) and the endothelium (curved arrow) (Figs. 2B and 2D), which were significantly ($p<0.0001$) reduced by TDZD-8 in the COII+TDZD-8 group (Figs. 2C and 2D). However, as compared to the control group of rats, the level of iNOS in the COII+TDZD-8 group was much higher, indicating that TDZD-8 was only partially inhibiting the enzyme.

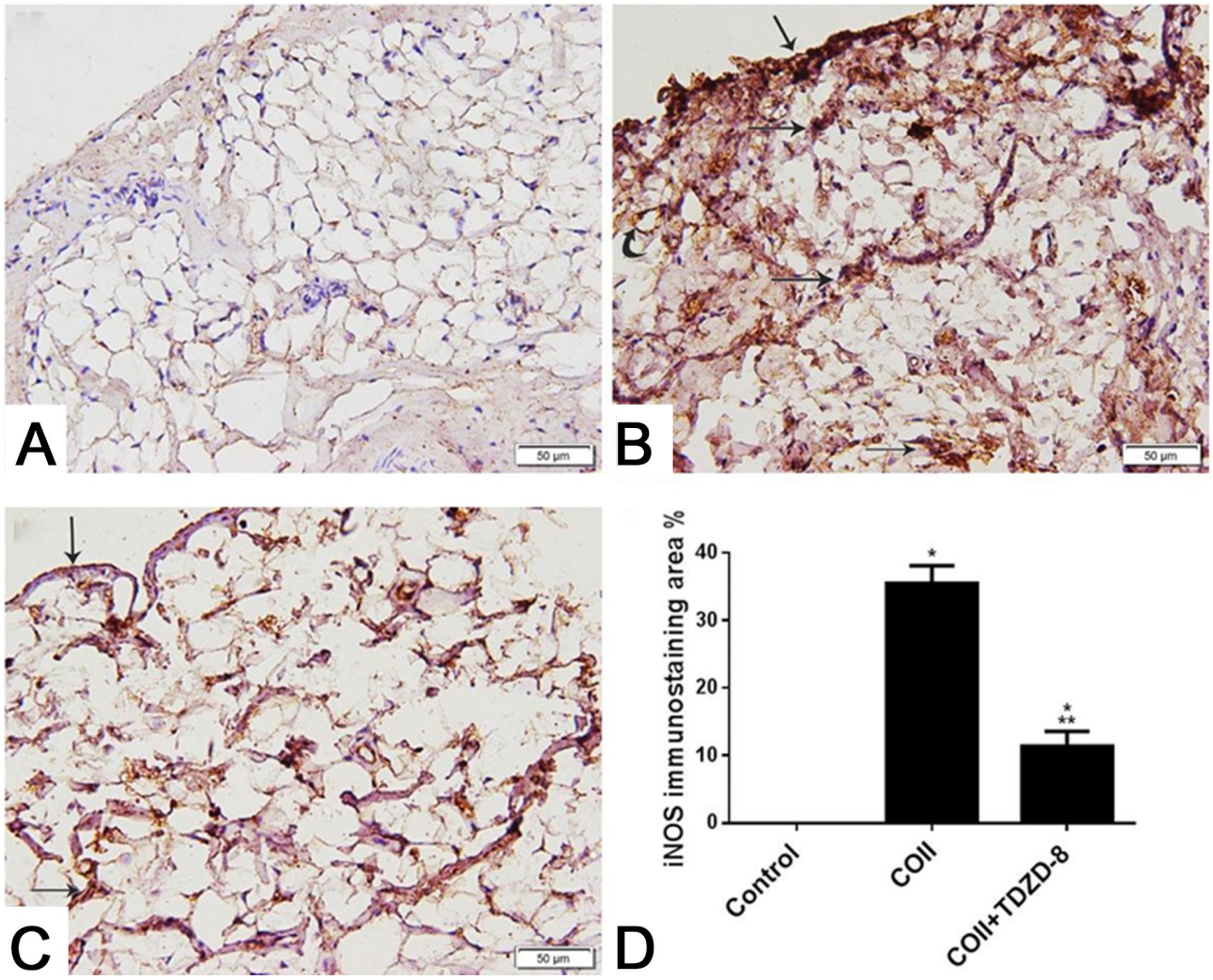

Fig. 2. In synovial tissue, TDZD-8 reduces rheumatoid arthritis (RA)-induced nitrosative stress (iNOS). Synovium sections (x200) obtained from the control (A), COII (B), and COII+TDZD-8 (C) groups of rats were immunohistochemically stained for iNOS. The histograms in (D) show a quantitative study of iNOS immunostaining area percent in these groups' synovium. For each group, the results are the mean (±SD); n=6. All of the p values shown are significant. * $p<0.0001$ in comparison to control, ** $p<0.0001$ in comparison to COII. COII: collagen type II; TDZD-8: thiadiazolidine derivative; iNOS: inducible nitric oxide synthase.

TDZD-8 inhibits biomarker of inflammation in injured synovium induced by rheumatoid arthritis. iNOS is located downstream of the inflammatory cytokine IL-1β in cell signaling (Zhou *et al*., 2020). We measured the gene and protein expression of synovial tissue IL-1β in all animal groups, as well as synovial inflammatory biomarkers IL-6 and TNF-α proteins, which are also induced when IL-1β is activated, to investigate the role of the IL-1β -iNOS axis in rheumatoid arthritis with and without TDZD-8 (Zhou *et al*., 2020). In the model group (COII), RA induced a significant ($p<0.0001$) rise in synovial IL-1β (Figs. 3A and 3B), IL-6 (Fig. 3C), and TNF-α (Fig. 3D) in comparison to the control group

(Control) (Control and TDZD-8). However, the levels of these parameters in the treated group of rats were still significant ($p<0.0001$) compared with the control in (C) and (D).

Furthermore, no significant difference between the untreated control and TDZD-8 control in all tested parameters (data not shown).

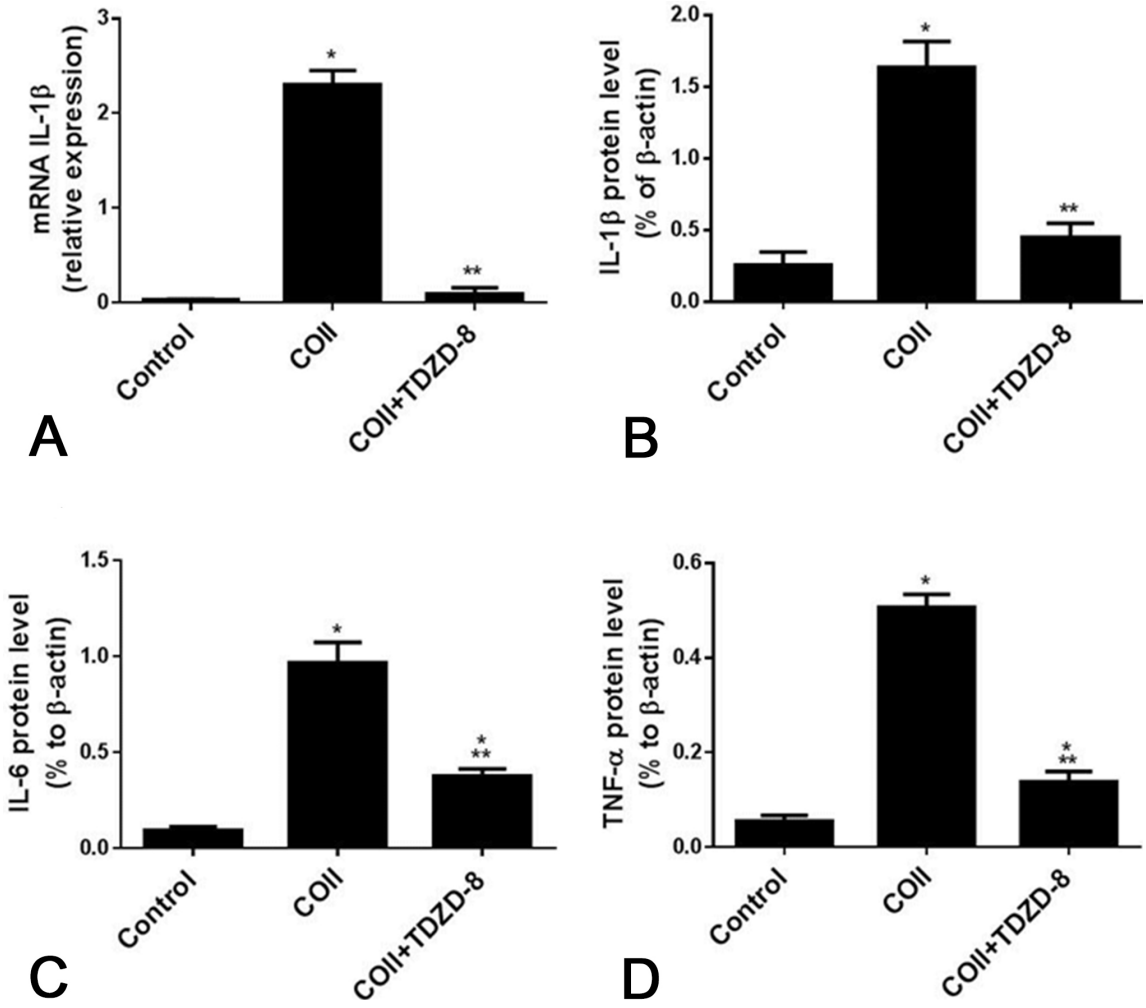

Fig. 3. TDZD-8 inhibits rheumatoid arthritis (RA)-induced inflammation in synovial tissue. synovium levels of IL-1β mRNA (A), IL-1β protein (B), IL-6 protein (C), and TNF-α protein (D) were measured at day 42 in the rats' groups; Control, COII, and COII+TDZD-8. Results represent the mean (±SD); n=6 for each group. Presented *p* values are all significant. * $p<0.0001$ *versus* control, ** $p<0.0001$ *versus* COII. IL-1β: interleukin-1β; IL-6: interleukin-6; TNF-α: tumour necrosis factor- α; COII: collagen type II; TDZD-8: thiadiazolidine derivative.

Correlation between nitrosative stress and biomarkers of rheumatoid arthritis and inflammation. To draw a link between nitrosative stress and inflammation and biomarker of RA in an animal model of RA, we evaluated the correlation between iNOS score and RF (Fig. 4A), IL-1β (Fig. 4B), IL-6 (Fig. 4C), and TNF-a (D). iNOS score displayed positive correlation with RF ($r = 0.978$) ($p<0.0001$), IL-1b ($r = 0.947$) ($p<0.0001$), IL-6 ($r = 0.986$) ($p<0.0001$), and TNF α ($r = 0.969$) ($p<0.0001$).

DISCUSSION

The main objective of this study was to examine nitrosative stress and IL-1β-iNOS mediated arthritis in rat knee joint synovium with and lacking the GSK3β inhibitor, TDZD-8. Similarly we investigated a correlation between nitrosative stress score and biomarkers of RA. Therefore, we modelled RA in rats to test the above mentioned working

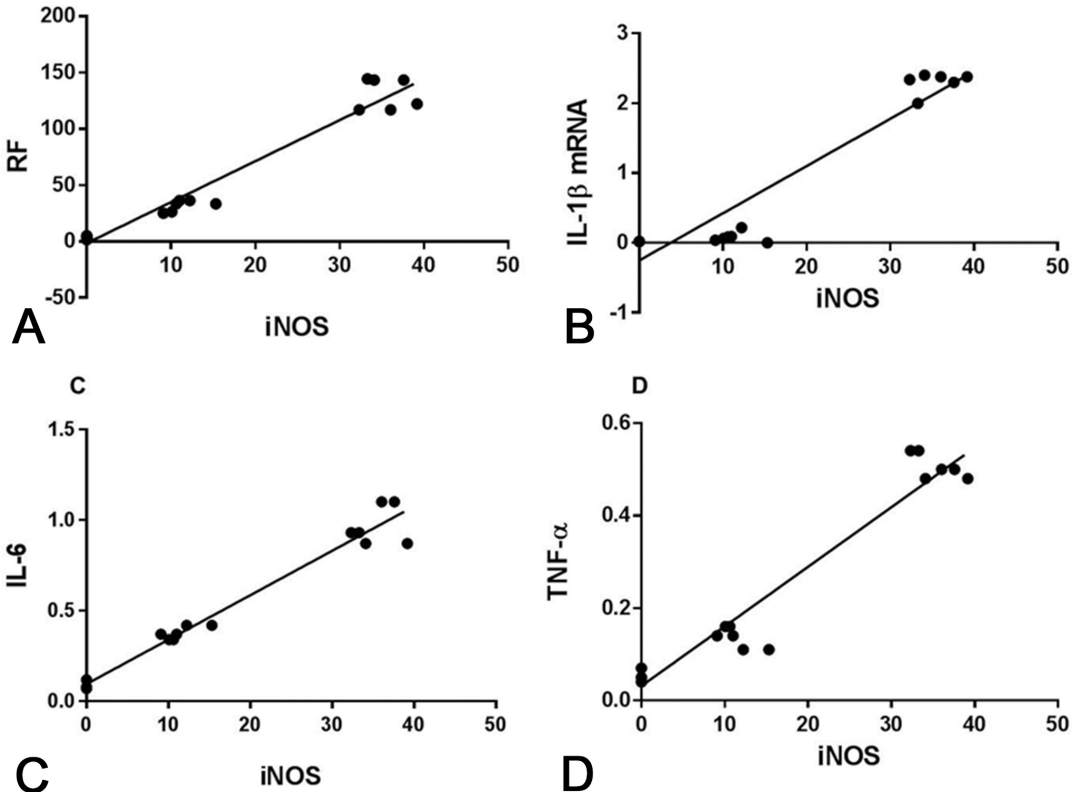

Fig. 4. Correlation between the nitrosative stress score and biomarkers of rheumatoid factor (RF) and inflammation. Degree of nitrosative stress (iNOS) in synovium was assessed in rats after the completion of the experiment, at day 42 in order to link between iNOS and RF (A), IL-1β (B), IL-6 (C), and TNF-α (D). iNOS: inducible nitric oxide synthase; RF: rheumatoid factor; IL-1b: interleukin-1β; IL-6: interleukin-6; TNF-α: tumour necrosis factor- α.

hypothesis. Here, we demonstrate that RA induction in rats via COII active immunization after 42 days caused a substantial augmentation of the knee synovial tissue IL-1β-iNOS axis mediated arthritis, which was ameliorated by TDZD-8 (Figs. 2 and 3). In addition, our data shown in Fig. 4 revealed a significant linkage between nitrosative stress (iNOS score) and biomarkers of inflammation and RA (IL-1β, IL-6, TNF-α, and RF). Therefore, our results are in agreement with the aim of this work in that COII immunization induced the activation of IL-1β-iNOS mediated arthritis in knee joint synovial membrane and TDZD-8 was able to ameliorate the action of COII.

Immune cells like macrophages and monocytes produce inflammatory mediators (Firestein, 2003; Cuda *et al*., 2016), and iNOS was testified to be elevated in the blood of RA patients (Mäki-Petäjä *et al*.). In addition, (i) deletion of the iNOS gene decreased joint inflammation and destruction in a rat with RA (Bell *et al*., 2019); and (ii) therapies that block IL-1β and TNF-α decreased the number of macrophages in the synovial sublining, inflammation, and bone erosion in RA patients (Liang *et al*., 2016; Bouta *et al*., 2017). These findings support our findings, which show that the IL-1β-iNOS axis is important in mediating arthritis associated with inflammation, immune cell infiltration into the knee synovium, and tissue injury (Figs. 1-3). Furthermore, our data that showed TDZD-8 caused a significant reduction in iNOS and inflammation (Figs. 2 and 3) which supports previous studies in RA mice that reported an inhibition of iNOS in inflamed joints and blood IL-1β, IL-6, and TNF-α (Cuzzocrea *et al*., 2006; Kwon *et al*., 2014).

In conclusion, we have demonstrated in a rat model of rheumatoid arthritis that causes knee joint synovial membrane injury, the activation of IL-1β-iNOS axis mediated arthritis, which is significantly inhibited by TDZD-8 treatment for 21 days.

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RESUMEN: La artritis reumatoide (AR), es una enfermedad autoinmune inflamatoria que causa la degradación del cartílago y la destrucción del tejido, pudiendo afectar las articulaciones sinoviales, como la articulación de la rodilla. No se ha estudiado el vínculo entre la óxido nítrico sintasa inducible por la enzima del estrés nitrosativo (iNOS) y la citocina interleucina-1 (IL-1β) en el daño de la membrana sinovial de la articulación de la rodilla provocado por AR con y sin la incorporación del inhibidor de GSK3β TDZD-8. Utilizamos el método de inmunización activa con colágeno tipo II (COII) durante veintiún días para inducir AR en ratas. Se administró TDZD-8 (1 mg/kg; i.p.) diariamente a ratas inmunizadas emparejadas durante tres semanas después del día 21 (COII+TDZD-8). Se tomaron muestras de sangre y tejido 42 días después de la inmunización. Se observó un gran aumento de los niveles sanguíneos del factor reumatoideo (FR), así como un daño considerable del tejido sinovial e infiltración de células inflamatorias en la membrana sinovial, para validar la aparición de la AR después de la inmunización con COII. La inmunización con COII aumentó los niveles tisulares de la proteína iNOS y la expresión de proteína y ARNm de IL-1β, que TDZD-8 suprimió considerablemente ($p<0,0001$). Además, hubo una correlación positiva significativa ($p<0,001$) entre iNOS, biomarcadores inflamatorios y FR. Concluimos que TDZD-8 redujo la artritis mediada por el eje IL-1β-iNOS inducida por la AR en la sinovial de la articulación de la rodilla de rata.

PALABRAS CLAVE: Artritis reumatoide; Eje IL-1β-iNOS; TDZD-8; Estrés nitrosativo; Modelo de rata.

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