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Norisoboldine Reduces Arthritis Severity by Attenuating Inflammation, Oxidative Stress, and Extracellular Matrix Degradation in a Rat Model of Rheumatoid Arthritis

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Introduction: Rheumatoid arthritis (RA) is a chronic autoimmune disorder marked by persistent joint inflammation, pain, and tissue degradation. This study evaluates the therapeutic potential of Norisoboldine (NOR), an isoquinoline alkaloid from Lindera aggregata, in a rat model of RA.

Methods: Rats were divided into five groups: normal control (G1), RA model (G2), NOR-treated groups at 15 mg/kg (G3) and 30 mg/kg (G4), and methotrexate-treated group (G5). NOR's anti-arthritic effects were assessed by measuring clinical arthritis scores and inflammatory markers (RF, CRP, TNF-α, IL-6, IL-10). Oxidative stress markers (MDA, SOD, catalase, GPx) and pathways (NF-κB/IKKβ and Nrf2/Keap1) were also evaluated. Histopathology assessed synovial inflammation and tissue degradation.

Results: NOR treatment significantly reduced arthritis severity, as evidenced by decreased clinical arthritis scores and inflammatory markers in RA rats. NOR also exhibited strong antioxidant effects, demonstrated by decreased MDA levels and enhanced SOD, catalase, and GPx activities. NOR further downregulated matrix metalloproteinases (Mmp-2, Mmp-3), aggrecanases (Adamts-4, Adamts-5), and PCNA expression. Histopathology confirmed marked reductions in synovial inflammation and tissue damage in NOR-treated groups.

Discussion: These findings suggest that NOR's anti-inflammatory and antioxidant properties contribute to reducing both inflammation and the overall severity of RA. NOR's multifaceted actions support its potential as a novel therapeutic agent for RA.

Conclusion: NOR demonstrates protective effects in RA rats by reducing inflammation, oxidative stress, and extracellular matrix degradation, offering promise as a therapeutic option to manage RA pathology comprehensively.

Keywords: Norisoboldine, methotrexate, rheumatoid arthritis, inflammation, oxidative stress

Introduction

Rheumatoid arthritis (RA) is a chronic disease that primarily affects the joints and is characterized by painful inflammation of the synovium.[1] If left untreated, it can have devastating effects such as cartilage breakdown, bone erosion, disability and organ damage.[2,3] According to Almutairi, Nossent, Preen, Keen and Inderjeeth,[4] an estimated 18 million people worldwide live with RA and incidence rate from 1980 to 2019 was reported to be around 5% (460 per 1,00,000 members) at a global level. Although the pathophysiology is still unclear, inflammatory factors such as pro- and/or anti-inflammatory cytokines and inflammatory mediators play a crucial role in the development and progression of this disease.[1,5] Although the main pathway in the pathogenesis of RA is high-grade inflammation, numerous scientific evidence has shown the linkage of oxidative stress and inflammation in this process.[6,7] Moreover, the subsequent activation of nuclear factor-κB (NF-κB) has been reported to stimulate the expression of various genes responsible for

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the maintenance of inflammation.[8] In recent years, dysregulation of matrix metalloproteinases (MMPs)[9] and proteoglycans, ie aggrecanases, which contribute to the pathophysiology of RA have been discovered, representing an important target for improved targeted RA therapy.

Although several lines of treatment options such as antirheumatic drugs, non-steroidal anti-inflammatory drugs, biological agents, and glucocorticoids are currently available to treat RA, these treatment options would only offer temporary relief, presented with adverse effects and cause immunosuppression, thereby prone to opportunistic infections.[10,11] Considering efficacy and safety concerns with the currently available drugs, exploring a critical alternative approach, ie, natural medicine, is undoubtedly warranted due to its health-promoting effects and fewer side effects.[12,13] Nowadays, research on natural compounds, including flavonoids, polyphenols, terpenes glycosides, and alkaloids, is gaining momentum to explore treatment options, particularly for treating RA.

Since ancient times, alkaloids have been considered a remarkable class of natural molecules to treat various health complications, including inflammation-mediated diseases or disorders.[14] Norisoboldine (NOR) is one such alkaloid, a primary isoquinoline isolated from the roots of *Lindera aggregata*, a well-known traditional Chinese medicine. Previously, studies conducted on NOR have demonstrated its therapeutic efficacy against various inflammation-mediated auto-immune diseases through unique mechanisms such as attenuation of collagen-induced arthritis through gut-associated lymphoid tissues in rats,[15] amelioration of ulcerative colitis[16] and collagen-mediated induction of arthritis in mice[17] through differentiation and development of Treg cells (sub-population of T cells), which are known for its crucial role in protection of immune system.

Although NOR is effective in various arthritis models, an in-depth study revealing the possible roles of different signaling pathways, such as NF-κB/Nrf2-ARE, MMPs, aggrecanase, and PCNA, is still needed. Hence, adjuvant-induced RA model rats were created to examine NOR's protective role through a comprehensive assessment using clinical, biochemical, molecular, and histopathological parameters.

Materials and Methods

Drugs and Chemicals

Norisoboldine (NOR), methotrexate and complete Freund's adjuvant (CFA) were procured from Sigma chemicals, USA. While other chemicals of the highest analytical grade were obtained from locally approved vendors.

Animals

Male Sprague-Dawley (SD) strain rats with 160–180 grams body weight were obtained from an authorized laboratory animal vendor. After receiving animals, they were strictly monitored for health conditions in a quarantine room by a study veterinarian, and animals that proved healthy were further considered for the conduct of the study. Acclimatization of animals to experimental room conditions for an initial five days was considered before the start of the study. During the acclimatization period, individual animals were identified by temporary numbers at the tip of the tail using a marker pen and once observed for ophthalmological and detailed health examinations by the study veterinarian. Animals had *ad libitum* free access to rodent pellet diet (Adequate nutritional components and free of microbes and contaminants) and drinking water (free of microbes) with sterilized clean corn cob for bedding purposes. Animals were maintained in well-controlled experimental room conditions such as temperature: 22 ± 3°C; relative humidity: 50–60%; and light/dark cycle: 12/12 hours. The studies were approved by the Institutional Animal Welfare and Ethics Committee (IAWEC) of the First Affiliated Hospital of Xingtai Medical College (Xingtai First Hospital) in Xingtai. The procedures were examined and approved, and the approval number: FAHXM-2023-107. The committee ensures that the research meets ethical standards regarding animal welfare and treatment during the research.

Induction of Adjuvant Arthritis (AA) in Rats

The experimental AA rat model was induced by treating animals with complete Freund's adjuvant (CFA) on day 1 (0.1 mL) and on day 7 (0.05 mL) through a subcutaneous route in the left posterior paw.[18] The model was evaluated on day 14 for the induction of arthritis by means of arthritis score and swelling of the paw. Arthritis index scores were

given blindfolded by an observer to all the groups at a scale of 0–4, with 0 being no swelling or erythema and 1–4 being slight, low to moderate, pronounced (limited usage of joints) and severe (rigidity in joints) erythema and/or edema. Paw volume was measured by using a plethysmometer.

Experimental Design and Treatment

Animals were grouped into five (5) groups of 10 in each group.

Group 1 (G1) was considered a normal control (NC) group.

Groups 2–5 rats were experimentally induced with rheumatoid arthritis (RA).

No rats were lost during the study period. The animals from group 2 (G2) were continued without treatment and considered a Rheumatoid arthritis (RA) model. While group 3 (G3) and group 4 (G4) animals were administered daily via oral route for 28 days with doses of 15 and 30 mg/kg NOR respectively.[19] On the other hand, group 5 (G5) rats were dosed with methotrexate (MTX) at 0.3 mg/kg twice a week.[20] Measurements of arthritis score (on days 0, 14, 28 and 42) and paw volume (on days 0, 14, 28 and 42) were determined. After the experimental period, ie, blood samples were collected from all animals and plasma was separated and stored until further analysis. Animals were euthanized under CO_2 asphyxiation. Also, joint tissue was collected and stored in 10% neutral formalin saline till further processing of tissues for histopathology.

Biochemical Analysis

After completion of the experimental period, animals before being sent for necropsy, blood collection was done in tubes with anti-coagulant tubes and subjected to centrifugation in a refrigerated centrifuge by setting conditions such as speed at 2500 rpm, temperature at 4°C and time for 10 minutes to separate plasma. Plasma was then used to determine arthritis markers such as levels of rheumatoid factors (RF) and C-reactive protein (CRP) with the help of the ELISA kit (CSB-E13666r and CSB-E07922r respectively, Cusabio Technology Llc, Houston, TX 77054, USA) based method.

Histopathology

The histopathological changes in joint tissues of normal controls as well as treated animals were studied using Hematoxylin and Eosin (H & E) staining method. Tissues were trimmed, processed (alcohol washes), embedded (paraffin fixation), sectioned (3–5 μm thick sections), and stained (H&E) and Masson trichrome (MT). The study pathologist performed a quality check of the slides in a blindfolded manner and read the slides using an Olympus phase contrast microscope (Tokyo, Japan).

Immunohistochemistry

For the immune-histochemical analyses, sections were submitted to deparaffinization, rehydration, blocking of endogenous peroxidase activity and antigen retrieval process. Sections were kept for overnight incubation at 4°C with respective primary antibodies such as anti-Nrf2, anti-TNF-α, anti-MMP2 and anti-PCNA later subjected to one-hour incubation with appropriate biotinylated HRP-conjugated secondary antibody. After that, sections were then submitted to treatment with 3.3'-diaminobenzidine and counterstaining with hematoxylin. The immuno-histochemical staining was imaged under an Olympus phase contrast microscope (Tokyo, Japan) and the intensity of the same was measured with the help of an image analyzer (Image-Pro Plus version 6.0 software). Simultaneously negative control slides were also maintained.

Evaluation of Markers of Inflammation

The markers of inflammation, ie, pro-inflammatory markers: nuclear- NF-κB p65 (E-EL-R0674, Elabscience, Wuhan, China), IL-6 (P20607, Elabscience, Wuhan, China), and anti-inflammatory marker: IL-10 (ab214566, Abcam, Cambridge, United Kingdom), were assessed in plasma by using an ELISA kit-based protocol, and their levels were measured as pg/mL.

Oxidative and Anti-Oxidative Status

Measures of oxidative stress status in the form of the levels of lipid peroxidation products, ie, malondialdehyde (MDA), and measures of anti-oxidative enzyme activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined using the kit-based method (Elabscience Biotechnology Co., Ltd., Wuhan, China) with the help of manufacturer's information. The units for MDA levels were nmol/mg protein, while SOD, CAT, and GPx were determined as U/mg protein, and U/mg protein respectively.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

RT-PCR was carried out to quantify the gene expression analyses of Ikbkb, mmp-2, mmp-3, ADAMTS-4, and ADAMTS-5 in joint tissue. To perform this, an RNA isolation kit was used to extract total RNA from the joint tissue with the help of the manufacturer's directives. RevertAid™ cDNA Synthesis kit (Thermo Fisher Scientific™ Fermentas) was then used to reverse transcribe mRNA to cDNA. Post reverse transcription, RT-PCR was run by taking SYBR Green PCR master mix using a thermocycler (Applied Biosystems, Thermo Fischer Scientific, USA), and relative expression of studied gene expressions was normalized with respect to house-keeping gene, ie, β-actin. The following primer sets are used for the reactions. Ikbkb-(F5'CGGGAGAATGACGTGAAGGT3'; R5'ATCGGGCTCCTCTGTAGGTC3'); Mmp-2-(F5'GCAGAAGTTCTTTGGGC TGC'; R5'TGGGTTGCCACATCTTGGTT3'); Mmp-3-(F5'CCTCTGAGTCTTTTCATGGAGGG'; R5'TCAGTGCGCCAA GTTTCAGA3'); Adamts4-(F5'CGTTCCGCTCCTGTAACACT'; R5'TTGAAGAGGTCGGTTCGGTG'); Adamts5-(F5'AT GCACTTCAGCCACGATCA'; R5'CCAGAATCTGCTTCCGTGGT3').

Statistical Analysis

Results were presented as mean ± S.D. of respective groups. The statistical method of analysis followed was a one-way analysis of variance (ANOVA) with Tukey's post hoc test to determine the statistical significance ($p < 0.05$) between different groups.

Results

Arthritis Score (AS) and Paw Volume (PV)

On days 14, 28, and 42, significantly increased AS and PV were observed in the RA group compared to the normal control group. On days 28 and 42, a significant reduction was observed in AS and hind paw volume in the NOR 15, NOR 30, and MTX treated group of rats in comparison to the same parameters on respective days in RA-induced groups of rats (Figure 1A and B).

Levels of RF and CPR in Plasma

After the experiment period was completed, significant increases in the plasma levels of RF and CPR were observed in adjuvant-induced RA rats compared to control group rats. Conversely, RA rats treated with NOR 15, NOR 30, and MTX for 28 days showed significant amelioration in plasma levels of RF and CPR when compared to the same levels in the RA group without any treatment (Figure 1C and D).

Histological Evaluation

The joint tissue was evaluated for histological changes (Figure 2). Normal control rats presented with normal histological arrangements. RA-model rats showed inflammation and features of osteoarthritis, such as synovial hyperplasia, severe inflammatory cell infiltration, and cartilage degeneration. Conversely, RA-model rats treated with NOR at 15 or 30 mg/kg and MTX demonstrated reduced inflammation and an overall controlled arthritis condition compared to RA-model rats, as shown in Figure 2.

Figure 3 displays the results of Masson trichrome (MT) staining of joint tissue for both normal control rats and RA model rats. The normal control rats exhibited typical cartilage structures without any inflammation or damage, while the RA model rats showed noticeable cartilage damage and inflammation, consistent with arthritis pathology. The treatments with 15 and 30 mg/kg/bw NOR showed some restoration in cartilage structure and reduced inflammation. However,

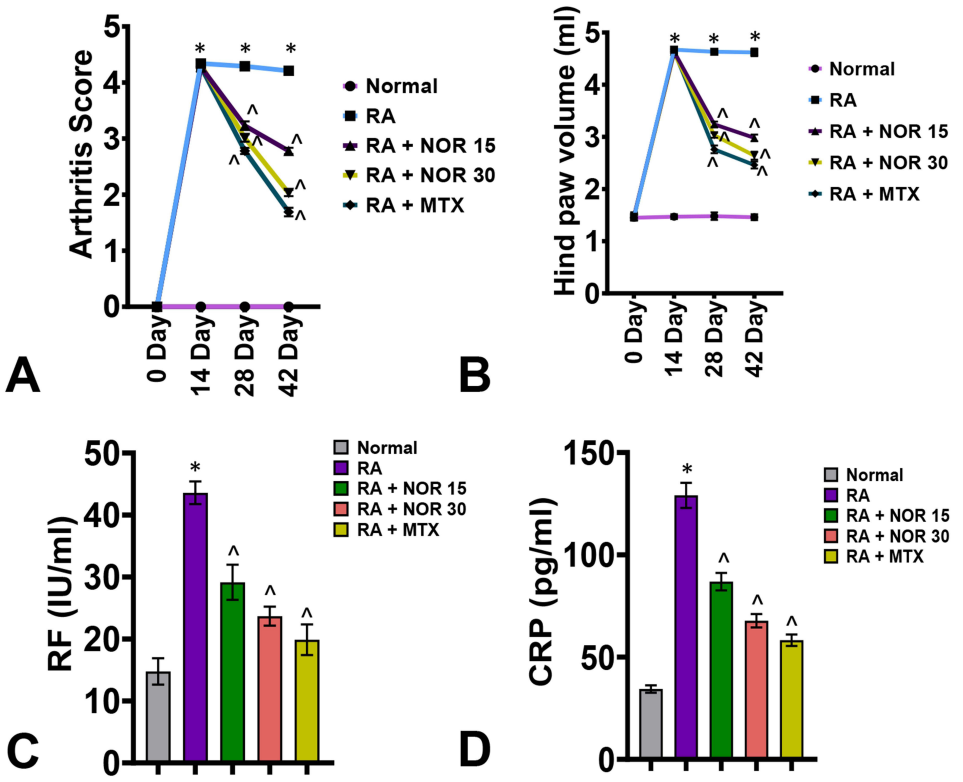

Figure 1 Effect of Norisoboldine on arthritis score, and paw volume in adjuvant-induced rheumatoid arthritis model rats. (**A**): Arthritis score; (**B**): Paw volume; (**C**): Rheumatoid factors (RF); (**D**): C-reactive protein (CRP). Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA+ NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. The results are expressed as Mean ± SEM (n = 6). *p < 0.05 versus normal control group, ^p < 0.05 versus Rheumatoid Arthritis model group.

MTX treatment primarily demonstrated a reduction in inflammatory signs and prevention of further cartilage degradation, rather than inducing cartilage repair.

Effect of nor on Oxidative and Antis-Oxidative Status in Joint Tissue of RA-Model Rats

The levels of MDA (Figure 4A) were increased in a significant ($p<0.05$) manner in association with reduced enzyme activities of SOD (Figure 4B), CAT (Figure 4C), GPX (Figure 4D) in the joint tissue of RA-induced rats. However, RA-induced rats treated with either 15 or 30 mg/kg body weight of NOR daily or 0.3 mg/kg of MTX twice in a week significantly ameliorated changes in the above oxidative and anti-oxidative stress markers in comparison with the same levels observed in only RA-induced rats.

The Nrf2 (Figure 5A), Nqo-1 (Figure 5D) and Ho-1 (Figure 5C) mRNA expressions were significantly downregulated with elevated gene expression of Keap1 (Figure 5B) in the joint tissue of only RA-model rats with its respective mRNA expressions in normal control rats. While significant upregulation in Nrf2, Ho-1, and Nqo-1 gene expressions along with downregulation of Keap1 gene expression was noticed in RA rats administered with NOR or MTX with that of only RA-treated rats without any further treatment.

During the study, the joint tissue of rats was examined to investigate the immunohistochemical staining of Nrf2. The findings revealed that the distribution of Nrf2 protein in the joint tissue of rats with RA was lower compared to the control group. However, RA animals that received either NOR or MTX treatment showed higher distribution of Nrf2 protein in the joint tissue compared to untreated RA animals (Figure 6A and B).

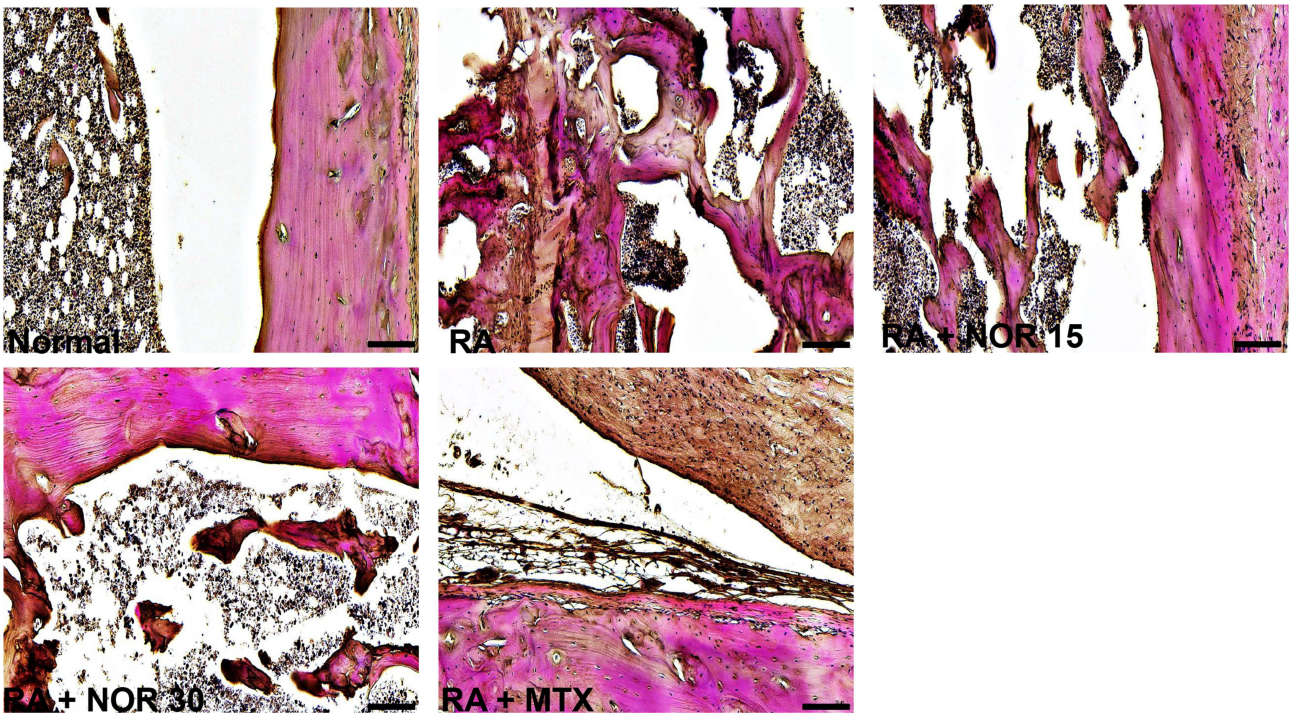

Figure 2 Effect of Norisoboldine administration on histopathological changes in joint tissue of adjuvant-induced Rheumatoid Arthritis model rats. Hematoxylin and eosin (H&E) staining; Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA+NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. (n = 6). Magnification = x40; Scale bar = 100μm.

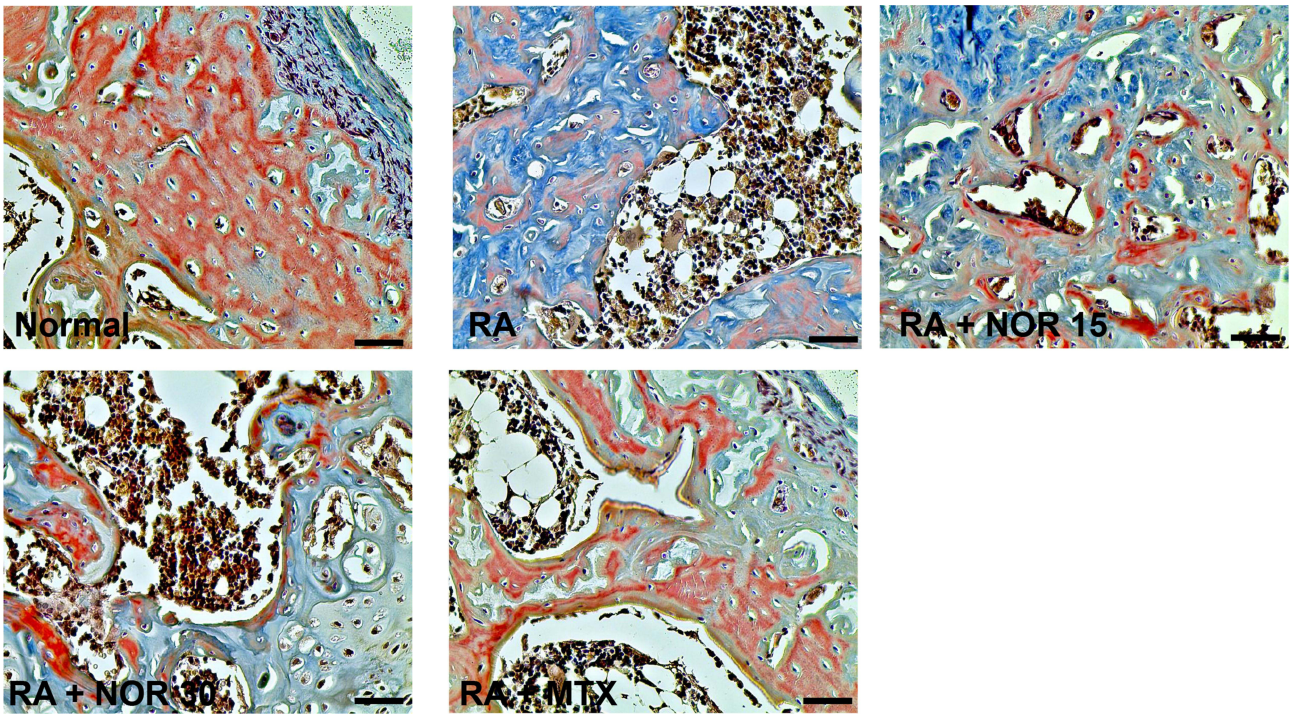

Figure 3 Effect of Norisoboldine administration on histopathological changes in joint tissue of adjuvant-induced Rheumatoid Arthritis model rats. Masson trichrome (MT) staining; Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA +NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. (n = 6). Magnification = x40; Scale bar = 100μm.

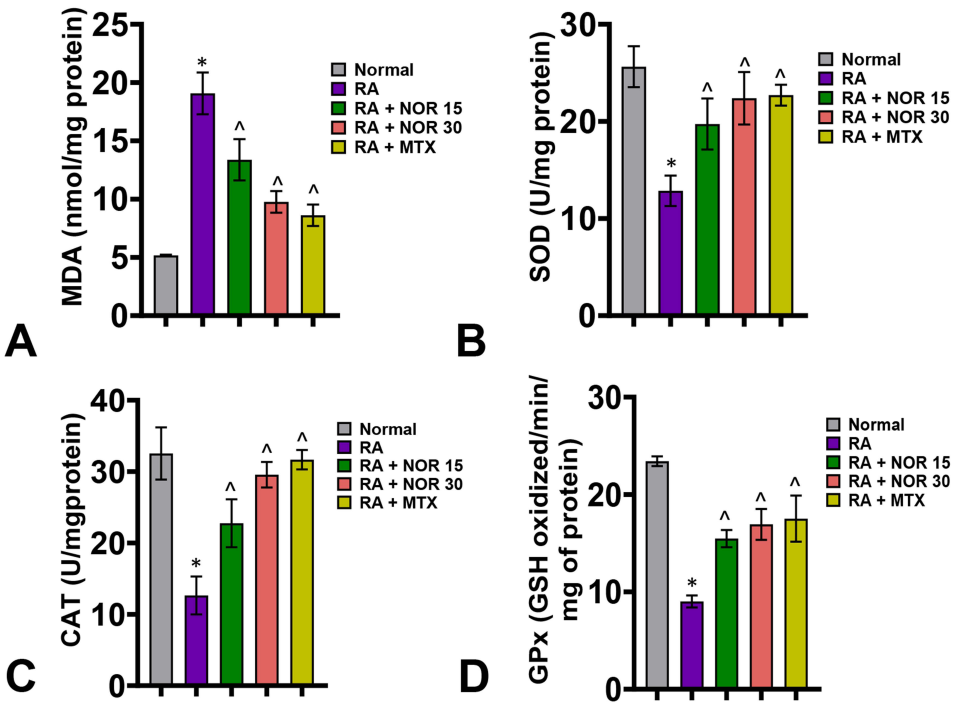

Figure 4 Effect of Norisoboldine administration on oxidative stress and anti-oxidative stress markers in joint tissue of adjuvant-induced Rheumatoid Arthritis model rats. (**A**): Malondialdehyde (MDA) levels, an indicator of lipid peroxidation; (**B**): superoxide dismutase (SOD); (**C**): Catalase (CAT); (**D**): Glutathione peroxidase (GPx). Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA+ NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. The results are expressed as Mean ± SEM (n = 6). *$p < 0.05$ versus normal control group, ^$p < 0.05$ versus Rheumatoid Arthritis model group.

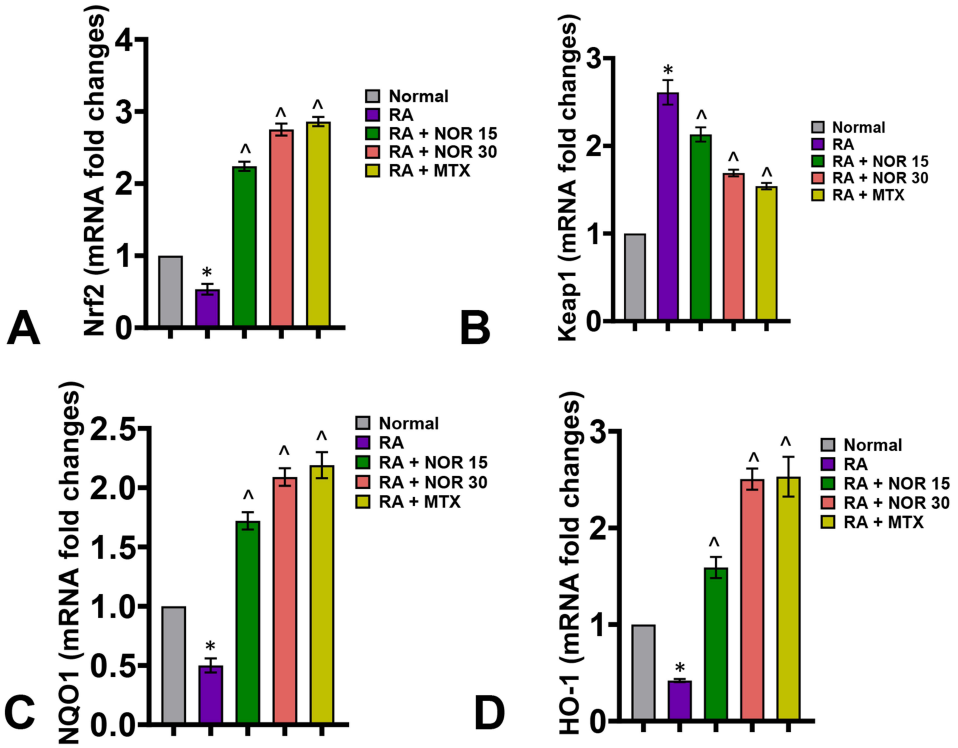

Figure 5 Effect of Norisoboldine administration on Nrf2-Keap1 signaling pathway in joint tissue of adjuvant-induced Rheumatoid Arthritis model rats. Relative mRNA expression of (**A**): *Nrf2*; (**B**): *Keap 1*; (**C**): *Nqo1;* (**D**): *Ho-1* in joint tissue measured by RT-qPCR analysis. Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA+ NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. The results are expressed as Mean ± SEM (n = 6). *$p < 0.05$ versus normal control group, ^$p < 0.05$ versus Rheumatoid Arthritis model group.

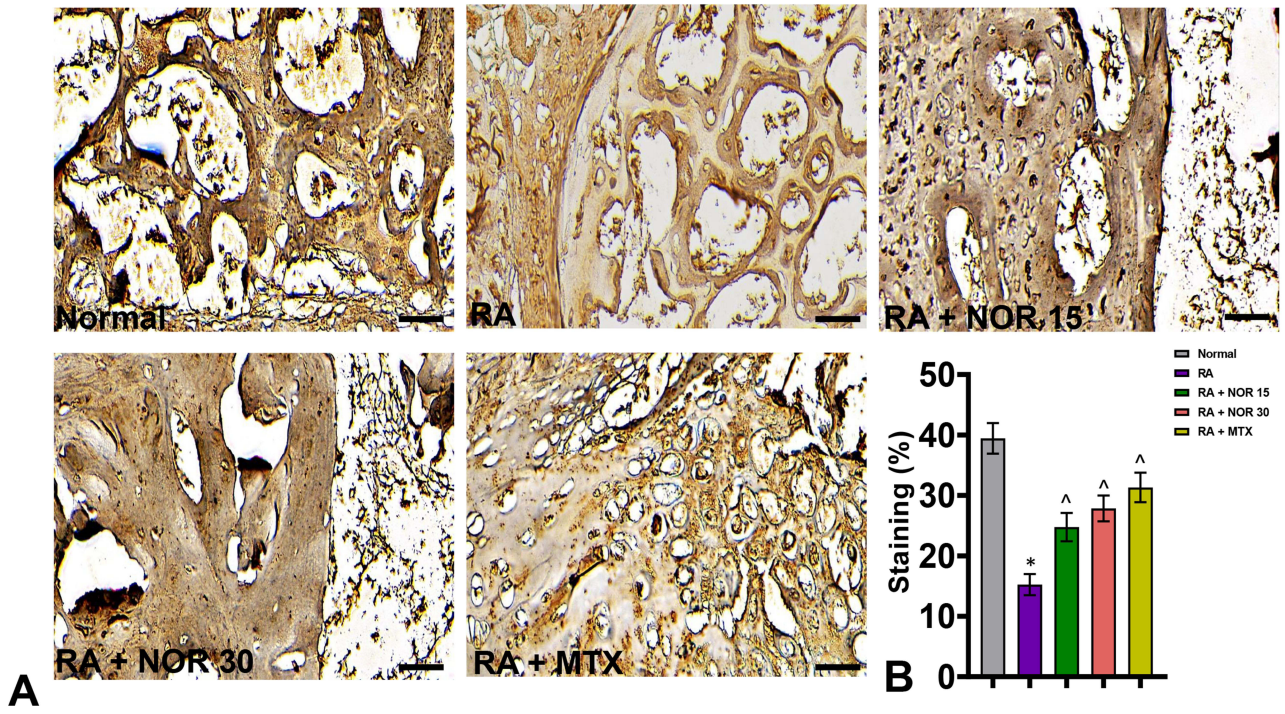

Figure 6 Effect of Norisoboldine on immunohistochemistry staining of (**A**) Nrf2 in joint tissue of adjuvant-induced Rheumatoid Arthritis model rats. (**B**) Nrf2 staining (%). Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA+ NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. The results are expressed as Mean ± SEM (n = 6). *$p < 0.05$ versus normal control group, ^$p < 0.05$ versus Rheumatoid Arthritis model group. Magnification = x40; Scale bar = 100μm.

Effect of nor on Inflammatory Markers in RA-Model Rats

The levels of NF-κB p65 (Figure 7A) and mRNA expression of IKKß (Figure 7B), levels of pro-inflammatory cytokine such as levels of IL-6 (Figure 7C) were significantly elevated in association with reduced levels of IL-10 (Figure 7D) in RA-treated rats in comparison with same levels in control animals. Meanwhile, administration of NOR or MTX significantly attenuated alterations through a significant decrease in NF-κB p65 levels, gene expression of IKKß, and levels of IL-6 and TNF-α significant increase in IL-10 levels with respect to the same levels observed in RA-induced rats.

The study was also broadened by studying the immuno-histochemical staining of TNF-α (Brown color) (Figure 8A and B) on the joint tissue of rats. These results revealed increased protein distribution of TNF-α in the joint tissue of only RA-induced rats with respect to controls. At the same time, treatment of RA animals either with NOR or MTX showed reduced protein distribution of TNF-α in the joint tissue of rats compared to RA animals without any treatment.

Matrix Metalloproteinases, Aggrecanases and PCNA

The *Mmp-2* (Figure 9A), *Mmp-3* (Figure 9B), *Adamts-4* (Figure 9C), and Adamts-5 (Figure 9D) gene expressions were significantly upregulated in RA-model rats, while in RA-model rats treated with NOR or MTX, the expression of the same genes in joint tissue showed significant attenuation.

The immunohistochemical staining of MMP-2 (Red color) (Figure 8) and PCNA (Figure 10A and B) and showed increased protein distribution levels of the same in RA-model animals, while on the other hand, NOR 15, NOR 30 and MTX treatment to RA animals depicted reduced distribution of MMP-2 and PCNA protein in joint tissue when compared to RA animals with no further treatment.

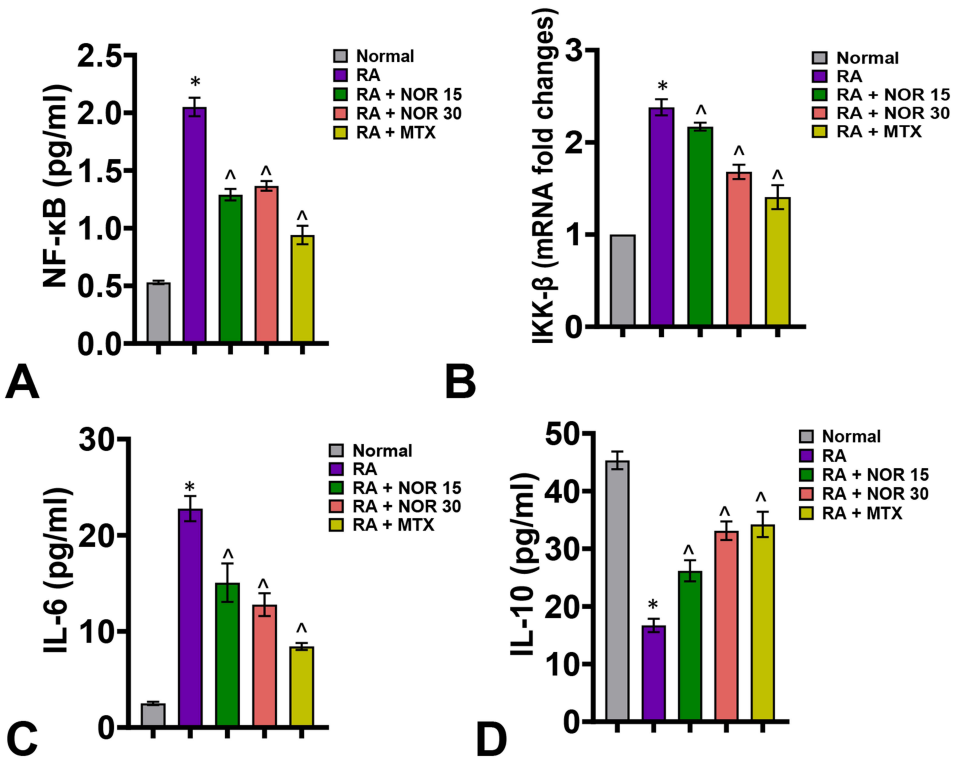

Figure 7 Effect of Norisoboldine on inflammation markers in adjuvant-induced Rheumatoid Arthritis model rats. (**A**): NF-kB; (**B**): Ikkß; (**C**): IL-6; (**D**): IL-10. Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA+ NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. The results are expressed as Mean ± SEM (n = 6). *$p < 0.05$ versus normal control group, ^$p < 0.05$ versus Rheumatoid Arthritis model group.

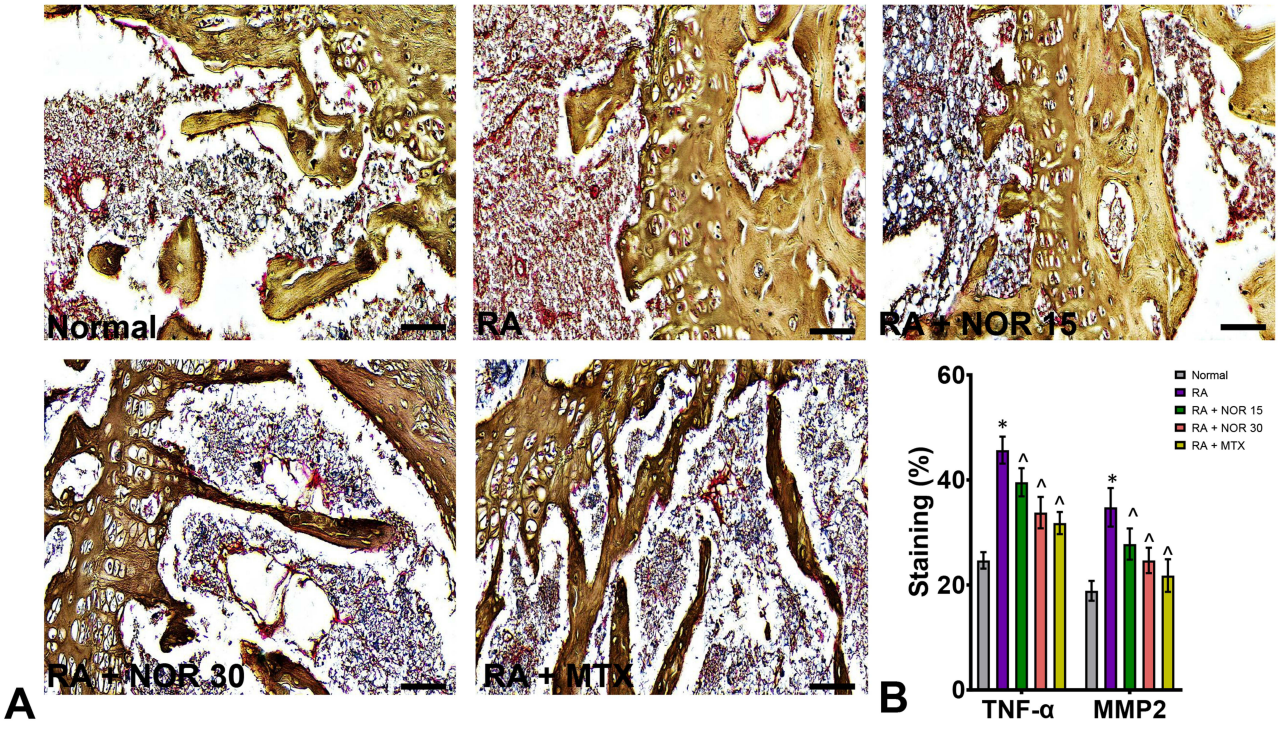

Figure 8 Effect of Norisoboldine on immunohistochemistry staining of (**A**) TNF-α (Brown)/MMP2 (Red) in joint tissue of adjuvant-induced Rheumatoid Arthritis model rats. (**B**) TNF-α (Brown)/MMP2 (Red) staining (%). Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA+ NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. The results are expressed as Mean ± SEM (n = 6). *$p < 0.05$ versus normal control group, ^$p < 0.05$ versus Rheumatoid Arthritis model group. Magnification = x40; Scale bar = 100μm.

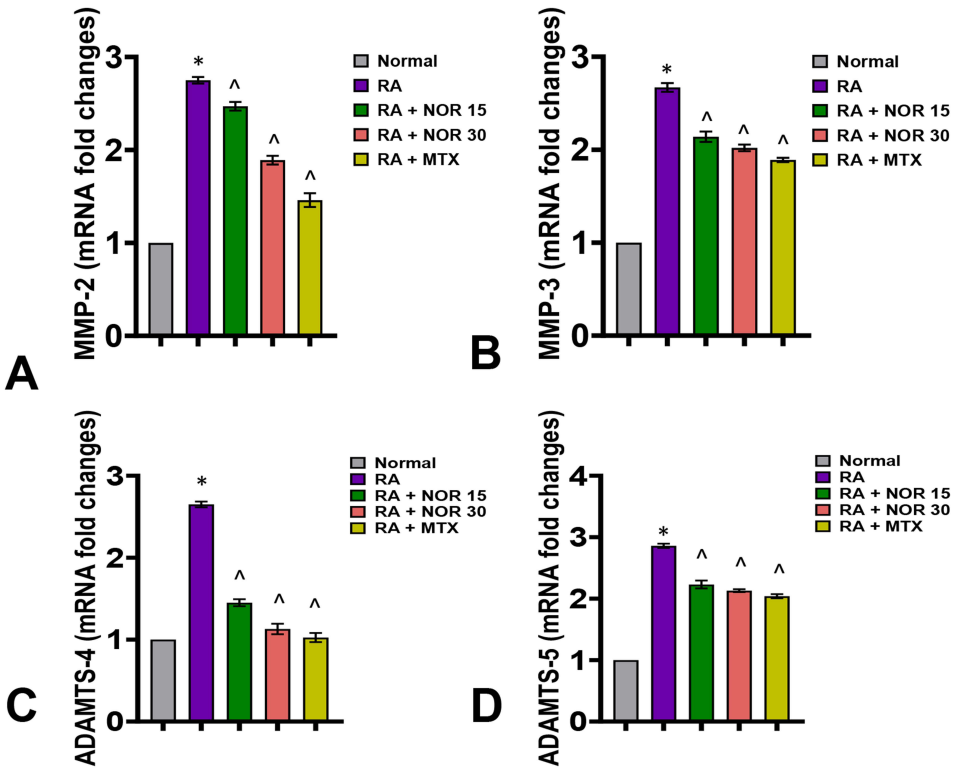

Figure 9 Effect of Norisoboldine administration on matrix metalloproteinases and aggrecanase in joint tissue of adjuvant-induced Rheumatoid Arthritis model rats. Relative mRNA expression of (**A**): *Mmp-2*; (**B**): *Mmp-3*; (**C**): *Adamts-4* (**D**): *Adamts-5*. Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA+ NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. The results are expressed as Mean ± SEM (n = 6). *$p < 0.05$ versus normal control group, ^$p < 0.05$ versus Rheumatoid Arthritis model group.

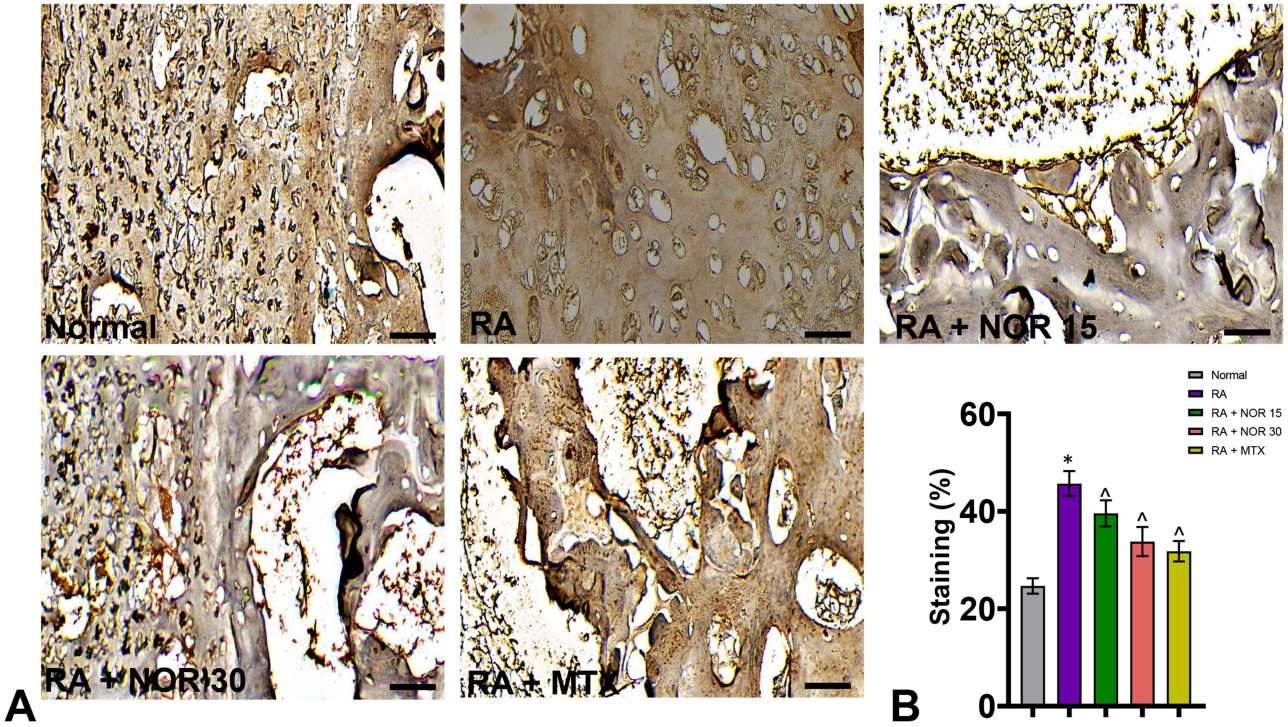

Figure 10 Effect of Norisoboldine on immunohistochemistry staining of (**A**) PCNA in joint tissue of adjuvant-induced Rheumatoid Arthritis model rats. (**B**) PCNA staining (%). Normal: Normal control rats; RA: Rheumatoid arthritis model rats; RA+NOR 15: Rheumatoid arthritis model rats treated with 15 mg/kg/bw of Norisoboldine; RA+ NOR 30: Rheumatoid arthritis model rats treated with 30 mg/kg/bw of Norisoboldine; RA+MTX: Rheumatoid arthritis model rats treated with Methotrexate. The results are expressed as Mean ± SEM (n = 6). *$p < 0.05$ versus normal control group, ^$p < 0.05$ versus Rheumatoid Arthritis model group. Magnification = x40; Scale bar = 100μm.

Discussion

This study highlights the protective role of Norisoboldine (NOR), an isoquinoline alkaloid from *Lindera aggregate*, in treating rheumatoid arthritis (RA) in an adjuvant-induced rat model. The therapeutic potential of NOR is demonstrated through significant improvements across various pathological markers of RA, including reduced arthritis scores (AS), paw volume (PV), and lowered levels of systemic inflammation markers like rheumatoid factor (RF) and C-reactive protein (CRP). NOR treatment notably attenuated these parameters, highlighting its multi-faceted anti-inflammatory effects. Additionally, NOR's modulation of inflammatory cytokines, NF-κB signaling, and antioxidant defenses underscores its therapeutic promise.

Arthritis score (AS) and paw volume (PV) have been considered vital parameters to assess inflammation in the joints as a measure of the severity of arthritis.[21,22] Model rats had significantly high AS and PV observed at different time points; however, NOR or MTX treatment in arthritis rats showed a significant reduction in the same parameters as an indication of an anti-inflammatory effect. Furthermore, plasma levels of RF and CRP have been considered as standard biomarkers to evaluate the systemic inflammation status.[23] The levels of RF and CRP were elevated markedly along with severe pathological alterations in adjuvant-induced model rats, while after treatment of model rats with either NOR or MTX showed significant attenuation in levels of the same markers and pathological alterations as an indication of anti-RA effects through reduced systemic inflammation and controlled arthritic condition. Supporting this evidence, Luo, Liu, Xia, Dai, Chou and Wang[24] demonstrated the protective effect of Norisoboldine in arthritic mice model (induced with collagen) through its alleviation of clinical indicators, ie, reversal of body weight loss, inflammatory swelling, and erythema and, thereby, disease progression in mice.

It has been well demonstrated that chronic inflammation is considered a hallmark feature of RA disease condition.[25] NF-κB is known to be a chief mediator of inflammation, particularly in RA disease conditions, which is evidenced by its presence in the synovial tissue of RA patients.[26] In normal biological conditions, NF-κB lies inside the cytoplasm in association with IkB (inhibitor of kB). In contrast, in stimulatory conditions, IkB kinase (IKKB) ubiquitinates IkB, thereby translocating NF-κB into the nucleus to induce transcription of pro-inflammatory genes.[27] The cytokines produced from the activation of NF-κB can activate themselves in other immune cells of synoviocytes, thereby inhibiting the expression of additional cytokines and aggressively promoting inflammation.[28] Also, in this study, the levels of NF-kB along with TNF-α, and IL-6 levels were markedly elevated in association with reduced levels of IL-10 and upregulated mRNA expression IKKß in adjuvant-induced RA-model rats when compared to the same parameters in control rats. Previously, it was reported that injection of IKKβ (dominant negative) through intra-articular mode reduced nuclear translocation of NF-κB, thereby protecting arthritic damage.[29] Also, decoy oligonucleotide, an inhibitor for NF-kB or IKK inhibitor, BMS-345541 attenuated adjuvant-induced arthritis.[27,30] A Plethora of scientific evidence in the form of in vitro and in vivo approaches also proved the Norisoboldine-mediated inhibition of inflammation through its inhibitory action on inflammatory mediators.[16,31]

In addition to inflammation, oxidative stress, a dysregulated cellular redox state that is the result of an excess of oxidants, antioxidants deficiency, or a combination of both, is another pathogenic hallmark phenomenon in RA,[32] as evidenced by the presence of multifold elevation in ROS generation in blood cell components of RA patients.[33] Furthermore, it was also demonstrated that oxidative stress is strongly linked to clinical parameters of disease outcome in RA condition.[34] ROS are required to maintain a cellular redox state in a normal biological state to perform various cellular functions. However, increased concentrations of ROS are detrimental to cells and affect essential biomolecules in cells.[35] In the current study, the dysregulated cellular redox system was evidenced by the presence of significantly elevated levels of oxidative stress markers with a significant reduction in activities of a pool of studied antioxidant defenses in RA model rats in comparison with respective markers in control animals. However, the enhanced levels of MDA and NO and suppressed antioxidant defenses were significantly attenuated with the treatment of NOR or MTX in RA-model rats compared to RA-model rats without any treatment.

Since the Nrf2-Keap-1 signaling pathway is crucial in cellular oxidative defense systems, this study was further broadened to know the possible involvement in disease progression.[36] Nrf2 is a critical factor at the transcription level to regulate genes responsible for anti-oxidation and detoxification roles.[6,37] In healthy conditions, Nrf2 is bound to Keap1 in cytoplasm; however, in stressed conditions, including oxidative stress effect, Nrf2 detaches from Keap1, translocates to the nucleus and activates the expression of protective genes thereby promoting cellular

defense against oxidative stress.[38,39] The results of the present study showed reduced Nrf2 gene expression and its down-regulated genes, such as Ho-1 and Nqo-1, and increased gene expression of Keap1 in RA-model rats. On the other hand, significant attenuation was observed in the same expression levels of Nrf2 and its regulated genes in RA-model rats administered with NOR or MTX concerning only RA-treated rats demonstrating the anti-oxidative effect is mediated through Nrf2/Keap1/ARE pathway. Earlier, Lv, Wang, Qiao, Yang, Xin, Dai and Wei[16] reported that Norisoboldine-induced elevation of Nrf2 level and suppression of levels of reactive oxygen species in lipopolysaccharide and ATP-stimulated THP-1 cells. Furthermore, Zhang, Suzuki, Adachi, Yoshida, Sakaguchi and Yamamoto[40] demonstrated that activation of Nrf2 improves experimental rheumatoid arthritis in mice.

Matrix metalloproteinases are a group of enzymes that break down extracellular matrix components, ie, collagen and proteoglycans. These enzymes are produced by various cells, including synovial fibroblasts and inflammatory cells, thereby contributing to the destruction of joint cartilage and bone functional impairment in rheumatoid patients.[41] Agggrecanases are a subset of MMPs that specifically cleave aggrecan, a major proteoglycan in cartilage. In RA condition, these enzymes degrade aggrecanase, leading to loss of cartilage integrity and joint function, thereby exacerbating inflammation.[42] MMPs and aggrecanases are implicated in the disease progression of rheumatoid arthritis and contribute to the destruction of joint tissues, leading to the disease's characteristic symptoms and progressive nature. Understanding the roles of these enzymes in arthritis provides significant insights for developing targeted therapies aimed at inhibiting their activity to slow down joint damage, thereby improving the quality of life of affected individuals. A case report by Song and Kim[43] reported that high titers of anti-proliferating cell nuclear antigen antibodies are linked to the pathogenesis of rheumatoid arthritis. The results of the current study demonstrated that RA model rats present with upregulated expression of MMP-1 and MMP-3, genes along with increased distribution of PCNA and ADAMTS-4 and ADAMTS-5 proteins.

Conclusions

In conclusion, this study highlights the protective effects of Norisoboldine (NOR) in managing rheumatoid arthritis (RA) using an adjuvant-induced rat model. NOR treatment significantly reduced arthritis scores (AS), paw volume (PV), and inflammation markers such as rheumatoid factor (RF) and C-reactive protein (CRP), demonstrating a strong anti-inflammatory effect. NOR's ability to modulate cytokines, inhibit NF-κB signaling, and boost antioxidant defenses underscores its therapeutic promise in RA. The reductions in NF-κB, TNF-α, and IL-6, along with increased IL-10, support NOR's potential in managing RA through anti-inflammatory and antioxidative pathways. However, further studies are warranted to substantiate NOR's efficacy in clinical settings.

Data Sharing Statement

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

Disclosure

The authors declare that they have no conflicts of interest.

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